Rheological Characterization of Dextran-Enzymatic Synthesis Media

A. TECANTE and A. LÓPEZ-MUNGUIA C. *Departmento de Alimentos, Division de Estudios de Posgrado, Facultad de Quimica, Universidad Nacional Autonoma de Mexico, Ciudad Universitaria,04510 Mexico DF, Mexico* A. GARCÍA-REJÓN *Instituto de Investigaciones de Materiales, Universidad Nacional Autonoma de Mexico, Departamento de Polimeros, Ciudad Universitaria, Apartado Postal 70-360, 04510, Mexico DF, Mexico*

Synopsis

The bacteria *Leuconostoc mesenteroides* (strain NRRL-B512) has been used for the fermentative production of the polysaccharide dextran from sucrose. The polysaccharide production was carried out via a four-step process involving (1) production of the enzyme dextransucrase (DS), (2) bacteria removal from the culture broth, (3) enzyme purification by gel permeation chromatography, and (4) enzymatic synthesis of dextran in a cell-free reaction medium.

The enzymatic reaction was followed measuring the change in rheological properties of the reaction medium with time at different initial substrate concentrations. The influence of other parameters, such as temperature, addition of acceptor molecules (maltose), and enzyme purity, were also considered.

The microbial extracellular polysaccharide is responsible for the non-newtonian character of the reaction media. At low shear rate values the flow behavior was newtonian, whereas at higher shear rates the reaction media exhibited a marked shear-thinning behavior. The Carreau equation appears to fit fairly well the experimental data over several decades of shear rate. The reaction media showed elastic behavior as well.

INTRODUCTION

Dextran is a glucose polymer joined mainly by $\alpha(1\text{-}6)$ glucosidic bonds when produced by *Leuconostoc mesenteroides*, dextransucrase. It has an important place among the extracellular polymers produced by biological processes. The different applications of this polymer justify its industrial-scale production and the necessity for its characterization, in order to produce polymers with consistent physical and chemical properties.

Nevertheless, in the industrial-scale production process, rheological changes during synthesis are rarely considered. The reaction is often followed by measurements of by-products (fructose). A method based on viscosity measurements that applies only to newtonian fluids has been proposed.[1]

It is obvious that the rheological properties of the synthesis media are influenced by such factors as polymer concentration and molecular weight; both increase as the reaction proceeds, and so the rheological behavior of the media will be non-newtonian.

The rheological properties of the fermentation broths or the enzymatic reaction media have a strong effect on the process itself as well as on the subsequent recovery operations. Furthermore, they can be very sensitive

indicators of the state of a process and could be useful for control and monitoring purposes.

However, there are not enough available data in the literature regarding the rheological characterization of the biological synthesis media in which polymers are produced. As a consequence, there is a lack of valuable information for optimal reactor design.

The available published data referring to exocellular polymers include such microorganisms as *Pullularia pullulans*,[2] *Hansenula holsti*,[3] *Rhinocladiella mansonii*,[4] *Penicillium funicolosum*,[5] and *Xantomonas campestris*.[6-9]

In the case of dextran, there seems to be no published work reporting the rheological characterization of the synthesis medium. Moreover, in published reviews[10,11] and recent reports on dextran production,[12] there is no particular reference to any work related to the rheological characterization of dextran synthesis solutions.

It is therefore the aim of this work to establish the relationship between different reaction conditions, such as initial sucrose concentration, reaction temperature, addition of alternate acceptors to the reaction medium, and enzyme purity, and the rheological behavior of the synthesis medium in which dextrans are produced.

EXPERIMENTAL PROGRAM

Polymer synthesis

Four steps were followed for dextran production:
1. Production of the transglucosidase enzyme dextransucrase, DS [EC. 2.4.1.5. α(1-6) D-glucan; D-fructose 2-glucosyltransferase]
2. Bacteria removal from the culture broth
3. Enzyme purification by gel permeation chromatography
4. Polymer synthesis in a cell-free reaction medium

Production of the Enzyme DS

The enzyme DS was obtained by culturing the bacteria *L. mesenteroides* (NRRL-B512) on a culture medium containing sucrose (20 g/L), yeast extract (10 g/L), K_2HPO_4(20 g/L), and minor nutrients, as has already been reported.[13] The initial pH of the culture medium was adjusted to 6.9 with orthophosphoric acid. The medium was sterilized for 20 min in an autoclave at 15 psi (120°C) and then inoculated. The fermentation conditions were 28 $\pm$ 0.5°C, 300 rpm, and 0.66 vvm. The fermentation was considered complete when the bacteria were in the stationary phase, usually 4.5 h after inoculation. The pH was then adjusted to 5.0, at which maximum enzyme stability is found.

Bacteria removal

The *L. mesenteroides* cells were removed from the fermentation broth by centrifugation at 3500 rpm for half an hour. The supernatant, hereinafter designated as "crude enzyme" (CE), was stored at 4°C. The enzymatic activity

of the CE was determined by measuring the initial rate of fructose production using the dinitrosalicilic acid (DNS) method.[14] The resulting activity was between 35 and 40 DSU/mL, with 1 DSU defined as the quantity of enzyme that transforms 1 mg sucrose into dextran in 1 h under reaction conditions.

