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Structure and Properties of Xanthan Versus Fermentation Time in an Emulsion System

M. Milas

Centre de Recherches sur les Macromolécules Végétales, Laboratoire propre du CNRS associé à l'Université Joseph Fourier, BP 53 X, 38041 Grenoble-Cedex, France

H. Viehweg & A. Weiss

Henkel KGaA, TFB-Biotechnologie, Henkelstrabe 67, 4000 Düsseldorf, FRG

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ABSTRACT

The concentration of xanthan produced in the culture broth during fermentation in an emulsion system is higher than concentrations obtained during classical fermentations. From an economic point of view, this could be of interest when the fermentation broth is used directly for a specific industrial application.

This work concerns the hydrodynamic and rheological properties of xanthan produced at various times during fermentation in an emulsion system. It was suggested that two kinds of xanthan are produced throughout the course of the fermentation. At the beginning, before and just after emulsification, a xanthan with a lower molecular weight is synthesized, while the properties of xanthan produced later in the emulsion system are very similar to those obtained from fermentation in aqueous medium. The acetate and pyruvate substituent levels remain constant during the entire fermentation. The xanthan is synthesized as a native, low viscous ordered conformation but after denaturation the renatured conformation restored is much more viscous. Some degradation may appear at the end of the fermentation. The intrinsic viscosity and weight average molecular weight laws are given for the two ordered conformations.

INTRODUCTION

The bacterial polysaccharide xanthan, produced by *Xanthomonas campestris*, has great industrial interest because of its unique solution pro-

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perties. The most striking of these properties is the very high viscosity of dilute solutions even in the presence of external salts or divalent counterions. Because of these properties, xanthan gums are considered viable alternatives to hydrolyzed polyacrylamides for use in enhanced oil recovery operations. Nevertheless industrially available products are known to have plugging tendencies (Kohler & Chauveteau, 1978). Many methods have been proposed to improve the filterability of xanthan e.g. enzyme clarification (Colegrove, 1977; Rinaudo *et al.*, 1983, 1984; Schröder *et al.*, 1985) or chemical treatments (Patton, 1973). Since a large part of this plugging behavior, however, can be attributed to microgels formed when the polysaccharide powder is dissolved, direct use of the fermentation broth has been proposed (Kohler & Chauveteau, 1978). The xanthan concentration normally obtained in culture broths is low, about 2%, which leads to expensive stocking and carrying charges. Ultrafiltration methods have been used to increase the xanthan gum concentration in the broth. Fermentation in an emulsion system can be used as an alternative.

In this work the structures and properties of xanthans produced at different fermentation times in an emulsion system were determined and then compared with those obtained via classical fermentation.

EXPERIMENTAL

The organism used was *Xanthomonas campestris* ATCC 31602. The culture medium contained the following concentrations (grammes per kilogramme of medium): soja, 6·0; corn steep liquor, 10·0; $(NH_4)_2HPO_4$, 0·4; Na_2HPO_4, 0·8; $Na_2HPO_4.12H_2O$, 0·9; $MgSO_4.7H_2O$, 0·2.

The glucose concentration was kept between 5 and 20 g/liter. The fermentations were carried out at 30°C in a 300 liter reactor (Chemap, FRG) with a working volume of 200 liters and four six-blade turbine impellers. The pH value was maintained at pH 7·0 by adding 4N KOH.

Emulsification begins after 20 h fermentation in a water based system. The preparation of the emulsion for a 200 liter fermentation was obtained by mixing oil (Isopar M, Esso AG) and emulsifier (Comperlan $F^{(R)}$, Henkel KGaA) for 1 min. This mixture was added to the fermentation broth over a period of 45 min. The final content of the broth was: 25% (w/v) oil, 1% emulsifier and 74% aqueous fermentation medium.

To test the reproducibility of xanthan production, two xanthan fermentations were characterized with respect to the xanthan concentration in the broth. Samples from the first fermentation were designated, 1–13, and from the second fermentation 1′–13′.

