Rheological and Other Physical Characteristics of Polysaccharides from Two Black Yeast-like Fungi

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Synopsis

Two black yeast polysaccharides show similar non-Newtonian behavior when the effects of polysaccharide concentration, shear rate, pH, salt, and temperature on the viscosity of dispersions are examined. Both polysaccharides readily form viscous aqueous dispersions and can be cast into flexible films, and both stabilize oil–water emulsions. Each of these two polysaccharides occurs extracellularly in the culture fluids of two different black yeast-like fungi—*Rhinocladiella elatior* Mangenot (strain NRRL YB-4163) and *Rhinocladiella mansonii* (Castellani) Schol-Schwarz (strain NRRL Y-6272). This report appears to be the first on the rheology of an extracellular polysaccharide which contains the unusual sugar N-acetyl-D-glucosaminuronic acid.

INTRODUCTION

In our search for new types of microbial polysaccharides, we have found several black yeast-like fungi that produce extracellular polysaccharides composed of amino sugars. Two of these strains have been identified[1] as *Rhinocladiella elatior* Mangenot[2] (NRRL YB-4163) and *Rhinocladiella mansonii* (Castellani) Schol-Schwarz[2] (NRRL Y-6272). Both strains failed to elicit pathogenicity.[3] Polysaccharide (PS) Y-6272 and PS YB-4163 both appear to have industrial potential since they disperse readily in water to give extremely viscous solutions and since both contain acidic (carboxyl) and masked basic groups (N-acetyl). Whereas the polysaccharide from NRRL Y-6272 is composed[4] of 1 part N-acetyl-D-glucosaminuronic acid (GlcNAcUA) to 2 parts N-acetyl-D-glucosamine, (Glc NAc), PS YB-4163[5] is composed almost entirely of GlcNAcUA. In this work, various rheological and physical characteristics of these two fungal polysaccharides are examined.

EXPERIMENTAL

Production and Laboratory Purification of Polysaccharides

Polysaccharides were produced by culturing *Rhinocladiella elatior* NRRL YB-4163 and *Rhinocladiella mansonii* NRRL Y-6272 on a three-part medium[6] containing D-glucose (5%), K_2HPO_4 (0.5%), $MgSO_4$ (0.1%), $ZnSO_4$ (200 µg/75 ml whole medium), L-asparagine (0.75%), and yeast autolyzate (0.37%) at 25°C. Cultures were harvested at maximum viscosity—YB-4163 at seven days (3500–5000 cps) and Y-6272 at four days (4000–6000 cps). The extracellular polysaccharide from both black yeasts was purified by removal of cells from di-

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luted broth (1 part broth to 3 or 4 parts water) by centrifugation (35,000× g, 30 min) and by precipitating the cell-free material with ethanol (95%, 2 vol) in the presence of KCl (1%). Contaminating neutral material was removed by precipitating the acidic polysaccharides with hexadecyltrimethylammonium bromide and recovering the acidic polysaccharide as its potassium salt in the usual fashion.[6] The yield (based on glucose converted to polysaccharide) of purified material in the potassium salt form was 10% for PS YB-4163 and 16% for PS Y-6272.

In some experiments, two PS YB-4163 samples of differing behavior were compared. Although both samples were cultured and isolated under apparently identical conditions, sample A may be of higher molecular weight since with some cultures prolonged contact of the extracellular polysaccharide with microbial cells leads to lowered culture viscosities because of enzymatic depolymerization.

Viscosity

Viscosity measurements were made with a cone–plate microviscometer (Wells-Brookfield, Model RVT, 4.7 mm diam and 1.565° cone angle) at 25°C and 1 rpm unless otherwise indicated. Dispersions for viscosity–concentration curves were prepared by volumetric serial dilution although the same results were obtained from individually prepared dispersions. Salt effects were observed by incremental addition of small amounts of solid salt to homogeneous, completely dispersed solutions of the polysaccharide. Readings usually were made after ten revolutions or when the values had become constant.