Gel Permeation Chromatography

A sample of the CE was partially purified in order to eliminate the short dextran chains synthetized during the enzyme production. The CE was eluted in a column packed with Bio-Gel A-M54, using 0.01 M pH 5.0 buffered citrate as eluant. Fractions containing the eluted enzyme (EE) were collected in the void volume. The elution was carried out at 4°C, and the EE was stored at the same temperature.

Polymer Synthesis

The dextran synthesis was carried out using both the CE and the EE. The enzymatic activity was lowered to a final value of 7 DSU/mL for the CE and 1 DSU/mL for the EE. Sucrose was added in crystalline form to the enzymatic solutions to prevent further dilution of the enzymatic activity. The reaction conditions in both cases were those shown in Table I. The reaction progress was followed by measuring the reducing power of the synthesis media using the DNS method.

The reaction was stopped when the reducing sugars between two consecutive samples did not show a further increase.

RHEOLOGICAL CHARACTERIZATION
OF THE SYNTHESIS MEDIA

Once the reactions started, samples were taken at known time intervals. All samples were heat-treated (90°C) for 3 min in order to inactivate the DS. Sodium azide was added to each sample to prevent contamination. All the samples were stored at 4°C before their rheological characterization. Steady shear measurements were made, in duplicate, on all samples using a Weissenberg Rheogoniometer with the cone and plate goemetry ($R = 3.75$ cm, $\theta_0 = 2°$). All measurements were carried out at $21 \pm 1°C$ in a shear-rate range between 10 and 1400 s^{-1}.

TABLE I
Reaction Conditions for Dextran Synthesis

Initial sucrose concentration C_{is} % (P/v)	Reaction temperature T_r (°C)
20	30
20	4
10	30
10[a]	30
10	4

[a] 1% Maltose as alternate acceptor.

RESULTS AND DISCUSSION

Shear Stress—Shear Rate Relationship

As expected, the rheological behavior of the synthesis media changed with reaction time. As an example of this behavior the relation between shear stress and shear rate for the polymers synthesized with $C_{is} = 20\%$, $T_r = 30°C$ using the CE is shown in Fig. 1. This figure shows three curves, each representing the behavior of the synthesis medium at a specific dextran concentration C_d obtained at different reaction times. As can be observed, the synthesis medium showed a typical newtonian behavior early in the reaction, for $C_d = 17.7$ mg/mL (curve A). At this stage both the average molecular weight ($\overline{MW}$) and C_d are relatively low, and consequently there is a combined effect between them, giving as a result the above-mentioned behavior. As the reaction proceeded, the rheological behavior changed from newtonian to non-newtonian, showing shear-thinning characteristics (curve B). Finally, near the reaction end ($C_d = 93$ mg/mL), shearing-thinning characteristics were more pronounced (curve C). This gradual change in
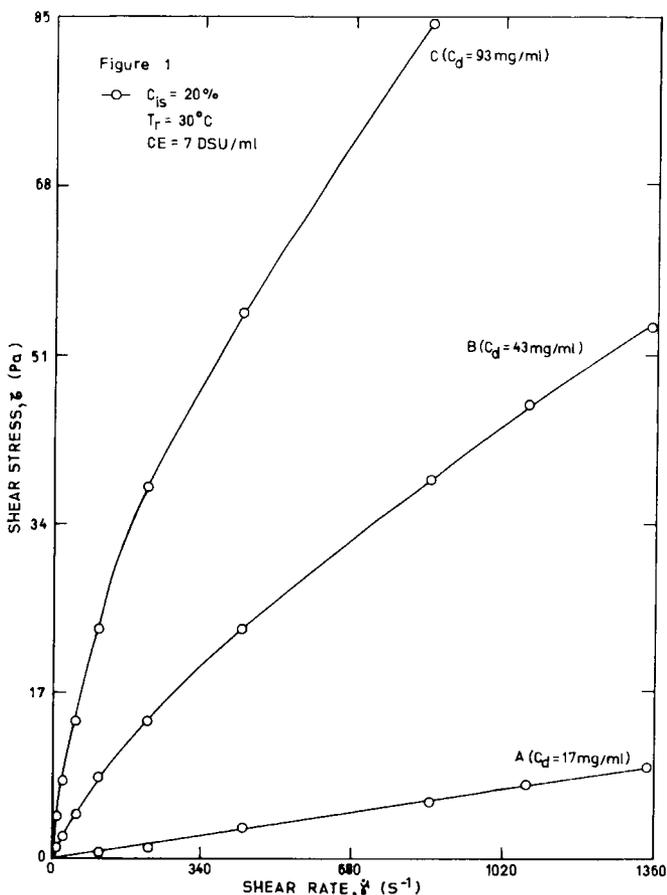
Fig. 1.   Shear stress versus shear rate. Evolution of rheological behavior as reaction proceeds.

behavior along the reaction can be explained in terms of an increase in polymer concentration and a simultaneous growth of the polymer chains or, more precisely, of the average molecular weight ($\overline{MW}$). It is known that under these reaction conditions the $\overline{MW}$ of dextrans is well over 10^6.[14] Hence, the rheological behavior of the synthesis media is determined by a combined effect between C_d and the MWD.

As previously mentioned, all the synthesis media exhibited this behavior. As will be seen later, only the synthesis medium containing maltose as an alternate acceptor did not exhibit this behavior. Likewise, none of the synthesis media exhibited a yield stress value.