Broth samples containing an estimated 250 mg xanthan were diluted in 250 ml of 0·01 M NaCl containing 0·4 g/liter NaN_3 and centrifuged for 2 h at 20 000 *g*. These solutions were filtered through 3 μm Millipore filters and 25 g of NaCl added. The xanthan was precipitated by addition of ethanol to a concentration of 50% (v/v). The precipitates were washed by increasing EtOH-H_2O mixtures from 70 to 100% (v/v), and finally dried for 48 h at 30°C under vacuum. These conditions avoid aggregate formation. The method for the recovery of xanthan from broth preserves the native (N) ordered structure (Milas & Rinaudo, 1984, 1986) and gives the sodium xanthan salt. The xanthan powders were stored at ambient conditions. Their moisture contents when prepared under these conditions were about 15%, (measured by thermogravimetry with a Setaram balance; Model G70). The weights recovered allowed the determination of the xanthan concentration in the broth.

The disordered or denatured conformation is obtained at temperatures above T_m, the melting temperature of the native (N) ordered conformation, which is dependent on ionic strength. When the N ordered conformation is heated above T_m, the ordered conformation restored by cooling is different to the N one and is termed the renatured (R) conformation (Milas & Rinaudo, 1986).

Techniques

The degree of pyruvate substitution and acetylation were measured by ^1H NMR. Dried xanthan was dissolved in 5×10^{-3} M sodium acetate solution (in D_2O) at a polymer concentration of about 5 g/liter. Sodium acetate (5×10^{-3} M) was used as an internal standard. The spectra were obtained with a Brucker WP 100 spectrometer at 85°C. The OH signals were suppressed by homonuclear presaturation. Two hundred scans were accumulated with a repetition time of 7 s and a sweep width of 1125 Hz. The acetate and pyruvate yields were expressed as an average number per side chain.

Optical rotation was determined for xanthan concentrations equal to 1 g/liter at $\lambda = 300$ nm in a Spectropol 1b from Fica (France). A 5 cm quartz cell, thermostated at 25°C was used. In order to follow the conformational transition, the temperature was varied from 10 to 80°C at 0·4°C/min using a temperature programmer (Haake PG 10, FRG). The change in the optical rotation was recorded continuously.

Intensities of light scattered from the xanthan solutions (polymer concentrations from 0·02 to 0·15 g/liter in 0·01 M NaCl) were measured at an angle of 6° with a low-angle laser light scattering apparatus (Chromatix KMX6, USA) at 25°C. Optical clarification of xanthan solutions was

achieved by filtration through 0·22 μm Millipore filters. The value of dn/dc was taken as 0·155 ml/g (Milas & Rinaudo, 1986).

Viscosity measurements at the low shear-rate limit were carried out also at 25°C by using a Contraves (Switzerland) Low Shear LS 30 viscometer equipped with a Haake thermostat.

RESULTS AND DISCUSSION

Xanthan concentration in the broth

From the weight of broth used and the amount of xanthan recovered, an estimation of the xanthan concentration in the broth can be made. The xanthan concentration increased until a fermentation time of 150 h, almost reaching 100 g/liter in the first fermentation (Fig. 1). These values are larger than those achieved in the second fermentation, in which 65 g/liter were obtained in 110 h.

Xanthan characteristics relative to fermentation time

Conformational transitions

The thermally driven conformational change by optical rotation for the different samples in 0·01 N NaCl was followed. The results are in agree-

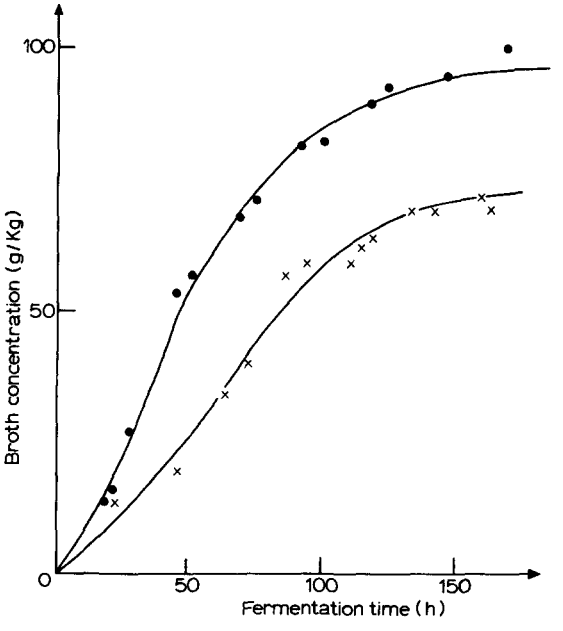

Fig. 1. Broth concentration versus fermentation time. • First fermentation; × second fermentation.

