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Ultrasonic depolymerization of an exopolysaccharide produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid

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Abstract:

Low frequency ultrasound was used to depolymerize a high-molecular-weight exopolysaccharide (EPS) produced by a deep-sea hydrothermal bacterium *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane. The influence of several parameters was examined including the duration of ultrasonic irradiation, EPS concentration, reaction temperature and volume of the sonicated solution. With the aim of optimizing the depolymerization, the native EPS was simultaneously treated with hydrogen peroxide and ultrasound. This study identified the sonication conditions that produce low-molecular-weight derivatives from the native EPS (>10^6 Da) with good reproducibility.

Keywords: Ultrasound; Depolymerization; Exopolysaccharide

1. Introduction

Since the discovery of the first deep-sea hydrothermal vent, many microorganisms of biological interest, especially those producing polysaccharides, have been isolated from such extreme environments (*Alteromonas sp* [1], *Alteromonas macleodii* subsp. *fijiensis* [2], *Vibrio diabolicus* [3], *A. infernus* [4] and *Pseudoalteromonas* sp [5]). The bacterium *Alteromonas macleodii* subsp. *fijiensis* biovar deepsane, collected during the "Hydronaut" trip [6], was shown to produce an EPS, designated HYD 657, of biological interest with applications in the manufacture of cosmetics as an active ingredient in the Deepsane ^{TM} operation. HYD 657 was selected for development in the cosmetic industry after screening tests proved its immunomodulatory action. Thanks to this biological activity, Deepsane ^{TM} enables the skin to optimize its immune defense system [7]. This EPS is composed of neutral sugars along with acidic sugars and an unusual sugar identified as a diacidic hexose.

Obtaining an EPS with a reduced molecular weight is the first step in improving its activity and its bioavailability. The depolymerization must be carefully controlled, as the chemical structure, in particular the presence of this unusual sugar, must be preserved.

Several methods can be used in order to depolymerize an EPS, such as acid hydrolysis, enzymatic depolymerization or free-radical depolymerization with metallic catalysts. For instance, extended acidic hydrolysis of an exopolysaccharide was shown [8] to cause severe depolymerization without preserving the monosaccharide ratios. On the other hand, enzymatic depolymerization requires a good knowledge of the structure of the EPS and makes each EPS an individual case, involving long time-consuming experiments. Free-radical depolymerization with metallic catalysts, which requires different stages of purification, is under investigation and will be reported elsewhere.

The effect of ultrasound on the depolymerization of polysaccharides is well described. In most cases, the ultrasonic treatment has been performed either in acidic solutions at relatively low temperature [9] or in aqueous alkaline media [10] or in the presence of sodium hypochlorite [11]. Ultrasonic irradiation has been used to depolymerize various biopolymers, including dextran, DNA, and starch, without modification of their chemical structure [12]. The current opinion is that low-frequency cavitation generates hydrodynamic shear forces that are able to split macromolecules mid-chain in a non-random way [13]. It is well established that the rate of depolymerization induced by ultrasonic irradiation depends on the viscosity of the solution. The best conditions must be found for EPS having a very high average molecular weight. Thus, the influence of several parameters was examined: duration of ultrasonic irradiation, EPS concentration, reaction temperature and volume of the sonicated solution. Sonication was also tested simultaneously in the presence of hydrogen peroxide with the aim of optimizing the depolymerization.

2. Experimental

2.1 Production and characterization of native EPS

The isolation procedure and chemical characteristics of the HYD 657 strain have previously been reported [14]. Optimal growth occurs between 30 and 35°C, at a pH between 6.5 and 7.5 and at ionic strengths between 20 and 40 $g.l^{-1}$ NaCl. HYD 657 is composed of neutral sugars (glucose, galactose, rhamnose, fucose and mannose) along with acidic sugars (glucuronic and galacturonic acids) and an unusual sugar, identified by GC and GC-mass

spectrometry analyses as a 3-0-(1 carboxyethyl)-D-glucuronic acid. This polysaccharide, like most EPS, presents a high molecular weight (>10^6 Dalton).