Effect of Initial Concentration of Sucrose C_{is}

Although both C_d and MWD have influence on the rheological behavior of the synthesis media, their effect is different. In general, an increase in C_d has an influence on the viscosity values and consequently on the relative position of the curves in a plot of viscosity against shear rate; an increase in $\overline{MW}$ and a concomitant change in MWD has influence on the rheological behavior of the fluid as a whole. This last factor determines the shape of the flow curve. Therefore, in order to compare the curves obtained, it is necessary to eliminate the influence of either of these two factors.

Because of the great influence of the MWD on the rheological behavior, it is more convenient to analyze the relation between viscosity and shear rate in terms of an equal value of C_d for a different value of C_{is}.

Figure 2 shows the relation between viscosity and shear rate for those polymers synthetized with $C_{is} = 20\%$ and $C_{is} = 10\%$ with $T_r = 30°C$ using the CE. Figure 3 shows the same relation for $T_r = 4°C$. Comparing the curves for a C_d value between 38 and 43 mg/mL (Fig. 2), a large difference in both the viscosity and rheological behavior of the two synthesis media can be observed. The difference in rheological behavior cannot be explained in terms of the difference between C_d values, and therefore it can be assumed that it must be related to changes in $\overline{MW}$ and MWD. For a $C_{is} = 10\%$ the behavior was newtonian over more than two decades of shear rate. For $C_{is} = 20\%$ the flow behavior is typical of polymer solutions. At low shear rates (< 100 s^{-1}) the behavior was newtonian. At higher shear rate values (> 100 s^{-1}), the change in viscosity was described by a relation of the power-law type. This situation suggests that, for those polymers synthetized with $C_{is} = 20\%$, the $\overline{MW}$ was higher and the distribution was possibly wider than for those polymers synthetized with $C_{is} = 10\%$. This last assumption seems to be confirmed by the fact that the fluid deviates from newtonian behavior at lower shear rates and shows a more gradual viscosity decrease as shear rate is increased.

Near the end of the synthesis ($C_d = 93$ mg/mL), the curves for both $C_{is} = 20\%$ and $C_{is} = 10\%$ showed newtonian and non-newtonian behavior. Nevertheless, the medium with $C_{is} = 10\%$ showed a similar behavior with respect to the medium with $C_{is} = 20\%$ when $C_d = 43$ mg/mL. This suggests that, for the synthesis with $C_{is} = 20\%$, when the reaction is half-completed, the structural properties of the polymers are similar to those of the polymers synthetized with $C_{is} = 10\%$, at the end of the reaction.
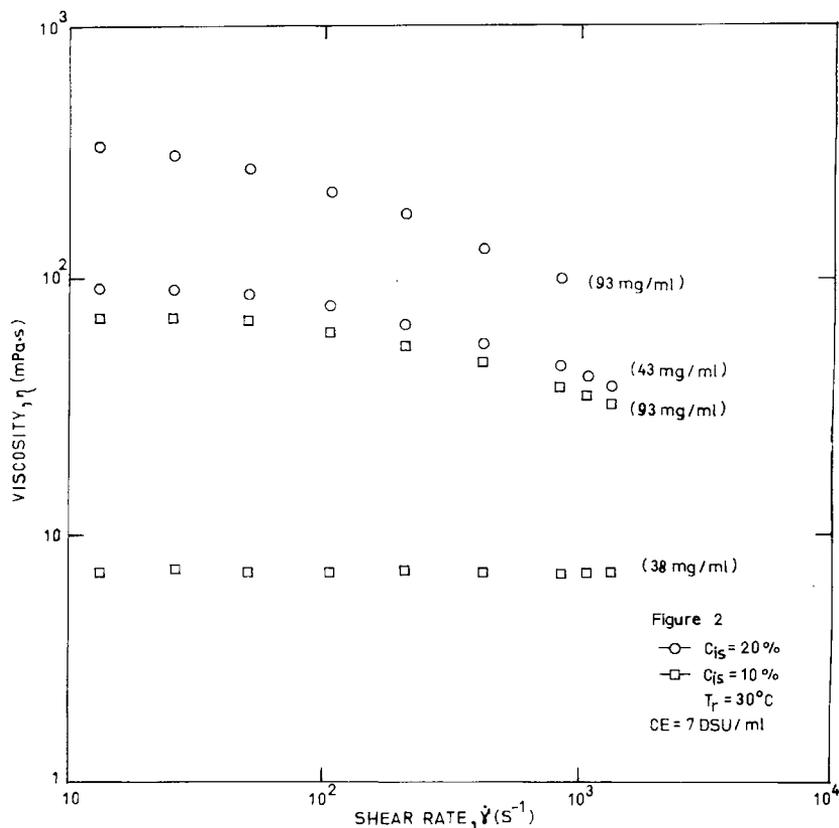
Fig. 2.   Viscosity versus shear rate (the numbers in parentheses refer to the polymer concentration in the medium).

The curve for $C_{is} = 20\%$, at the end of the synthesis, did not show the plateau clearly shown for $C_{is} = 10\%$. This behavior maybe associated with the existence of a wider MWD. Likewise, the difference in viscosity values between both curves may be related to the size of the polymer chains and to their interaction with the solvent, so that, for these polymer concentrations, perhaps the polymer-polymer and the polymer-solvent interactions are more significant. As will be discussed in the following section, for those reactions carried out at 4°C a similar pattern was observed, so that, independently of T_r, the polymers obtained using higher C_{is} exhibited a qualitative indication of broader MWD.