The spring relaxation technique of Patton[7] for obtaining viscosity profiles in the ultralow shear-rate range utilized the RVT cone–plate viscometer as described previously.[8] A cone–plate rheometer (Rheometrics Mechanical Spectrometer, Union, N.J., described by Macosko and Starita[9]) was used with a cone (72 mm in diameter and angle of 0.04 radians) to measure primary normal stress difference ($P_{11} - P_{22}$) at 73°F. The shear stress–shear rate values obtained with the Mechanical Spectrometer agreed well with those obtained from the Brookfield cone–plate viscometer.

A Wells-Brookfield viscometer (Model LVT) fitted with a No. 4 spindle was used to measure viscosities (30 rpm) over the temperature range of 2–95°C (above 95°C bubbles appear which lead to erratic readings). Polysaccharide dispersions were heated in a 8-mm (inside diameter) tube in which a thermocouple was placed to measure temperature.

Whereas the repeatability of viscosity measurements was good (±5%) for various aliquots of a particular polysaccharide dispersion, occasionally differences in viscosity measurements (sometimes as high as ±10%) were noted for dispersions made up at different times. These differences are attributed to small variations in concentration, age of dispersion and sample, and amount of microbial pigment[6] present.

Water–Oil Emulsions

The polysaccharides were dispersed in water containing 0.2% benzoic acid as a preservative. Aliquots were emulsified with various oils using a Virus 45 ho-

mogenizer at a control setting of 60 for 4 min. The resulting emulsions were stored in screw-cap glass vials at room temperature and observed visually.

Films

The polysaccharides were dispersed in water usually at 0.5%–1% concentration. The viscous but bubble-free solutions were poured on Plexiglas plates and allowed to air dry.[10] The resulting films were stripped from the plates and equilibrated at 20°C and 50% relative humidity, and physical tests were run under the same conditions. Tensile strength and elongation were both determined with a Scott IP2 incline plane serigraph having at zero load and a 40-mm distance between jaws. Double folds were measured by use of a Schopper double-fold machine in which the test strip is given 120 double folds per min while under a constant tension of 1 kg.

RESULTS AND DISCUSSIONS

General Properties

Gum Form. Both PS YB-4163 and PS Y-6272, when precipitated from solution with ethanol or hexadecyltrimethylammonium bromide, wind on the stirrer as a partially dehydrated, fibrous, cohesive material.

Solid Form. As now prepared on a laboratory scale, the freeze-dried solids vary from pure white to dark grey, depending on how the samples were isolated.[8] When equilibrated under conditions of 50% relative humidity and 20°C, both freeze-dried PS YB-4163 and PS Y-6272 contain 18%–20% moisture.

Aqueous Solutions. The specific optical rotation of PS YB-4163 and PS Y-6272 is $[\alpha]_D^{25}$ −75°C (c, 0.5, H_2O) and $[\alpha]_D^{25}$ −6°C (c, 1.0, H_2O), and suggests
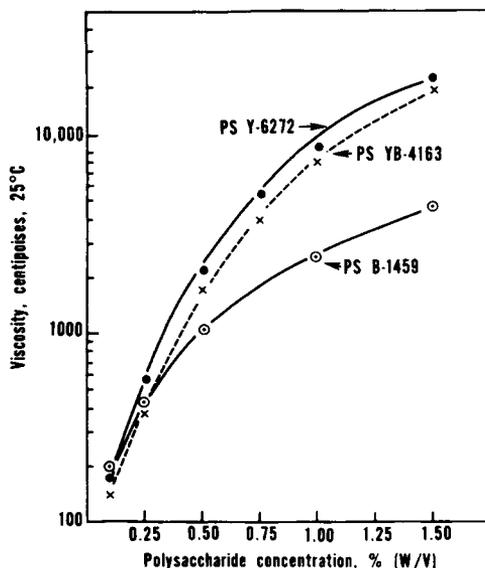