ment with previous work (Milas & Rinaudo, 1986) and no difference in the melting temperature of the ordered N and R structures was observed, as had been previously reported (Milas & Rinaudo, 1986) in contrast to the results published by Lecourtier *et al.* (1986). This discrepancy is difficult to understand because similar results have been obtained with purified xanthan samples from different companies (Shell, Rhone-Poulenc, Henkel). At wavelength 300 nm the specific optical rotation $[\alpha]^{300nm}$ is equal to -41 ± 3 and -113 ± 5 for the disordered and ordered N and R conformations, respectively.

Acetyl and pyruvyl contents

The average numbers of acetyl and pyruvyl groups per side chain are $0{\cdot}6 \pm 0{\cdot}05$ and $0{\cdot}5 \pm 0{\cdot}04$ for acetyl and pyruvyl groups respectively. The pyruvyl content is slightly lower in the second fermentation $0{\cdot}4 \pm 0{\cdot}05$. No significant variation with respect to the fermentation time was observed, contrary to the results of Tait *et al.* (1986) obtained with fermentations in aqueous medium.

Weight average molecular weights $\bar{M}_w$

As reported previously, (Milas & Rinaudo 1984, 1986) there were some differences between the hydrodynamic behavior of xanthan in the N and R ordered conformations. Thus, the $\bar{M}_w$ of xanthan in these two forms was measured; the results are given in Table 1. The N form exhibited a constant $\bar{M}_w$ up to 45 h fermentation time, which was followed by an increase in $\bar{M}_w$ with time. When the fermentation time was over 140 h, $\bar{M}_w$ of R was found to be lower than in the N state. As $\bar{M}_w$ of N and R xanthan are measured on the same solutions (which have been heated to obtain the R forms) a difference higher than 10% between these two forms is significant. This difference is felt to be due to stabilized breaks in the backbone of the N state similar to that reported for DNA (Thomas, 1956). These breaks can appear at the end of the fermentation due to the presence of a degradative enzyme, but they are stabilized by strong interactions between the main chain and the side chains. During the denaturation process, these interactions are destroyed leading to the separation of the different parts of the chain. As a result they would no longer exist in the R state (Milas & Rinaudo, 1986).

The second virial coefficient A_2 is different for the two states ($1{\cdot}5 \times 10^{-3}$ and $1{\cdot}8 \times 10^{-3}$ cm^3 mol/g^2, respectively, for the states N and R in 0·01 M NaCl). It is suggested that this difference is due to a modification of the water structure around the xanthan chain, especially that involved in the solvation of the side chains.

TABLE 1
Fermentation Time Versus Weight Average Molecular Weight and Intrinsic Viscosity in the N and R Xanthan Conformations[a]

Sample	*Fermentation time (h)*	$\bar{M}_w \times 10^{-6}$ *(N)*	$\bar{M}_w \times 10^{-6}$ *(R)*	*[η](ml/g) (N)*	*[η](ml/g) (R)*
1	19	2·5	2·5	2000	3600
4	28	2·0	2·0	1700	2850
5	45	3·15	3·07	2450	4300
7	69	4·15	4·0	3080	6000
10	100	5·0	4·6	3260	6550
12	123	5·2	4·75	3600	7200
13	146	5·2	4·5	3600	6700
1′	24	1·8	2	1220	2600
3′	48	4·9	5	3400	7000
5′	87	5·7	5·7	4250	8500
8′	115	6·6	6·7	4700	9300
10′	136	5·9	6·0	4300	7500
13′	164	6·2	5·4	4250	6800

[a]Solvent: 0·01 M NaCl at 25°C. First fermentation: samples 1–13; second fermentation: samples 1′–13′.

