African Journal of Biotechnology Vol. 6 (22), pp. 2603-2611, 19 November, 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Characterization of the exopolysaccharide produced by a whey utilizing strain of *Klebsiella oxytoca*

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Accepted 20 August, 2007

Physical, chemical and rheological properties of a polysaccharide produced by an isolate of *Klebsiella oxytoca* were characterized. Freeze dried samples of the polysaccharide were neutral and were completely soluble in water. Samples did not form gels even in the presence of salt treatments. The major monosaccharide constituents of the polysaccharide were rhamnose (37%, w/w) and glucose (34%, w/w). Residues of cellobiose were detected, suggesting that the polysaccharide had a cellulose backbone. The gum was more comparable to broth apparent viscosities of xanthan gum than to gellan gum. The *K. oxytoca* polysaccharide (KOP) produced high solution viscosity at low concentrations. At a gum concentration 0.5% (w/v), an apparent viscosity of 400 cP at 24 s^{-1} was obtained. Rheological behavior showed that the KOP formed non newtonian fluids, indicating that it is a pseudoplastic biopolymer. Although the KOP solutions displayed pseudoplastic behavior, increases in shearing time did not result in significant changes on the apparent viscosity. This indicated that the gum is neither thixotropic nor rheopectic. The conclusion reached about the potential application of the gum was that it could be suitable for use as a stabilizing or suspending agent rather than a gelling agent.

Key words: Apparent viscosity, exopolysaccharide, *Klebsiella oxytoca*, pseudoplastic, rheology, rhamnose, whey.

INTRODUCTION

Over the past few decades, several microorganisms have been investigated for the production of exopolysaccharides using whey or lactose as fermentation substrate (Shams and Jaynes, 1983; Cerning et al., 1992). Xanthan and gellan gums are examples of well accepted bacterial exopolysaccharides that have significant industrial importance. Apart from bacteria, attempts have been made to produce exopolysaccharides from submerged fungi cultures (Fang et al., 2002; Hwang et al., 2003; Joo et al., 2004).

Over the past three decades, attempts have been made to improve the utilization of whey by using it as a fermentation substrate for producing value added products such as ethanol and microbial exopolysaccharides (Stauffer and Leeder, 1978; Shams and Jaynes, 1983; Schwartz and Bodie, 1985; Dlamini and Peiris, 1997a; Fialho et al., 1999). Whey is a by-product of the cheese industry produced at a rate of nine kg per kg of sweet cheese. Although several uses of whey have been reported (Coton, 1985; Zadow, 1988), there is evidence that whey disposal presents serious problems (Tyagi et al., 1991; Horton, 1993). Whey has very high biological oxygen demand (> 45 000 ppm) and high lactose content (4.5%, w/v). For this reason, if discharged to the environment, whey can cause severe pollution since it is poorly metabolized by most environmental organisms (Ryder, 1988).

Research on producing commercial bacterial gums using whey or lactose based media, with the goal of improving whey utilization, have been done with little success (Schwartz and Bodie, 1985; Dlamini and Peiris, 1997a; Fialho et al., 1999). Attempts to isolate lactose metabolizing exopolysaccharide producing microorgan-

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sms have been made (Flatt et al., 1992; Dlamini and Peiris, 1997b). Under optimized conditions, *Klebsiella oxytoca* produced copious amounts of exopolysaccharide, 15 g/l, that had very high apparent viscosity, 20 000 cP at 0.6 s^{-1} (Peiris et al., 1998). It has been reported that the location of genes for expolysaccharide production in this *K. oxytoca* are plasmid encoded (Peiris and Dlamini, 1999), thus prompting favorable recombinant DNA technology manipulation with lactic acid bacteria. This is paramount because several researches have shown that lactic acid producing bacteria produces very low exopolysaccharide yields (Cerning et al., 1992; Bouzar et al., 1997; van Schalkwyk, et al., 2003; Aslim et al., 2005).

No reports, however, have been made about the physicochemical and rheological characteristics of the exopolysaccharide produced by *K. oxytoca*, yet such information is prerequisite to determining the potential industrial application of this biopolymer. Depending on their chemical composition and ability to modify rheology of aqueous solutions, gum polysaccharides can be of variable industrial application (Flatt et al., 1992; Marszalek et al., 1998). The purpose of this research was to determine the monosaccharide composition and the rheological properties of the exopolysaccharide produced by *K. oxytoca* so that its industrial application can be ascertained.