Fig. 1. Viscosity vs polysaccharide concentration. Viscosity of aqueous dispersions of the potassium salt form of PS YB-4163, PS Y-6272, and PS B-1459 (xanthan gum, cell free) was measured at 1 rpm (3.84 sec^{-1}) and 25°C.

that PS Y-6272 must contain mostly β-linkages while PS YB-4163 must contain a mixture of α- and β-linkages. Their neutral equivalent weights are 255 (PS Y-6272) and 622 (PS YB-4163), and are consistent with the compositional data which indicate PS Y-6272 is made up of 1 part GlcNAcUA and 2 parts GlcNAc while PS YB-4163 is composed entirely of GlcNAcUA. Both polysaccharides are assumed to be linear rod-like molecules, but there is no evidence to support this notion. Solutions (e.g., 1%) of both polysaccharides are extremely viscous. PS YB-4163 displays elastic behavior; that is, the entire solution flows together when poured from a container. This property is often lost with continued "work-up" of the polysaccharide.
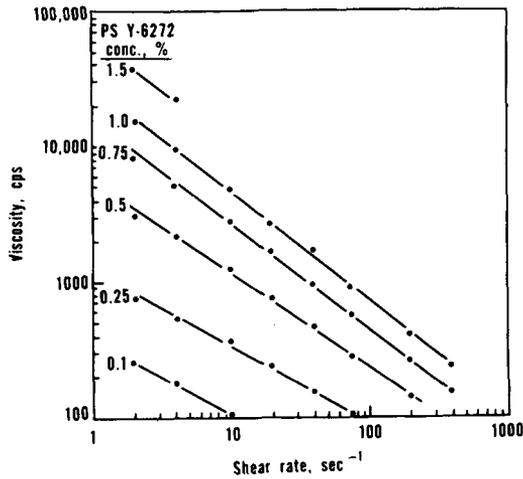
Fig. 2. Viscosity of PS Y-6272 (potassium salt form) dispersions vs shear rate in sec^{-1} at various polysaccharide concentrations (w/v). Measurements made at 25°C.
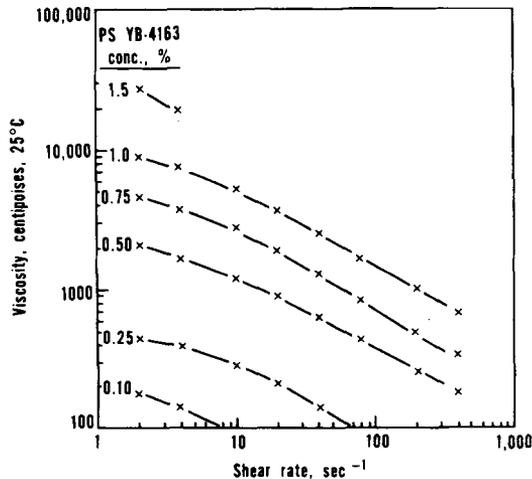
Fig. 3. Viscosity of PS YB-4163 (potassium salt form) dispersions vs shear rate in sec^{-1} at various polysaccharide concentrations (w/v). Measurements made at 25°C.

Factors Affecting Viscosity

As will be seen in various figures, the rheological behavior of PS YB-4163 and Y-6272 are quite similar.

Effect of Polysaccharide Concentration. As shown in Figure 1, both PS YB-4163 and PS Y-6272 produce very viscous solutions and even at low concentrations compare favorably with xanthan gum, a commercially used microbial polysaccharide.[11] Between 0.5% and 1.5% polysaccharide concentration, 1.5% level, both polysaccharide dispersions are gel-like and are generally not uniformly dispersed.

Effect of Shear Rate. The effect of shear on the viscosity of various concentrations of PS YB-4163 and Y-6272 is shown in Figures 2 and 3. As can be seen in these two figures, both polysaccharides are shear thinning at all concentrations of polysaccharides tried.