Intrinsic viscosities

Relative viscosities in the low shear-rate Newtonian region and 0·01 M aqueous NaCl solution were measured as a function of polymer concentration (0·0125–0·200 g/liter). The corresponding reduced specific viscosities were extrapolated to zero concentration to yield the intrinsic viscosities $[\eta]$. The variations in the $[\eta]$ were found to be related to the $\bar{M}_w$ changes (Table 1). When the $[\eta]$ and $(\bar{M}_w)$ values for the two xanthan states were plotted on a double logarithmic scale, slopes equal to 0·84 and 1·18 were found for the N and R states respectively (Fig. 2). Despite the change of polydispersity expected between N and R conformations, and as the Mark-Houwink exponents are not too far from one, it was felt that the difference between the exponents is real and therefore do indeed provide genuine evidence for the more extended conformation of the R state, as previously demonstrated (Milas & Rinaudo, 1986). The $[\eta]$ and $(\bar{M}_w)$ values for the R conformation, are very similar to those obtained from Kelco (Sato *et al.*, 1984), Shell (Milas & Rinaudo, 1986; Milas *et al.*, 1986; Callet *et al.*, 1987) or Rhône-Poulenc (Sato *et al.*, 1984, Callet *et al.*, 1987) products in the same conformation. In the N conformation, it has been shown that $[\eta]$ depends on the xanthan samples (Callet *et al.*,

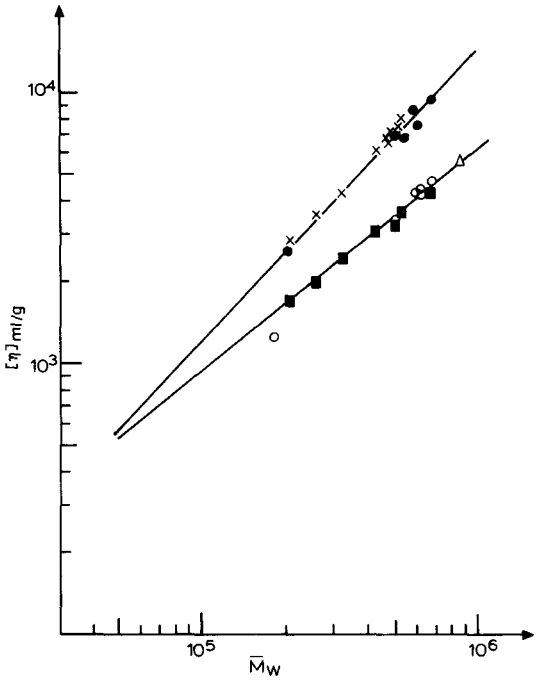

Fig. 2. Double logarithmic plot $[\eta]$, $\bar{M}_w$ for the different xanthan samples in the N and R conformations. At 25°C in 0·01 M NaCl. First fermentation: ■, N; ×, R. Second fermentation: ○, N; ●, R. △, Species 2 in the N conformation from relations 1 and 2.

1987). The values obtained in this work are of the same order of magnitude with respect to $\bar{M}_w$.

Relative viscosity

In Fig. 3 the relative viscosities at 1 g/liter and 25°C in 0·01 M NaCl in the lower Newtonian plateau of the two ordered states, N and R, were plotted as a function of the fermentation time. The dissolution of the xanthan powder in 0·01 M NaCl solutions preserves the N state. In order to obtain the R state, therefore, these solutions were heated for 1 min at 80°C. This temperature and time are found to be enough to obtain total denaturation (Milas & Rinaudo, 1986). The same differences that have already been obtained for $\bar{M}_w$ and $[\eta]$ were found, but with larger variations due to the sensitivity resulting from reduced viscosity on C_p, and $\bar{M}_w$.