2.2 Ultrasonic treatment

For volumes lower than 200 ml, sonolysis was carried out using a 20 kHz ultrasound generator (Vibracell VCX 600). 10 ml experiments were performed with an ultrahigh intensity microprobe (amplitude 228µm, 100W) in a flask with a conical bottom. 200 ml experiments were performed with a high intensity probe (124µm, 250W) in a home-made glass flow vessel.

For larger volumes, experiments were carried out using a specific set-up (Figure 1). This was composed of a home-made sonochemical reactor operating in the low frequency range and included in a circulation loop. The temperature of the solution was kept constant by using a double-jacketed vessel.

2.3 Ultrasonic treatment and H_2O_2

Twenty milligrams of EPS was dissolved in 5 ml of water prior to ultrasonic irradiation. When the temperature of the polymer solution reached 60°C, a 0.24 % (w/w) hydrogen peroxide solution was added (0.05 ml/min for 100 min). The reaction was stopped after two hours of ultrasonication. The same experiment was also performed with a 0.48% (w/w) hydrogen peroxide solution.

These experiments were repeated with 400 mg of EPS dissolved in 150 ml. When the temperature of the polymer solution reached 60°C, a 0.24 % or 0.48% (w/w) hydrogen peroxide solution was added (0.5 ml/min during 100 min). The reaction was stopped after three hours of sonication.

2.4 Molecular weight determination

Each fraction (2 mg/ml) was analyzed by high-performance size-exclusion chromatography (HPSEC) in 0.1 M ammonium acetate at a flow rate of 0.5 ml/min using a Superdex 200 (Amersham Pharmacia Biotech) column. Calibration was performed with pullulan standards, which are neutral glucans, whereas our EPS is a charged polysaccharide, so that such calibration does not allow exact measurement of the polysaccharide molecular weight. Area measurements and calculations of M_p (peak-average molecular mass), M_w (weight-average molecular mass), M_n (number-average molecular mass) and I (polydispersity) were carried out using Aramis software (JMBS Développements, Le Fontanil, France).

2.5 Composition of EPS

The molar ratio of polysaccharides was determined according to Kamerling et al [15] and Montreuil et al [16]. The monosaccharide residues were analyzed after acidic methanolysis of the polymer and subsequent GC analyses as trimethylsilyl derivatives.

2.6 Viscosity determination

Viscosity was determined by a rotational viscometer Visco Tester GL/R (Haake); the result is a direct reading of the viscosity value in mPas. For larger scale experiments, viscosity was estimated by the measurement of the flow rate in a 10 ml graduated pipette.

3. Results and discussion

3.1 Effect of ultrasonication time

Figure 2 shows the effect of ultrasonication time, between 15 min and 4 h, on the changes in molecular weight of 10 ml of a 2.5 mg/ml aqueous solution of EPS at 60°C.
The results indicate that ultrasonic degradation of the 2.5 mg/ml EPS solution is most effective in the early minutes of treatment: after only 15 min, the weight-average molecular mass is divided by 3.5. The degradation rate approaches an asymptote during the remainder of the treatment time. These results are consistent with the existence of a limiting value of molecular weight, which would be for HYD 657, in these conditions, about 120,000 g/mol after ten hours of irradiation. At the end of the ultrasonic treatment, the viscosity is close to that of water. The best compromise between the duration of sonication and the reduction of the molar mass is found after 3 h.

Table 1 shows the constant composition of the EPS. Even after ten hours of irradiation, there is no alteration.

The depolymerization was found to decrease with increasing concentration. The higher the viscosity is, the more difficult it becomes to cavitate the solution and therefore the degradation effect is lower. Viscosity is dependent on the concentration of the polymer.