Neither polysaccharide shows thixotropic behavior since neither shows a time dependence in viscosity determinations. Viscosities measured at low shear rates were independent of the shear history before the measurement. Although the curves in Figures 2 and 3 are similar in appearance, particularly at high shear rates, the slopes of the viscosity–shear rate lines for PS Y-6272 (Fig. 2) are steeper than those for PS YB-4163 (Fig. 3) under similar conditions. Also at low shear rates, the viscosity–shear-rate lines for PS YB-4163 level off.

In Figure 4, the shear-stress–shear-rate relationships for both black yeast polysaccharides are plotted. Xanthan gum (PS B-1459) is included for comparative purposes. As can be seen in Figure 4, PS Y-6272 and sample B of PS YB-4163 exhibit more non-Newtonian behavior at high shear stress than at low stress where it approaches Newtonian behavior. The curve for PS YB-4163 (sample A) is more complex and displays more Newtonian behavior (i.e., it parallels the Newtonian viscosity lines) in the middle of the measured range of stress, but at lower stress, it levels off as shown in Figure 4. PS YB-4163 (sample A) and xanthan display plastic behavior and appear to have a yield stress of ~7 and 10 dynes/cm^2, respectively. This apparent yield stress may explain why PS YB-4163 (sample A) (but not sample B or PS Y-6272) pours as a "lump."

The effect of shear rate on the primary normal stress difference, $P_{11} - P_{22}$,
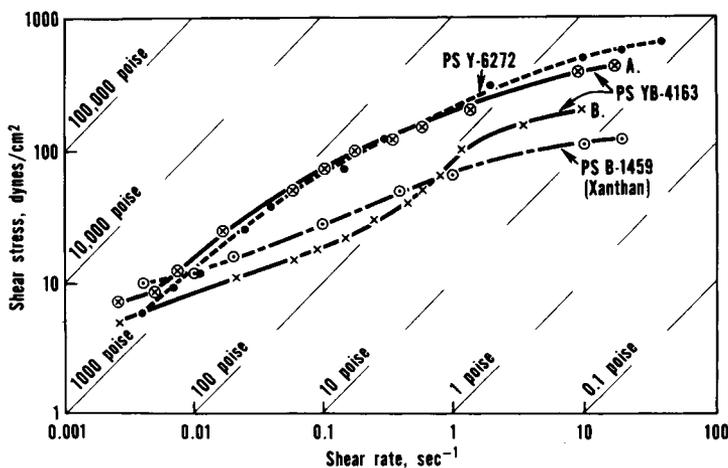

Fig. 4. Shear-stress–shear-rate–viscosity relations at 25°C for aqueous dispersions of three microbial polysaccharides (potassium salt form). Concentration, 1%.
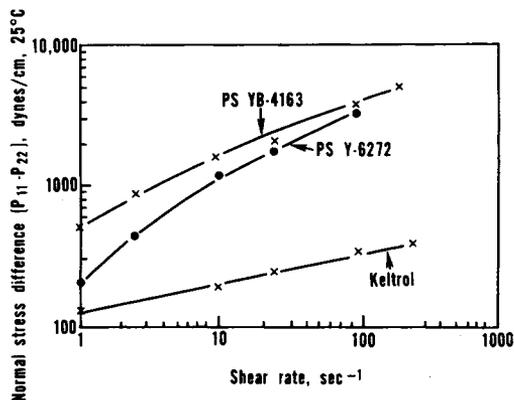
Fig. 5. Normal stress difference $(P_{11} - P_{22})$ vs shear rate in sec^{-1}. Concentration of potassium salt form, 1%; 25°C. Keltrol is a commercially available xanthan gum preparation.
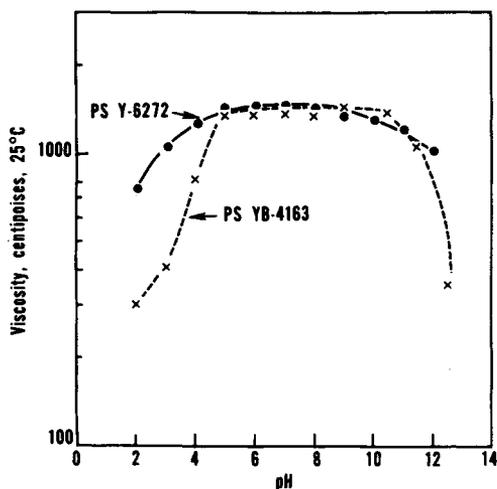