Particularly noticeable is the sharp increase in viscosity between 40 h and 120 h, especially for the R state. In contrast no change in viscosity appears before 40 h and a maximum is reached at the end of 120 h

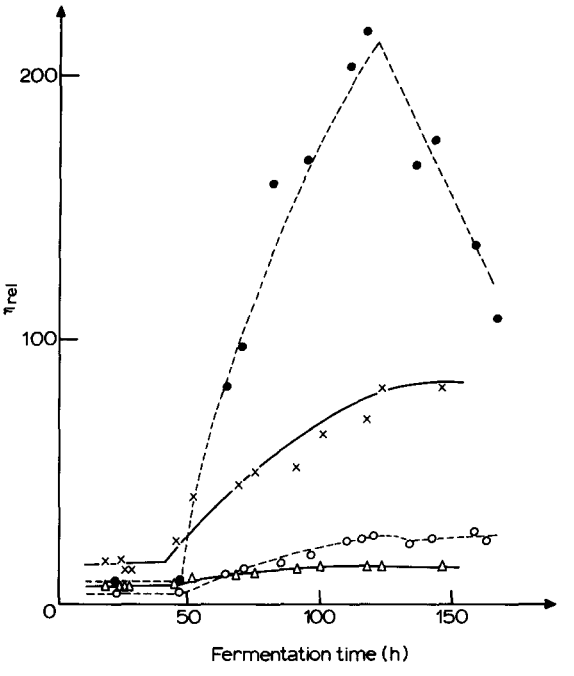

Fig. 3. Relative viscosities in the lower Newtonian plateau versus fermentation time for the different xanthan samples. Xanthan concentration 1 g/liter in 0·01 M NaCl at 25°C. First fermentation: △, N; ×, R. Second fermentation: ○, N; ●, R.

fermentation. The sudden increase in the viscosity and $\bar{M}_w$ of the xanthan samples after 40 h seems to indicate that two kinds of xanthan were produced relative to the fermentation time. To put this hypothesis into a more quantitative form (using data from the first fermentation), the following is proposed:

— An initial form of xanthan was synthesized during the first 40 h of fermentation (Fig. 3), yielding 47 g/liter of xanthan at the end of this period (Fig. 1). This polymer exhibited a low and constant $\bar{M}_{w1}$ $[\eta]_1$; Sample 4 is taken as the reference for this species.

— After 40 h a second type of xanthan was produced, which demonstrated higher and constant $\bar{M}_{w2}$ and $[\eta]_2$. In this time period, the weight fraction of Species 1 (w_1) decreased, while the weight fraction of Species 2 (w_2) increased with the fermentation time. From this assumption, $\bar{M}_w$ and $[\eta]$ at any time will be equal to:

$$\bar{M}_w = \Sigma w_i \bar{M}_i = w_1 \bar{M}_{w1} + w_2 \bar{M}_{w2} \quad (1)$$

$$[\eta] = \Sigma w_i [n]_i = w_1 [\eta]_1 + w_2 [\eta]_2 \quad (2)$$

In eqns (1) and (2), where i is the xanthan species of molecular weight M_i, there is an unknown parameter, $\bar{M}_{w2}$ and $[\eta]_2$ respectively. From the experimental results of Sample 4 (which is characteristic of the xanthan Species 1) and Sample 13 (which represents a mixture of Species 1 and 2) $\bar{M}_{w2}$ and $[\eta]_2$ can be estimated (the characteristics of the xanthan Species 2). This gives $\bar{M}_{w2} = 8{\cdot}4 \times 10^6$ and $[\eta]_2 = 5500$ ml/g in the N conformation.

From the two sets of values for $\bar{M}_{w1}$ and $\bar{M}_{w2}$ and $[\eta]_1$ and $[\eta]_2$ the $[\eta]$ and $\bar{M}_w$ values of the samples during the fermentation in the N conformations (eqns (1) and (2)) can be predicted.

The values are compared with the experimental data in Table 2. The $\bar{M}_w$ and $[\eta]$ found are in very good agreement with the experimental ones. So the assumption that two different species of xanthan are synthesized before and after 40 h of fermentation is plausible. Moreover, the $[\eta]_2$ and $\bar{M}_{w2}$ values fall on the straight line obtained for the N conformation (Fig. 2).

For the second fermentation and from the same $\bar{M}_{w1}$, $\bar{M}_{w2}$ and $[\eta]_1$, $[\eta]_2$ values the calculated values $\bar{M}_w$ and $[\eta]$ found are also in good agreement with the experimental values (Table 3). The higher $\bar{M}_w$ and $[\eta]$ values obtained at the end of this fermentation can be explained by a lower concentration of the xanthan Species 1, ie 20 g/kg instead of 47 g/kg for the first fermentation.