MATERIALS AND METHODS

Gum preparation

The gum was produced by fermentation in whey based media using a strain of *K. oxytoca* that was isolated by Dlamini and Peiris (1997b). Fermentation was done at 25°C in a Braun Biostat B, 2-l fermentor following the procedure described by Dlamini and Peiris (1997a). The pH was maintained at pH 7 using two solutions made of 10% (v/v) ammonium solution and 10% (v/v) orthophosphoric acid. The dissolved oxygen tension (DOT) was maintained at 60%. This ensured that optimum conditions for the production of the exopolysaccharide described by Peiris et al. (1998) were achieved. The gum was recovered from fermentation broths and purified as described before (Dlamini and Peiris, 1997a). Concentrated samples were precipitated by chilled absolute ethanol and washed twice in deionized water before final precipitation and lyomphylization.

Drying of polysaccharide and milling

The alcohol precipitated samples were transferred to sample jars and frozen at -20°C for 24 h. The frozen samples were freeze dried at -50°C and 100 kPa vacuum in a Dynavac Freeze drier FD6 (Dynavac High Vacuum Pty, Limited). Freeze drying was done until constant weights were achieved (30 to 48 h). Freeze dried samples were ground into fine powder using a Knifetec 1095 Sample Mill (Tecator). Milling was done in cycles of 10 s for 7 min. The ground product was stored at room temperature in a desiccator.

Physical analysis

To determine total dry matter of the gum, 5 g of sample were weighed in pre-dried tarred crucible dish. Then the weighed sample was dried at 105°C for 4 h. After that, the sample was cooled in a desiccator and re-weighed. Dry matter percentage was calculated as final weight divide by initial weight multiplied by 100. The particle size was determined by sieving 5 g of purified dry samples through a 200 mm diameter screens (Endcotts perforated wire mesh sieve, Selbys Scientific, Australia). The smaller aperture size used was 390 µm and the bigger one was 500 µm. Particle size was calculated as the percentage of the sample going through the sieve after three min over the initial weight.

Determination of polymer monomers

The method used for the preparation and analysis of samples with high performance liquid chromatography (HPLC) was adapted from Flatt et al. (1992). Purified samples of the gum were completely dissolved in milli Q water to form 1% (w/v) concentrations. A total of 700 mg of gum solution was transferred into ampoules followed by 700 mg of 2.5 M HCl. The samples were mixed and degassed with nitrogen before the tubes were sealed. Sealed ampoule tubes were placed in the oven at 100°C for 3 h. After hydrolysis, the samples were allowed to cool down for 10 min before drying at 45°C. After drying, the hydrolysis products were re-dissolved in Milli Q water, and filtered. Neutral sugars were quantified by HPLC (Hewlett Packard Series 1050) using the Aminex 87-P column (cation - exchange column, Aminex HPX - 87 P and anionic - exchange column, Bio-Rad Laboratories, Richmond, CA.). A 25 µl of filtered hydrolysis product was injected and a separation flow rate of 0.5 ml /min was used. The column temperature was kept at 85°C, and the Reflective Index (RI) detector temperature was 35°C. Milli Q water was used as the mobile phase.

Testing for gel formation

Abilities of samples to form gels were tested by heating the sample solutions at different temperatures (50, 85, 100, 110 and 121°C). After heating, samples were allowed to cool and were examined for gelling. To test the effect of pH on gelling, samples' pHs were varied from pH 2, to 12 using 5 M HCl or 5 M NaOH. To test the effect of salt type and concentration on gel formation, sample solutions were treated with sodium chloride, potassium chloride, calcium chloride and magnesium chloride. The salt concentrations used varied from 0.1% (w/v) to 5% (w/v). Samples treated with different salts and pH were heated at 115°C and 121°C and examined for gel formation after cooling.

Rheology measurements

Dry exopolysaccharide samples were prepared for rheology measurement by rehydrating 1% (w/v) of the purified gum in redistilled water. The sample was mixed by stirring using a magnetic stirrer until the gum was completely dissolved and then the rheology measurements were carried out. Viscosity was measured using a Wells-Brookfield cone and plate viscometer, model LVDV-1, version 2.0, cone sizes CP-41 or 52 (Brookfield Engineering Laboratories Stoughton MA). The viscometer was attached to a circulating water-bath maintained at 25°C. Cone size CP41 was used for samples with viscosities below 3,800 cP. For samples with viscosities above 3,800 cP, cone size CP52 was used.
Shear rate was calculated for the set speed as follows:
Shear rate (S^{-1}) = speed (RPM) x 2

For this Wells-Brookfield Viscometer, the relationships among viscosity, shear rate and shear stress were stipulated in the Wells-Brookfield Viscosity Manual:

Viscosity (cP) = shear stress x 100 / shear rate

Shear stress (dynes/cm^2) = Viscosity x shear rate / 100

Table 1. General characteristics of the exopolyasaccharide produced by *Klebsiella oxytoca* in lactose based media.