Fig. 6. Viscosity at 25°C vs pH of dispersions (0.5%) of black yeast polysaccharides (potassium salt form).

is shown in Figure 5. As can be seen, the values of $P_{11} - P_{22}$ for both PS YB-4163 and PS Y-6272 are similar in magnitude but both are higher than that measured for a commercial sample of xanthan (Keltrol). Although the $P_{11} - P_{22}$ values for PS YB-4163 are higher than those for PS Y-6272, the higher elasticity of PS YB-4163 observed visually over that of PS Y-6272 and xanthan led us to expect that the $P_{11} - P_{22}$ values of PS YB-4163 would be greater than found. The values for $P_{11} - P_{22}$ can be used to calculate the shear modulus G by use of the formula $G = 2\tau^2/(P_{11} - P_{22})$, where τ is the shear stress in dynes/cm^2. The modulus G is independent of shear rate for the three polysaccharides in Figure 5. The three averages of G from γ (shear rate) = 1–250 sec^{-1} are 211 dynes/cm^2 for Keltrol (xanthan), 219 for PS Y-6272, and about 200 for PS YB-4163.

Effect of pH. As can be seen in Figure 6 between pH of 5–10, the viscosity of both PS YB-4163 and PS Y-6272 is nearly constant, while at the very low and high pH values, viscosity is diminished, with PS YB-4163 being more sensitive to these extreme pH values than PS Y-6272. At these extreme pH values permanent loss of viscosity was found to occur with time.
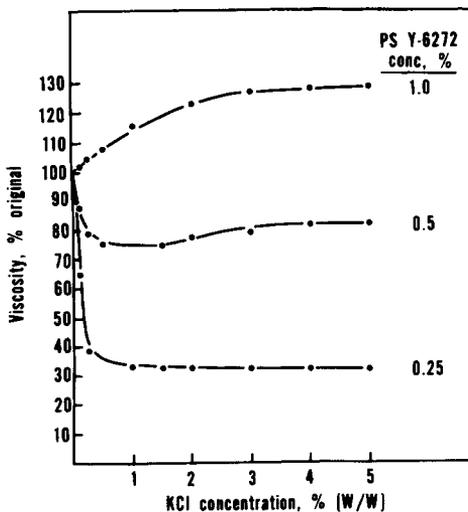
Fig. 7. PS Y-6272: effect of added salt (KCl) on viscosity (25°C). Shear rate, 3.84 sec^{-1}. Initial viscosity of salt-free PS Y-6272 (potassium salt form) dispersions: 1%, 7900 cps; 0.5%, 2050 cps; 0.25%, 560 cps.
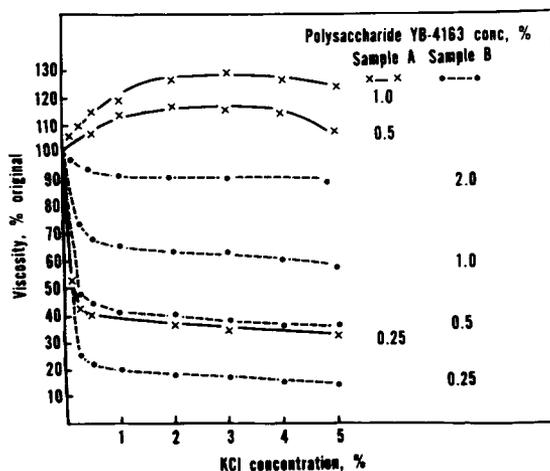

Fig. 8. PS YB-4163, samples A and B: effect of added salt (KCl) on viscosity (25°C). Shear rate, 3.84 sec^{-1}. Initial viscosity of salt-free of PS YB-4163 (potassium-salt form) dispersions were: 1.0% A, 7800 cps; 0.5% A, 1,775 cps; 0.25% A, 620 cps; 2.0% B, 12,700 cps; 1.0% B, 2,740 cps; 0.5% B, 652 cps; 0.25% B, 214 cps.