From $\bar{M}_{w1}$ and $\bar{M}_{w2}$ previously obtained and in the absence of degradation processes, the $[\eta]$ values for the xanthan in the R conformation can also be predicted. The $[\eta]_1$ and $[\eta]_2$ values in this conformation are estimated from the $[\eta]$ $(\bar{M}_w)$ curve (Fig. 2). The values are compared in Table 4 with the experimental ones for the first fermentation. The agreement is very good for the first four samples. But, for the next samples, when the xanthan concentration in the broth reached a plateau, the experimental values are lower, in contrast to the results obtained in the N conformation. This illustrates that during this step, stabilized breaks in the backbone in the N conformation are formed. This behavior explains the sharp peak in the relative viscosity for the R material after about 120 h of the second fermentation (Fig. 3).

The agreement between experimental and calculated data found for this model excludes the possibility of a continuous increase of $\bar{M}_w$ with fermentation time for xanthan synthesized after 40 h. Another possible model includes only one type of xanthan being produced but polymerized extracellularly which may be a function of fermentation kinetics especially in the emulsion phase. This model was not tested because it needs information which is difficult to obtain (mechanism and kinetic constants for example).

TABLE 2

Comparison between Weight Average Molecular Weight and Intrinsic Viscosity Measured and Calculated with the Assumption of Two Species of Xanthan Synthesized before and after 40 h Fermentation Time (values for the N conformation, first fermentation)

	Samples						
	1	*4*	*5*	*7*	*10*	*12*	*13*
Fermentation time (h)	19	28	45	69	100	123	146
Total xanthan broth concentration (g/kg)	14	25	54	68	81	93	94
Broth concentration of Species 1 (g/kg)	14	25	47	47	47	47	47
Broth concentration of Species 2 (g/kg)	0	0	7	21	34	46	47
Weight fraction of Species 1 (w_1)	1	1	0·87	0·69	0·58	0·505	0·50
Weight fraction of Species 2 (w_2)	0	0	0·13	0·31	0·42	0·495	0·50
Experimental weight average molecular weight ($\times 10^{-6}$)	2·5	2·0	3·15	4·15	5·0	5·2	5·2
Calculated weight average molecular weight ($\times 10^{-6}$) (eqn (1))	2·0	2·0	2·9	4·0	4·7	5·17	5·2
Experimental intrinsic viscosity (ml/g)	2 000	1 700	2 450	3 080	3 260	3 600	3 600
Calculated intrinsic viscosity (ml/g) (eqn (2))	1 700	1 700	2 160	2 873	3 294	3 600	3 600

TABLE 3

Comparison between Weight Average Molecular Weight and Intrinsic Viscosity Measured and Calculated with the Assumption of Two Species of Xanthan (native xanthan conformation, second fermentation)

	Samples			
	1'	*3'*	*5'*	*8'*
Fermentation time (h)	24	65	87	115
Total xanthan broth concentration (g/kg)	15	35	57	65
Broth concentration of Species 1 (g/kg)	15	20	20	20
Broth concentration of Species 2 (g/kg)	0	15	37	45
Weight fraction of Species 1 (w_1)	1	0·57	0·35	0·30
Weight fraction of Species 2 (w_2)	0	0·43	0·65	0·7
Experimental weight average molecular weight ($\times 10^{-6}$)	1·8	4·9	5·7	6·6
Calculated weight average molecular weight ($\times 10^{-6}$) (eqn (1))	2·0	4·7	6·2	6·5
Experimental intrinsic viscosity (ml/g)	1 220	3 400	4 250	4 700
Calculated intrinsic viscosity (ml/g) (eqn (2))	1 700	3 350	4 150	4 350

TABLE 4

Comparison between Weight Average Molecular Weight and Intrinsic Viscosity Measured and Calculated with the Assumption of Two Species of Xanthan (renatured xanthan conformation, first fermentation)

	Samples						
	1	*4*	*5*	*7*	*10*	*12*	*13*
Experimental intrinsic viscosity (ml/g)	3 600	2 850	4 300	6 000	6 550	7 200	6 700
Calculated intrinsic viscosity (ml/g) (eqn (2))	3 000	3 000	4 235	5 950	7 000	7 700	7 750
Experimental weight average molecular weight ($\times 10^{-6}$)	2·5	2·0	3·07	4·0	4·6	4·75	4·5
Calculated weight average molecular weight ($\times 10^{-6}$) (eqn (1))	2·0	2·0	2·9	4·0	4·7	5·17	5·2