3.2 Effect of EPS concentration

Bacterial polysaccharides are currently used in many industrial domains for their rheological properties in aqueous media. The effect of solution concentration on degradation has been investigated by several authors [17]. First, a 2 mg/ml aqueous solution was chosen in order to compare several methods of depolymerization. This concentration is low because of the high viscosity of HYD 657 in aqueous solution (Fig. 3).
The temperature of the ultrasonic reactor was adjusted to 60°C during sonication. At the end of the treatment, the M_w of the lower-molecular-weight EPS was 200,000 g/mol with a polydispersity of 2.
In the same conditions, for a 3 ml/mg aqueous solution, three hours was necessary to obtain the same molecular mass.
For the same concentration, temperature influences the viscosity of the solution (Fig. 3).

3.3 Effect of temperature

Several authors [18] have investigated the effect of temperature on depolymerization. In each case, the extent of the depolymerization has been found to decrease with an increase in temperature. Thus, considering the possibility of industrial development, and especially the expense of large-scale reactor cooling, we checked the influence of increasing temperature on the degradation process by ultrasonics. In our case, no change in the depolymerization process was observed in the studied temperature range. This could be the consequence of a compensation effect, due to the increase in viscosity with temperature.

3.4 Reproducibility of ultrasonic degradation

The reproducibility of the ultrasonic depolymerization of HYD 657 was studied for 10 ml of the solution with a 2 mg/ml concentration for 180 min at 60°C. The same experiment was carried out five times with the same production batch of the EPS.

The reproducibility was investigated with batches of different initial relative molecular mass, whereas the concentration of EPS was 2 mg/ml and the sonication lasted for 3 hours. The studies gave similar results, with M_w about 200,000 g/mol and I_p about 2. The existence of a limiting molecular mass allowed us to standardize the different batches with a similar molecular weight after depolymerization.
Several months after ultrasonic treatment, viscosity values were the same demonstrating that ultrasonic irradiation produced a permanent reduction in viscosity.

3.5 Effect of H_2O_2 addition

The first experiments showed a large decrease in viscosity to a limiting value. The combination of sonication with other methods of depolymerization may help overcome this limitation. Moreover, ultrasonic treatment could induce other mechanisms of decomposition.
The sonolysis of water is known to produce hydroxyl radicals and hydrogen atoms. This reaction can be followed by the formation of hydrogen peroxide. The frequency dependence of this cleavage is known and the efficiency appears to be improved by increasing the frequency [16].

The hydroxyl radical is most commonly generated through metal-catalyzed reactions of the Haber-Weiss type or more directly through a Fenton-type reaction [17]. These kinds of reaction are used in metal-catalyzed depolymerization.
Although, in the low frequency domain, mechanical effects usually predominate over free radical production, we tried to increase the production of $OH^{\cdot}$ by adding H_2O_2.
Thus, ideally, each H_2O_2 could produce two hydroxyl radicals and increase radical depolymerization by ultrasonic treatment. Thanks to this mechanism, mechanical and radical depolymerization could be combined. The final hydrogen peroxide excess can be eliminated by catalase.
The results show (Table 2) that hydrogen peroxide and ultrasonic irradiation in combination favored depolymerization: M_w was halved compared to ultrasonic treatment alone for the 0.24 % (w/w) H_2O_2 solution. Thus, the influence of ultrasound can be increased by the presence of H_2O_2.
However, when different amounts of hydrogen peroxide were added, it was found that a more concentrated solution of H_2O_2 does not increase depolymerization.
The possibility of the existence of traces of metals in our EPS cannot be dismissed. These could act as a catalyst for the Fenton reaction. The H_2O_2 produced during ultrasonication was very low and hence there was no Fenton reaction in the absence of H_2O_2 in addition to ultrasonic depolymerization.

3.6 First step to the scale-up of ultrasonic depolymerization

In order to carry out this project on an industrial scale, it was important to check the feasibility and the transposability of our first results.
The influence of the volume of the HYD 657 solution used for ultrasonic depolymerization was investigated with volumes of 10 ml, 200 ml and 9 liters, whereas the concentration of EPS was 2 mg/ml.
Due to the cost of the raw material, the first manipulation on a larger scale (9L) was performed in the best conditions determined for a smaller volume, even though the apparatus was not the same. For a large volume, the main problems encountered are the power required, and the efficacy and homogeneity of the solution. With a pseudo-batch operation and a loop circulation of the solution, the efficacy is better than with a batch but the processing time is longer.