Effect of T_r on Rheological Behavior

The lower the temperature T_r, the lower is the reaction rate. It has been reported that lower reaction temperatures result in a lower yield of high-molecular-weight dextran and higher yields of low-molecular-weight dextran.[15,16]

Figures 4 and 5 show the viscosity behavior for $C_{is} = 20\%$ and $C_{is} = 10\%$ respectively, at 4 and 30°C. At the same C_d value, lower viscosity values were obtained at 4°C. Likewise, in this case the shear-thinning be-
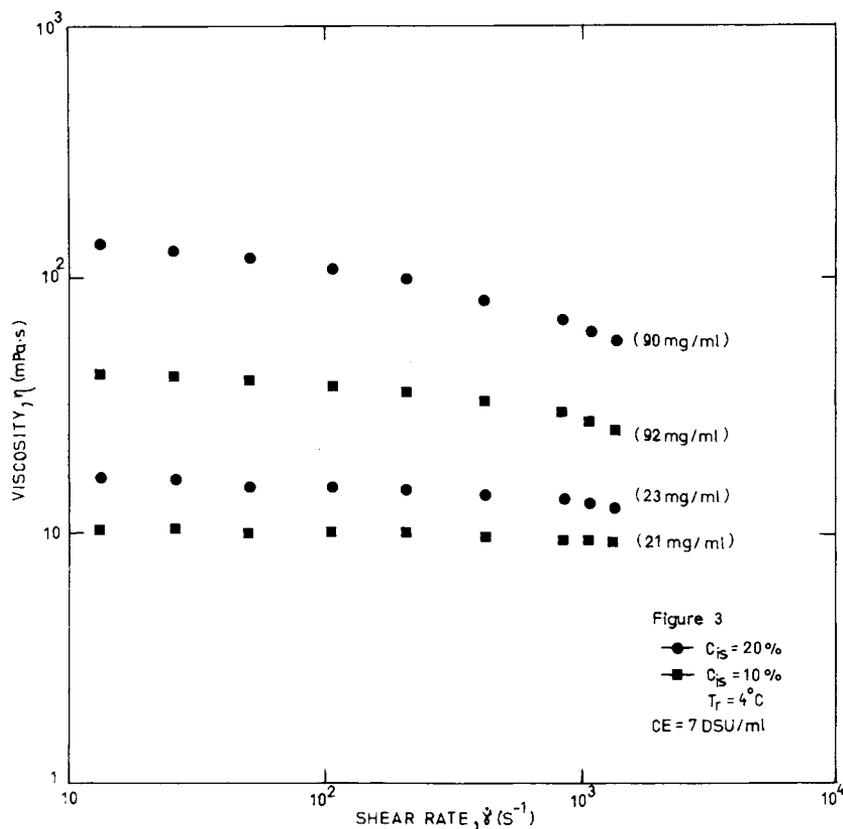
Fig. 3. Viscosity versus shear rate (the numbers in parentheses refer to the polymer concentration in the medium).

havior was less pronounced. The length of the newtonian plateau was greater than that for the synthesis at 30°C. The effect of T_r is clearly more significant than the effect of C_{is}. This can only be explained if we consider dextran synthesis as the result of two different types of reactions: on one hand, we have the growth of dextran chains always linked to the active site of the enzyme, and, on the other hand, the reaction between an acceptor molecule (mainly fructose and low MW dextran) and the dextranosyl-enzyme complex that stops the polymer chain growing process. The effect of temperature in the first process has already been quantified with an activation energy of 12.4 kcal/mol.[17] However, if the effect of temperature in the second reaction is lower, then the acceptor reaction would be carried out at a relatively higher rate; this would explain why under the same reaction conditions lower MW are obtained at lower temperatures.

As can be seen in Figs. 4 and 5 for the synthesis with $C_{is} = 20\%$ and $C_{is} = 10\%$ at 4°C, the media showed a slight shear-thinning behavior; for 30°C the decrease in viscosity was less gradual. It is possible to explain this difference in viscosity values by different $\overline{MW}$ rather than by differences in MWD; the $\overline{MW}$ for reactions at 30°C is probably higher than that at 4°C. The same behavior was observed for reactions with $C_{is} = 10\%$.
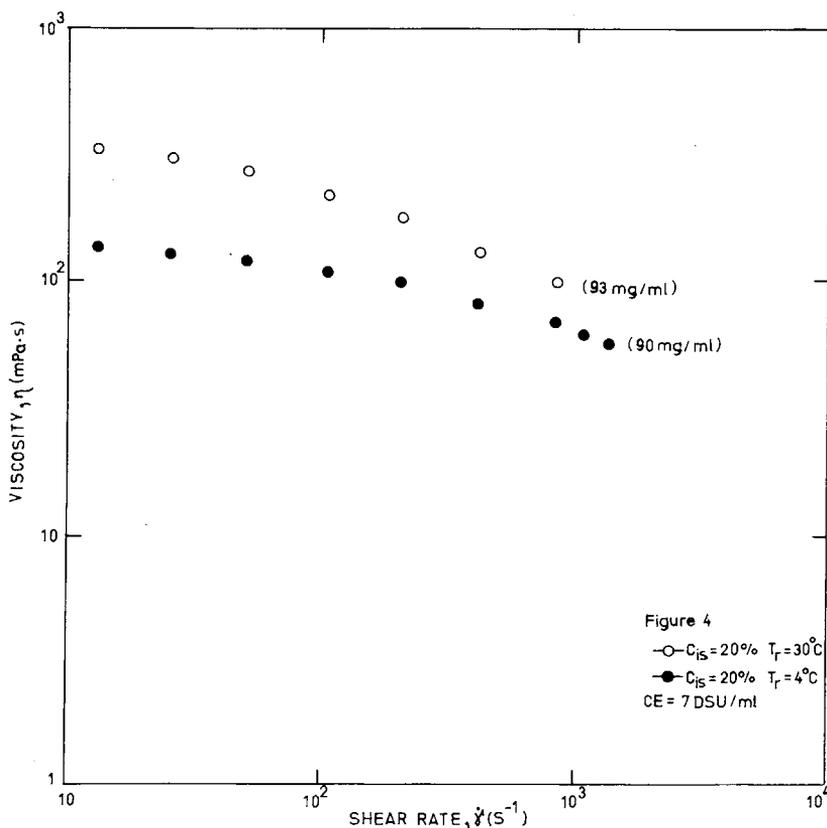
Fig. 4. Viscosity versus shear rate (the numbers in parentheses refer to the polymer concentration in the medium).