Effect of Salts. The effect of addition of KCl to solutions of PS Y-6272 on viscosity is shown in Figure 7. The curves shown here are also representative of the effect of other salts (K_2SO_4, K_2HPO_4, NH_4OAc, NaCl, $Na_2B_4O_7 \cdot 10H_2O$, Na citrate, $CaCl_2$, and $MgCl_2$) on the viscosity of PS Y-6272 solutions. As can be seen at a polysaccharide concentration of 1.0%, the viscosity increases with addition of salt. At the polysaccharide levels of 0.5% and 0.25%, addition of salt causes the viscosity to decrease, but after the addition of 1% salt the viscosity curves are essentially level. Trivalent metals, such as Fe^{3+}, cause both PS Y-6272 and PS YB-4163 to gel but rapidly precipitate the polysaccharides. The effect of KCl on PS YB-4163 solution viscosity is shown in Figure 8.

The majority of samples of PS YB-4163 tested behave like sample B in Figure
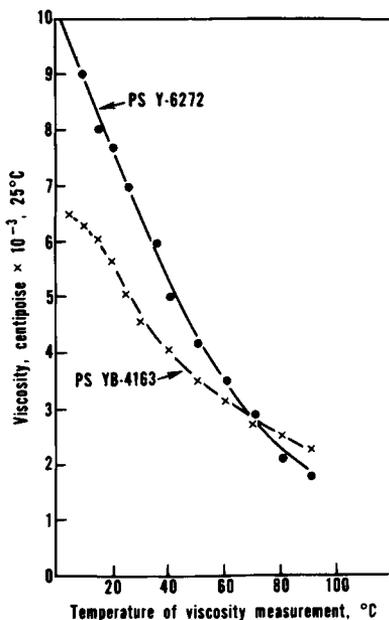
Fig. 9. Viscosity of PS Y-6272 and PS YB-4163 solutions (1%, potassium salt form) at various temperatures. (Wells-Brookfield LVT viscometer, spindle #4, at 30 rpm.)
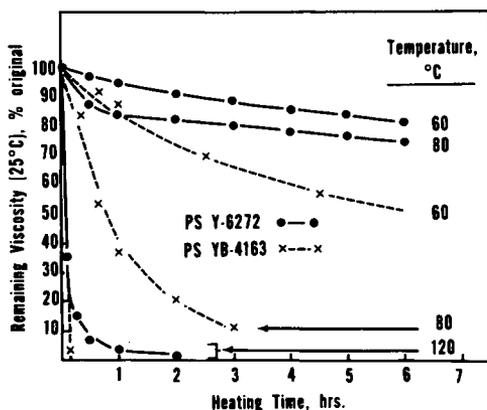

Fig. 10. Effect of extended heating on viscosity of individual dispersions (1%) of PS Y-6272 and PS YB-4163. Dispersions were heated at indicated temperatures for varying lengths of time before cooling and measuring the remaining viscosity (3.84 sec^{-1}, 25°C).

8. Sample A differed from sample B in that A gives a higher solution viscosity when compared on a weight basis. Sample A is included to show that a product of improved quality was obtained which is superior to the product that is now being isolated routinely. The viscosity of sample B decreased with increasing amounts of KCl at all concentrations of polysaccharides tested. With sample A, at the 1.0% and 0.5% levels, addition of KCl caused the viscosity to increase; but at the 0.25% level, KCl caused it to decrease. It is interesting to note that after reaching the 0.5%–1.0% level of salt, the further addition of salt to PS YB-4163 or PS Y-6272 solution does not cause significant change in solution viscosity.