During manipulation, the viscosity was estimated by the measurement of the time required for the flow of the solution in a 10 ml precision pipette. The experimental results were then expressed as the ratio of the time measured for the polymer sample solution over the time measured for water in the same conditions. The corresponding experimental results are given in Table 2. As can be seen, the ratio decreases and tends towards 1. It can therefore be considered that the viscosity of the solution is very close to that of water.
The composition was also determined (Table 1) and was found unaffected by the fifteen hours of exposure to ultrasound.
Sonication was most effective for small volumes, for which M_w was divided by 20 when the limiting value was reached. With the biggest volume (9 liters), sonication was the least effective; M_w was only divided by 7.5. However, in this manipulation the material was different. Looking at the initial molecular mass of HYD 657, the results for depolymerization by ultrasonic irradiation are good even for higher volumes. Moreover, it is important to emphasize that there was no optimization. Studies are currently underway to improve these first experimental conditions.

4. Conclusions

The aim of this research was to find the appropriate conditions for sonication to produce, with good reproducibility, our EPS with a reduced molecular weight. This work is a first step in a successful scale-up to an industrial application to obtain EPS derivatives with an average molecular weight comprised between 100 and 300 kDa. Additional studies are underway to improve these first results on an industrial scale. Furthermore, these results will be compared with others obtained by different methods of depolymerization currently under investigation.

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References

[1] P. Vincent, P. Pignet, F. Talmont, L. Bozzi, B. Fournet, J. Guezennec, C. Jeanthon, D. Prieur, Appl. Environ. Microbiol. 60 (1994) 4134.
[2] G. Raguénès, P. Pignet, G. Gauthier, A. Peres, H. Rougeaux, R. Christen, G. Barbier, J. Guezennec, Appl. Environ. Microbiol. 62 (1996) 67.
[3] G. Raguénès, R. Christen, J. Guezennec, P. Pignet, G. Barbier, Int. J. Syst. Bacteriol. 47 (1997) 989.
[4] G. Raguénès, A. Peres, R. Ruimy, P. Pignet, R. Christen, M. Loaec, H. Rougeaux, G. Barbier, J. Guezennec, J. Appl. Microbiol. 82 (1997) 422.
[5] H. Rougeaux, J. Guezennec, R.W. Carlson, N. Kervarec, R. Pichon, P. Talaga, Carbohydr. Res. 315 (1999) 273.
[6] J. Guezennec, P. Pignet, G. Raguenes, E. Deslandes, Y. Lijour, E. Gentric, Carbohydr. Polym. 24 (1994) 287.
[7] A.M. Vacher, M.C. Fritsch, US-Pat 2002187167, 2002.
[8] J. Guezennec, P. Pignet, Y. Lijour, E. Gentric, J. Ratiskol, S. Colliec-Jouault Carbohydr. Polym. 37 (1998) 19.
[9] R.H. Chen, J.R Chang, J.H. Shyur, Carbohydr. Res. 299 (1997) 287.
[10] D. Mislovičová, J. Masárová, K. Bendžálová, L. Šoltés, E. Machová, Ultrason. Sonochem. 7 (2000) 63.
[11] L. Callegaro, D. Renier, US-Pat 6020484, 2000.
[12] S.C. Szu, G. Zon, R. Schneerson, J.B. Robbins, Carbohydr. Res. 152 (1986) 7.
[13] G.J. Price, T.J. Mason, Advances in Sonochemistry, The use of ultrasound for the controlled degradation of polymer solutions, vol. 1, JAI Press Limited, London, 1990.
[14] M.A. Cambon-Bonavita, G. Raguenes, J. Jean, P. Vincent, J. Guezennec, J. Appl. Microbiol. 93 (2002) 310.
[15] J.P. Kamerling, G.J. Gerwig, J.F.G. Vliegenhart, J.R. Clamp, Biochem. J. 151 (1975) 491.
[16] J. Montreuil, S. Bouquelet, H. Debray, B. Fournet, G. Spik, G. Strecker, Carbohydrate Analysis: A practical approach, Glycoproteins, IRL Press Oxford, Washington, 1986.
[17] T.J. Mason, J.P. Lorimer, Applied Sonochemistry, Wiley VCH, 2002.
[18] T.J. Mason, J.P. Lorimer, Sonochemistry: Theory applications and uses of ultrasound in chemistry, Ellis Horwood, Chichester, 1988.
[19] C. Pétrier, A. Jeunet, J.L. Luche, G. Reverdy, J. Am. Chem. Soc. 114 (1992) 3148.
[20] A. Singh, Can. J. Physiol. Pharmacol. 60 (1982) 1330.