Effect of Maltose as an Alternate Acceptor

The addition of maltose to an enzyme-sucrose mixture deviates dextran synthesis towards short-chain polysaccharides, increasing the reaction rate at the same time.[15,16] Consequently, at the end of the reaction, the medium contains mainly oligosaccharides.

The presence of maltose in the synthesis medium has quite dramatic effects on rheological behavior. Figure 6 shows the viscosity function for this case. The result shows that the observed newtonian behavior is associated with the existence of short-chain–low MW molecules. Hence, it can be assumed that the newtonian plateau is closely related to the presence of short-chain molecules. On the other hand, as the reaction proceeded, newtonian behavior was maintained and only a difference in viscosity values with time was observed.

Effect of Enzyme Purity

The CE was eluted in a column packed with Bio-Gel in order to remove the low MW dextrans previously formed during enzyme production, which could act as acceptors. Because of the low enzymatic activity of the eluted enzyme EE, reaction time was almost three times larger than that for the
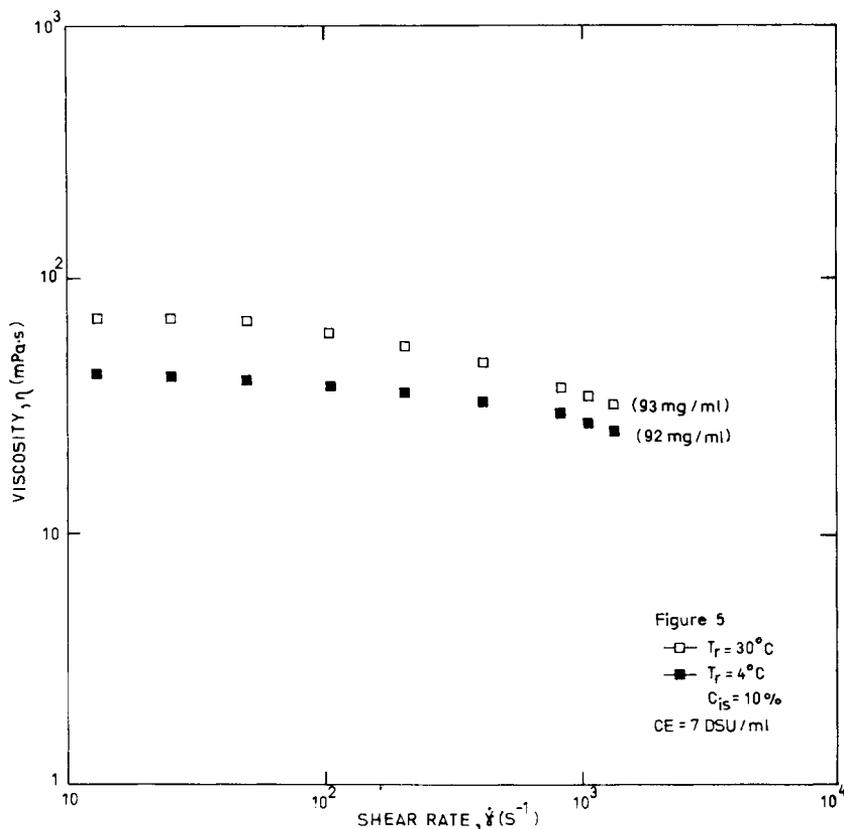
Fig. 5.   Viscosity versus shear rate (the numbers in parentheses refer to the polymer concentration in the medium).

CE under the same reaction conditions. Figures 7 and 8 show the effect of enzyme purification on the rheological behavior of the synthesis media with $C_{is} = 20\%$ and $C_{is} = 10\%$, respectively. In both cases T_r was 4°C. The rheological behavior of the synthesis media using the EE was quite different from those with CE under same C_{is} and T_r values. For example, when C_d was 6 mg/mL (Fig. 7) the medium with EE showed power-law behavior. As C_d increased (18.6 EE and 23.1 CE) the rheological behavior did not change for the medium with EE, but for that with CE, the viscosity function was characterized by two zones of viscosity variation. This difference becomes important if we consider that the C_d for the EE was 19% lower than the C_d for the CE. Figure 8 shows the same trend in rheological behavior when C_{is} was 10%. Higher viscosity values were obtained using the EE, and a very different behavior was observed. In any case, using the EE resulted in a stronger shear-thinning behavior (see Table II for flow behavior index n).