Effect of Temperature. As the temperature of dispersions of PS YB-4163

TABLE I
Emulsions Containing Black Yeast Polysaccharide in Equal Parts of Oil and Water

| | Extent of emulsions after 24 hr[a] | | | | | |
| | PS YB-4163 | | PS Y-6272 | | Xanthan | |
Oil	0.3%	0.5%	0.3%	0.5%	0.3%	0.5%
Paraffin	++	++	+	+	++	++
Soybean and cottonseed[b]	+	++	+	+	--	++
Soybean[c]	+	++	+01.+	++	++	
Corn[d]	++	++	+	+	--	++
Olive	++	--	+	++	--	++
Safflower	+	++	+	+	--	++

[a] ++ = After 24 hr, 100% emulsion; + = after 24 hr, some emulsion; -- = after 24 hr, no emulsion.

[b] Wesson, Hunt-Wesson Foods, Inc.

[c] Crisco, Procter and Gamble.

[d] Mazola, Best Foods.

TABLE II
Physical Tests on Polysaccharide Films[a]

Polysaccharide	Glycerol, %	Tensile strength, kg/mm[b]	Elongation, %	Schopper double folds
YB-4163	0	5.9	3	17
	15	3.3	6	34
Y-6272	0	4.7	6	30
	15	3.9	8	27
B-1459[c] (xanthan)	0	5.8	3	6[b]
	15	4.0	4	43[b]
Corn amylose	0	7.2	13	900
	15	5.4	20	280

[a] One percent solutions were cast on flat plates and allowed to air dry.

[b] Heating the solution to 80°C before pouring on the plate would increase the number of double folds.

[c] Cell-free laboratory sample.

and PS Y-6272 is increased to near their boiling point, the viscosity is significantly lowered, as seen in Figure 9. If the length of heating is short, the original viscosity is restored upon cooling the solutions. If, however, the length of heating is increased, permanent loss of viscosity is observed.

In Figure 10, the effect on viscosity of heating dispersions (1%) of PS YB-4163 and PS Y-6272 is shown. Dispersions were heated at 60°, 80°, and 120°C for various time intervals and then cooled to 25°C and the remaining viscosity was measured. As seen in Figure 10, under these conditions (1% dispersions, pH 6.5), PS Y-6272 is more stable to heat at the three temperatures tried than PS YB-4163. Dispersions of PS Y-6272 are tolerant of lengthy heating at 60° or 80°C but not at 120°C. Heating of dispersions of PS YB-4163 for periods beyond 1 hr, even at 60°C, causes considerable loss of viscosity. The PS YB-4163 sample is more pigmented and the pigment may contribute to the apparent lower stability to heat of PS YB-4163.

Stabilization of Emulsions

PS Y-6272 and PS YB-4163 were tested as oil–water emulsion stabilizers and were compared with xanthan, a gum considered to be an excellent emulsion stabilizer. A solution of each of the three polysaccharides at two different concentrations was blended with an equal volume of various commercially available oils (final concentration of polysaccharide was 0.3% and 0.5%). After blending the samples in the prescribed manner and transferring to vials, the height of the emulsified material as well as the height of water and oil layers were measured with time. Table I shows the results noted 24 hr after making the emulsions. As can be seen at the 0.3% and 0.5% levels, PS YB-4163 is the better stabilizer of the two black yeast products. At the 0.3% level, PS YB-4163 appears to be a better stabilizer than xanthan with some oils. At the 0.5% level, both PS YB-4163 and xanthan are excellent emulsion stabilizers.

Film Formation

Both black yeast polysaccharides form cohesive, flexible films that were cast from aqueous solutions on suitable Plexiglas plates. In Table II are listed various physical measurements made on films of not only PS YB-4163 and PS Y-6272 but also xanthan and corn amylose, an excellent film former. As seen in Table II, the films made from PS YB-4163 and PS Y-6272 are not as durable as those from amylose but are similar to the film from xanthan. The flexibility of black yeast polysaccharide films was poorer than anticipated and was not improved by additions of 30% glycerol as a plasticizer. Exploration of other plasticizers was not undertaken. Films of both fungal polysaccharides readily redisperse in water.

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