Table 1 : Monosaccharide ratios of HYD 657, native and depolymerized by ultrasonication on a small and a larger scale

Rha, rhamnose; Fuc, fucose; GlcUA, glucuronic acid; GalUA, galacturonic acid; Gal, galactose; Glc, glucose; Man, mannose.

EPS	Rha*	Fuc	GlcUA	GalUA	Gal	Glc	Man
Native	3.4	1.8	6.1	7.3	21.7	12.3	1.8
10 ml, 10 h ultrasonication	3.2	1.9	5.5	7.5	22.2	12	1.7
9l, 15 h ultrasonication	3.1	1.8	5.3	7.7	24.5	12.8	1.8

Table 2 : Molecular weight determination for combinations of ultrasound and H_2O_2 addition.

EPS		M_w (g/mol)	M_n (g/mol)	M_p (g/mol)	I_p
Native		3 010 020	762 150	2 355 590	3.95
20 mg EPS	2h ultrasonication	204 500	101 700	201 400	2
	2h ultrasonication, 0.24 % H_2O_2	112 700	36 800	102 400	3
	2h ultrasonication, 0.48 % H_2O_2	127 700	53 500	115 300	2.4
400 mg EPS	3h ultrasonication	239 140	112 140	214 680	2.1
	3h ultrasonication, 0.24 % H_2O_2	118 770	41 220	91 860	2.9
	3h ultrasonication, 0.48 % H_2O_2	145 650	43 530	118 160	3.3

Table 3 : Ratio of the time required for the solution to flow from a graduated 10 mL pipette over the time required for water at the same temperature

Sonication time (h)	***3***	***6***	***9***	***12***	***15***
$\Delta t_{solution}/\Delta t_{water}$	***1.17***	***1.12***	***1.095***	***1.075***	***1.06***

Fig. 1. Specific set-up used for larger volumes.