There seems to be a combined effect among reaction temperature, enzymatic activity, and enzyme purity that results in higher $\overline{MW}$ and probably narrower MWD than those obtained with the CE.

When dextran is separated from the enzyme, only fructose molecules remain as acceptors, so polymer can be formed from the very beginning of
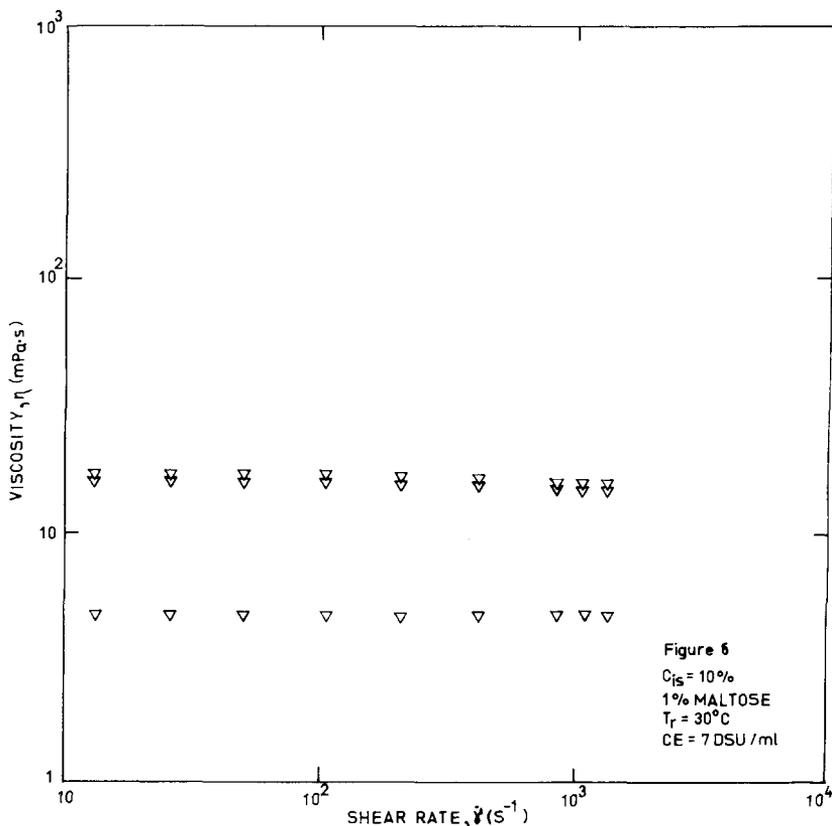
Fig. 6.   Viscosity versus shear rate (maltose as alternate acceptor).

the reaction and the effect of lowering the enzymatic activity seems to be associated with narrower MWD.

In order to establish a more accurate relationship between MWD and rheological behavior, dextrans were fractionated in a column packed with ultrogel A4 (500,000; 9×10^6). Nevertheless, the polymer was eluted in the void volume showing that the $\overline{MW}$ is well over 9×10^6. Techniques commonly used for $\overline{MW}$ determination are less accurate as the $\overline{MW}$ and sample polydispersity increase, and so it is necessary to find an adequate technique that allows a precise determination of these molecular properties.

Non-newtonian Behavior Modeling

The non-newtonian viscous behavior of those synthesis media in which the viscosity-shear rate curve showed two zones of viscosity variation was well represented by the Carreau equation[18]:

$$\frac{\eta - \eta_0}{\eta - \eta_{00}} = [1 + (\lambda \cdot \gamma)^2]^{-N} \quad 0 < N < 0.5$$

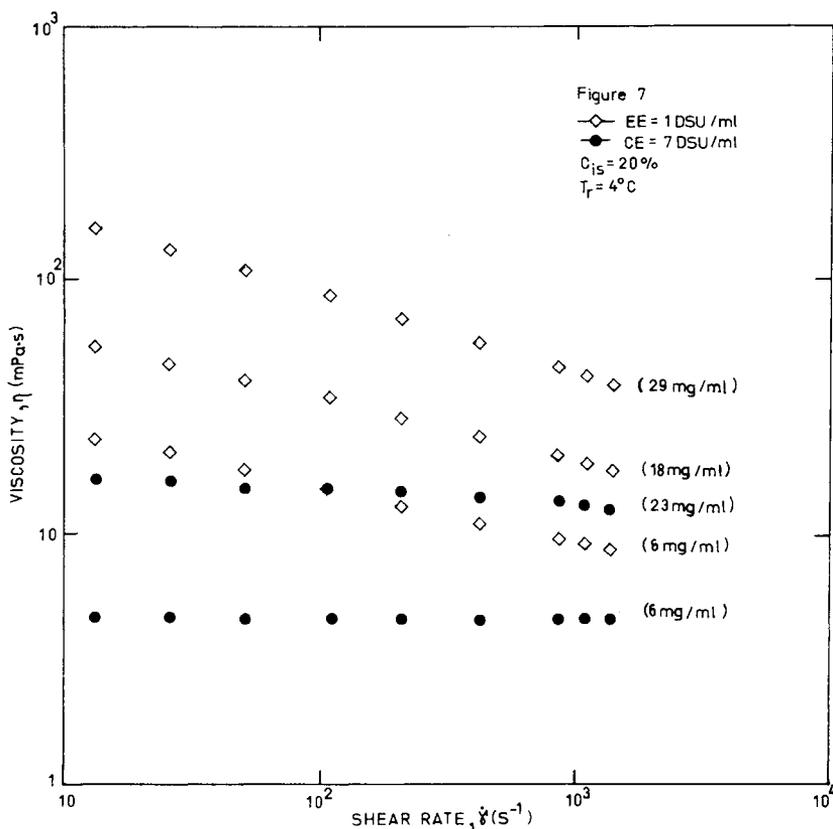where $N = (1 - n)/2$ and λ is a characteristic time.