Tait *et al.* (1986) have already shown that growth conditions may have an effect on the viscosity of *Xanthomonas campestris* exopolysaccharide. In particular xanthan viscosity was minimal during exponential growth and maximal in polysaccharide isolated as the growth rate fell. However, no relationship between $[\eta]$, molecular weight, or the nature of the xanthan ordered conformation (N or R) obtained after isolation or in the solutions of the sodium form xanthan used for the viscosity measurement are given. Thus, it was not possible to understand the origin of the viscosity change and the characteristics of the xanthan at the end of the fermentation.

CONCLUSION

The chemical structure (pyruvate and acetate contents) of the xanthan produced does not change throughout the fermentation process. For all the samples two ordered conformations, N and R, were found. The R ordered conformation gives relative viscosities about 10 times higher than the N one at 1 g/liter. The rheological and hydrodynamic studies of the xanthans and their solutions demonstrate three definite phases in the fermentation:

- The initial stage includes fermentation times up to 40 h, i.e. before and just after emulsification. In this phase, the characteristics of the xanthan produced are poor, and there is no increase in the relative viscosity of the xanthan solution.
- The second phase (between 40 and 120 h) which appears after emulsification of the system, corresponds to a large increase in xanthan molecular weight and solution viscosity. Whereas the xanthan synthesized in the first stage had a low $\bar{M}_w = 2 \times 10^6$, the xanthan synthesized in the second phase had a $\bar{M}_w = 8{\cdot}4 \times 10^6$. The $\bar{M}_w$ of xanthan synthesized in both phases was assumed to be constant.
- Since the xanthan concentration does not increase after 120 h fermentation time, the main characteristic of the final phase is the degradation of xanthan which has consequences especially in the hydrodynamic and rheological properties of the R state.

Thus the properties of the xanthan at the end of the fermentation depend on the degree of degradation, but more importantly on the relative fractions of the two kinds of xanthan produced in the first and second steps, i.e. before and after emulsification.

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REFERENCES

Callet, F., Milas, M. & Tinland, B. (1987). In *Gums and Stabilisers for the Food Industry 4*, ed. G. O. Phillips, D. J. Wedlock, & P. A. Williams. Pergamon Press, Oxford, UK, pp. 203–10.

Colegrove, G. T. (1977). US Patent No. 4 010 071, 1 Mar.

Kohler, N. & Chauvetau, G. (1978). Paper presented at the 53rd Annual Fall Conference, Houston, Texas, 1–3 October. Paper SPE 7425.

Lecourtier J., Chauveteau, G. & Muller, G. (1986). *Int. J. Biol. Macromol.*, **8**, 306.

Milas, M. & Rinaudo, M. (1984). *Polymer Bull.*, **12**, 507.

Milas, M. & Rinaudo, M. (1986). *Carbohydr. Res.*, **158**, 191.

Milas, M., Rinaudo, M. & Tinland, B. (1986). *Carbohydr. Polym.*, **6**, 95.

Patton, J. T. (1973). Paper presented at the 48th Annual Fall Conference, Las Vegas, Nevada, 30 September–3 October. Paper SPE 4670.

Rinaudo, M., Milas, M. & Kohler, N. (1983). US Patent No. 4 416 9980, 22 Nov.

Rinaudo, M., Milas, M. & Kohler, N. (1984). US Patent No. 4 431 734, 14 Feb.

Sato, T., Kojima, S., Norisuye, T. & Fujita, M. (1984). *Polym. J.*, **16**, 423.

Schröder, E. T., Kierulf, C. & Sutherland, I. W. (1985). 3rd European Symposium Enhanced Oil Recovery Rome 16–18 April.

Tait, M. I., Sutherland, I. W. & Clarke-Sturman, A. J. (1986). *J. Gen. Microbiol.*, **132**, 1483.

Thomas, C. A. (1956). *J. Am. Soc.*, **78**, 1861.