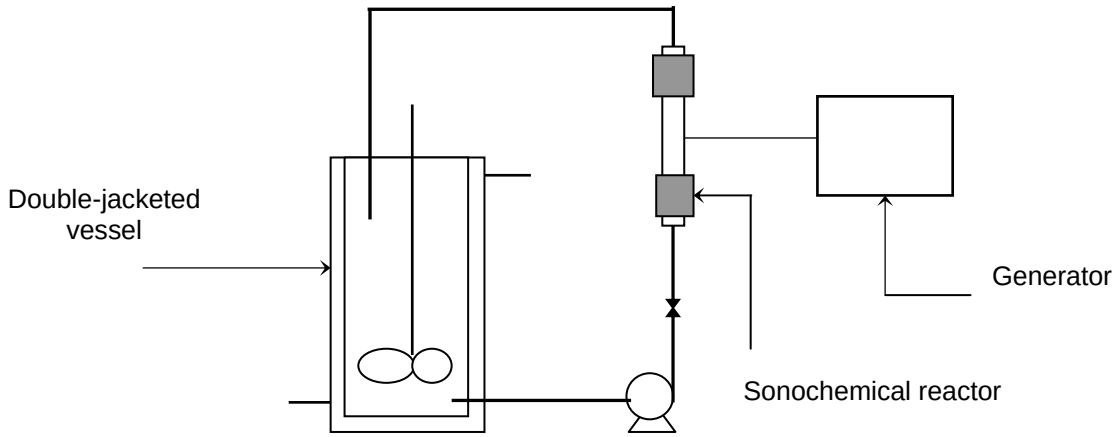
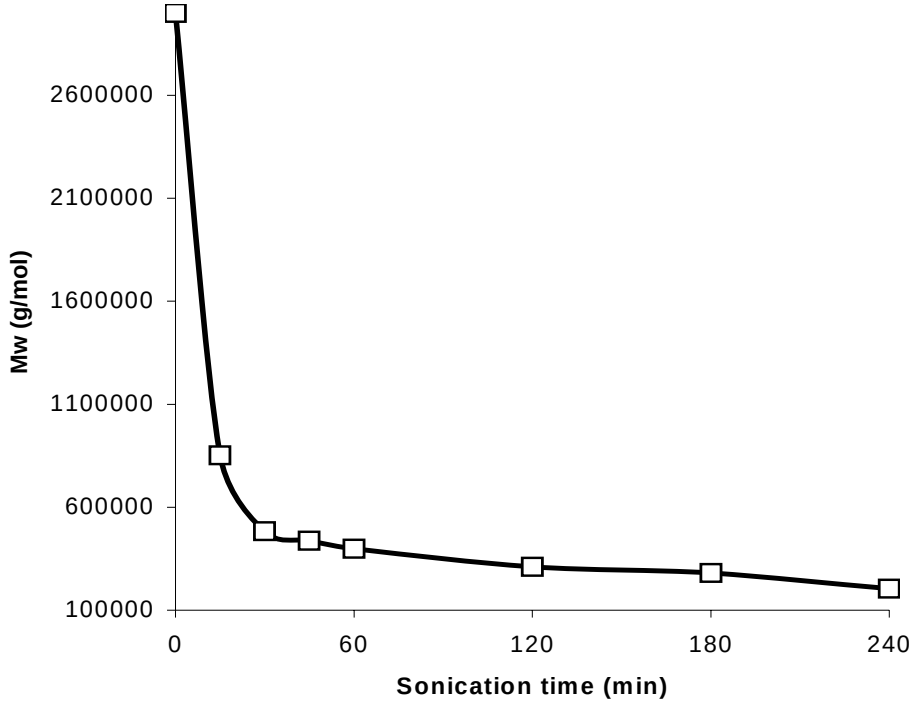


Fig. 2. The M_w of HYD 657 as a function of sonication time.