Fig. 7. Viscosity versus shear rate. Comparison between crude and purified enzyme.

This semiempirical model was selected to fit non-newtonian behavior, because it takes into account the transition between the newtonian plateau and the power-law region.

In this equation N is related to the flow behavior index n of the power-law. On the other hand, λ is a characteristic time associated with the elastic response of the fluid. It is a semiquantitative parameter as it gives only an estimate of the real relaxation time of the material.

The power-law equation was used for those synthesis media that exhibited only this type of behavior. In Table II, the values of N, λ, n, and m are shown, as given by the Carreau and power-law equations.

Parameters N and λ were computed as described in the literature;[19] η_{∞} was taken as the viscosity of water, and η_0 was estimated by extrapolating to a shear value of $1\ s^{-1}$.

The data show that λ was in the range of 10^{-2} s. Taking this as an approximate criterion for establishing the elastic characteristics of the synthesis media, it can be concluded that medium with $C_d = 93$ mg/mL obtained at $C_{is} = 20\%$ and $T_r = 30°C$ was the one with higher elastic characteristics. Its N value (0.18) indicated that this medium was more non-newtonian.
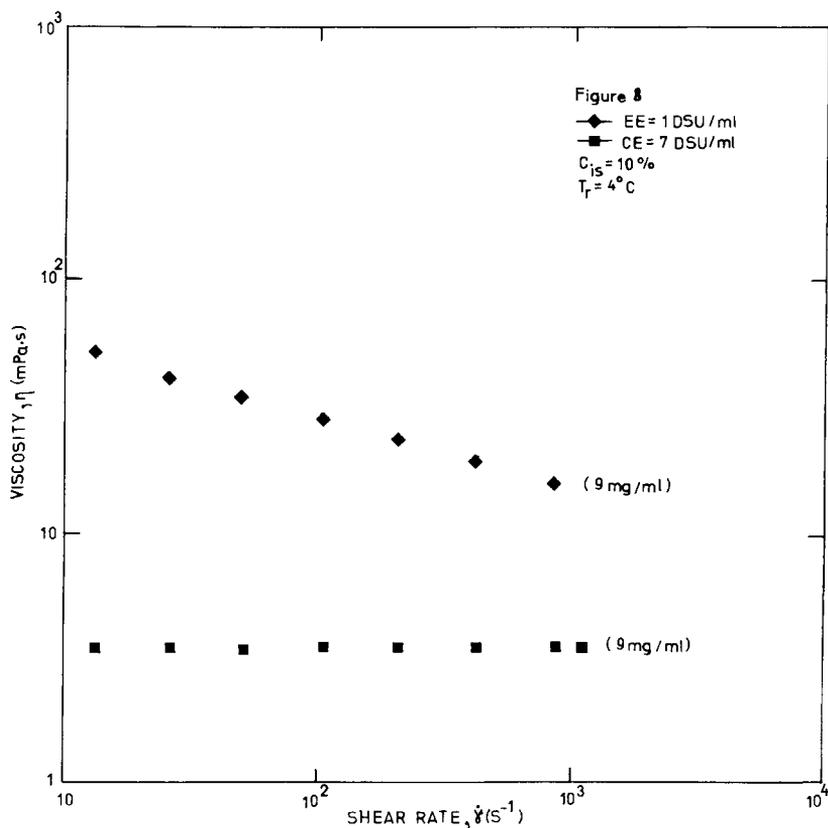
Fig. 8.   Viscosity versus shear rate. Comparison between crude and purified enzyme.

TABLE II
Carreau and Power Law Equation Parameters for Dextran Synthesis

C_{is} (%)	T_r (°C)	C_d (mg/mL)	N	λ (s)	η^a	m (Pa · s^n)
10[b]	4	8.8	0.0	—	1.0	0.00321
	4	21.3	0.0	—	0.98	0.0109
	4	92.8	0.07	0.022	0.86	0.0741
20[b]	4	6.0	0.0	—	1.0	0.00462
	4	23.0	0.05	0.015	0.90	0.0266
	4	90.0	0.14	0.14	0.72	0.4078
10[b]	30	38.0	0.0	—	1.0	0.00749
	30	93.7	0.13	0.012	0.74	0.200
20[b]	30	17.0	0.0	—	1.0	0.00563
	30	43.0	0.15	0.016	0.70	0.310
	30	93.0	0.18	0.038	0.64	1.09
20[c]	4	6.0	0.12	—	0.77	0.0427
	4	23.1	0.12	—	0.76	0.100
	4	90.0	0.14	—	0.73	0.273
10[c]	4	6.0	0.14	—	0.73	0.1014
	4	18.6	0.18	—	0.65	0.3834
	4	29.1	0.20	—	0.59	1.050

[a] In those cases where $n = 1$, m is in Pa·s.
[b] Reactions with crude enzyme.
[c] Reactions with purified enzyme.

These flow behavior characteristics are intimately related to polymer MWD. Nevertheless, the quantitative relation is far from being satisfactorily described, mainly in the case of polydisperse systems.

The existent theories relating the viscous behavior with $\overline{MW}$ for monodisperse systems, such as Ref. 20, are not valid when the sample is polydisperse. These monodisperse theories have been modified to take into account polymer polydispersity.[21] With these modifications it is possible to predict the shape of the viscosity function when the polymer $\overline{MW}$ is known. It is possible, in theory, to invert the above procedure and determine the $\overline{MW}$ when the viscosity versus shear rate curve is known. Unfortunately, these theories have been the object of very serious criticism.[22-24]

Furthermore, this procedure must be coupled with molecular weight experimental data in order to prove its validity. At present techniques to determine molecular weights are fairly elaborate, and even with its limitations the rheological characterization path appears to be a good alternative and should justify the need for further work.

Conclusions

During the enzymatic synthesis of dextran the rheological behavior of the synthesis media is strongly affected by changes in reaction conditions. Higher initial sucrose concentrations resulted in a accentuated non-newtonian behavior. independently of reaction temperature when using the CE.

During the first stages of dextran synthesis, the medium exhibits newtonian behavior until a certain polymer concentration is reached (C_d between 20 and 45 mg/mL). As the reaction proceeds the rheological behavior becomes non-newtonian and can be characterized by a power-law type of equation. Furthermore, rheological characterization has proven useful to establish similarities between the products obtained at different reaction stages. This result should be important in the industrial production environment. A decrease in reaction temperature gives as a result a newtonian response over the entire shear rate range studied, independently of reaction time.

Purification of DS and lower enzymatic activities affect the rheological response in such a way that power-law behavior was observed from the beginning of the reaction.

Changes in $\overline{MW}$ and MWD are strongly dependent on synthesis conditions and at the same time contribute to changes in rheological behavior. The relation between these factors is difficult and has yet to be determined.

References

1. G. H. Bixler, G. E. Hines, R. M. McGhee, and R. A. Shurter, *Ind. Eng. Chem.*, **45**, 692 (1953).
2. E. Knitting and J. E. Zagic, *Biotech. Bioeng.*, **14**, 379 (1972).
3. S. P. Rogovin, R. F. Anderson, and M. C. Cadmus, *Biotech. Bioeng.*, **1**, 51 (1961).
4. K. A. Burton, M. C. Cadmus, A. A. Lagoda, P. A. Sanford, and P. R. Watson, *Biotech. Bioeng.*, **18**, 1669 (1976).
5. N. Kosaric, J. E. Yu, and J. E. Zagic, *Biotech. Bioeng.*, **13**, 381 (1971).
6. Anonymous, *Mfg. Chem.*, **31**, 206 (1960).
7. A. R. Jeanes, J. E. Pittsley, and F. R. Senti, *J. Appl. Polym. Sci.*, **5**, 519 (1961).
8. R. A. Moraine and S. P. Rogovin, *Biotech. Bioeng.*, **13**, 381 (1971).

9. A. S. Szczesniack and E. H. Farkas, *J. Food Sci.,* **27**, 381 (1982).

10. M. Charles, in *Advan. Biochem. Eng.,* Vol. 8, T. K. Ghose, A. Fletcher, and N. Blake-brough., Eds., Springer-Verlag, Berlin, 1978, p. 1.

11. G. W. Pace and R. C. Righelato, in *Advan. Biochem. Eng.,* Vol. 15, A. Fletcher, Ed., Springer-Verlag, Berlin, 1980, p. 41.

12. P. Monsan and A. López, *Biotech. Bioeng.,* **23**, 2027 (1981).

13. A. R. Jeanes, *Methods in Carbohydrate Chemistry,* **5**, 127 (1965).

14. A. López and P. Monsan, *Biochimie,* **62**, 323 (1980).

15. J. F. Robyt, B. K. Kimble, and T. E. Walseth, *Arch. Biochem. Biophys.,* **165**, 634 (1974).

16. J. F. Robyt and H. Taniguchi, *Arch. Biochem. Biophys.,* **171**, 129 (1976).

17. M. T. Delgado and A. López, in *Biotecnología de Enzimas,* C. Huitrón, Ed., UNAM, México, 1983, p. 157.

18. R. B. Bird, R. C. Armstrong, and O. Hassager, *Dynamics of Polymeric Liquids,* Vol. 1, *Fluid Mechanics,* John Wiley & Sons, New York, 1977.

19. S. I. Abdel-Khalik, O. Hassager, and R. B. Bird, *Polym. Eng. Sci.,* **14**, 859 (1974).

20. F. Bueche, *J. Chem. Phys.,* **22**, 1570 (1954).

21. S. Middleman, *J. Appl. Polym. Sci.,* **11**, 417 (1967).

22. H. Yamakawa, *Modern Theory of Polymer Solutions,* Harper & Row, New York, 1971, p. 316.

23. A. Peterlin, *Adv. Macr. Chem.,* **1**, 252 (1968).

24. R. B. Bird, O. Hassager, R. C. Armstrong, and C. F. Curtiss, *Dynamics of Polymeric Liquids,* Vol. II, John Wiley & Sons, New York, 1977, p. 592.