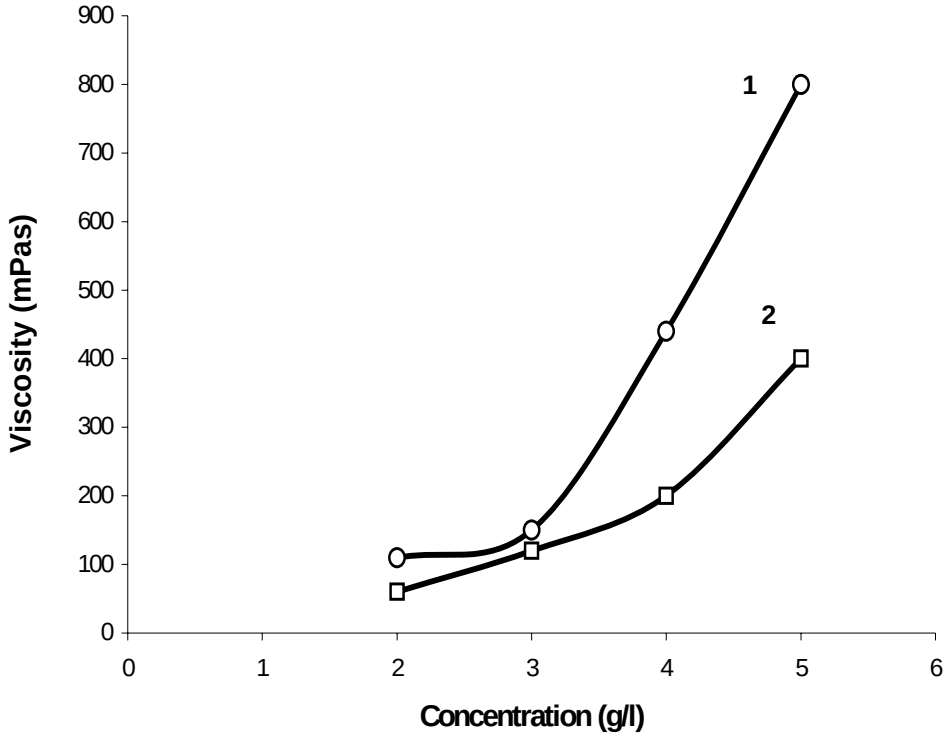


Fig. 3. Evolution of viscosity with concentration at two temperatures: (1) 30°C (2) 60°C.

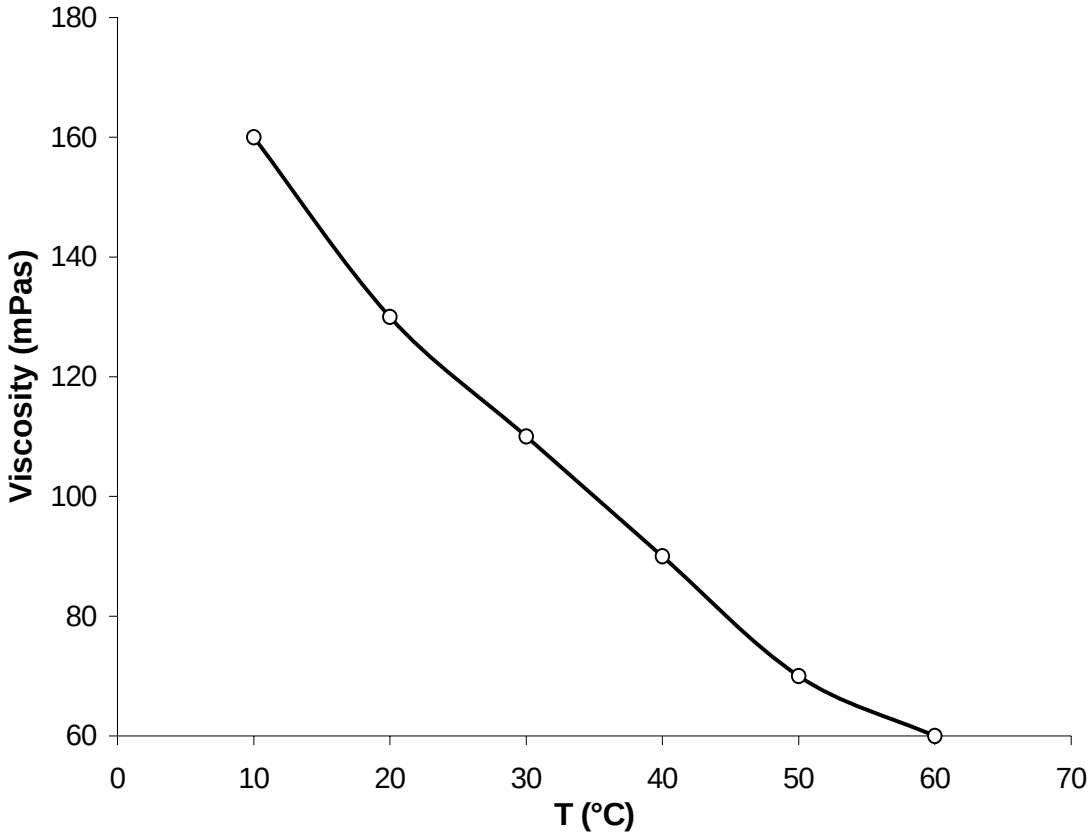


Fig. 4. Evolution of viscosity with temperature for a 2 g/l HYD 657 solution.