Characteristic	Results
Solubility in water (5 g/l at 25°C)	soluble
Dry matter	92 ± 0.9% (w/w)
pH	7 ± 0.2
Apparent viscosity, concentration 0.5% (w/v)	400 cP at 24 s^{-1}
Gelation	No gelation
Colour	white
Form	dry, free flowing
Particle size	99% (w/w) through 500 microns
	98% (w/w) through 390 microns

RESULTS

General characterization of the *Klebsiella oxytoca* polysaccharide

The general characteristics of the biopolymer produced by *K. oxytoca* were determined as described above. The results are shown on Table 1 and they showed that the *K. oxytoca* exopolysaccharide (KOP) was water soluble. Solubility of 0.5% (w/v) of this biopolymer at 25°C was found to be better than that of carrageenan, xanthan or gellan. The KOP was completely dissolved within 15 min. The pH of the dissolved biopolymer was neutral (pH 7 ± 0.2). The dry matter for the KOP (91.7%, w/w) was within the acceptable range (87 to 94%, w/w) for most industrial gums (Kelco international, 1987).

The results presented on Table 1 have also shown that the biopolymer produced high solution viscosity at low concentrations. At a gum concentration 0.5% (w/v), an apparent viscosity of 400 cP at 24 s^{-1} was obtained. This was comparable to broth viscosities of well-established industrial gums such as xanthan (Johns and Noor, 1991). Under conditions of this study, solutions of KOP did not form gels. Gel formation was tested in solutions of pH 2 to 12 and gum concentrations of 0.1 to 1.2% (w/v). Samples were heated from 25 to 121°C. They were also treated with monovalent and divalent salts of 0.1 to 5% (w/v) concentrations and still there was no gelation.

Sugars composition of the *Klebsiella oxytoca* polysaccharide (KOP)

The sugar constitution of the KOP was determined by the High Pressure Liquid Chromatophy (HPLC) method as described above. The cation exchange column in the lead form (Aminex HPX-87 P) was used to quantify neutral sugars for the polysaccharide. The HPLC Chromatograms of the standard sugar solutions and gum hydrolysate are presented on Figures 1 and 2. The first main peak in Figure 2a was identical to that of cellobiose on the standard chromatogram (Figure 1). The cellobiose peak was confirmed when a solution of cellobiose was spiked to the sample, there was a sharp rise on the peak at retention time 8.6 (Figure 2b). This confirmed that this first peak on the chromatogram was cellobiose. The second and the third main peaks on Figure 2a corresponded to those of glucose and rhamnose respectively on the standards (Figure 1a). These were again confirmed by spiking pure solutions of glucose (Figure 2c) and rhamnose (Figure 2d) to the sample. The last peak did not correspond to any of the monosaccharides tested. The peak areas indicated that rhamnose (36.6%, w/w) was the most predominant monosaccharide in this biopolymer and free glucose was present in smaller amounts (13.4%, w/w). Cellobiose was 20.3% (w/w) and the unidentified polysaccharide residue was 29.6% (w/w).

Rheological properties of the exopolysaccharide

The rheological properties of the *K. oxytoca* polysaccharide (KOP) were compared with those of gellan and xanthan gums. This was done to understand the behavior of the gum in aqueous solutions. The factors affecting rheology studied were pH, temperature, time, biopolymer concentration, salt type and concentration. Log_{10} transformations of apparent viscosity, shear stress, shear rate and polymer concentration were done to obtain linear plots (Wachenheim and Patterson, 1992). The results presented on Figures 3 and 4 compares the effect of polymer concentration on apparent viscosity of the three gums, xanthan, gellan and the *K. oxytoca* polysaccharide (KOP), at four shear rates. Apparent viscosities were measured from polymer concentrations of 0.1 to 1.2% (w/v). The results indicated that at lower gum concentrations, 0.1 to 0.5% (w/v), gellan had comparatively lower apparent viscosities than either KOP or xanthan. However, at higher concentrations (1.2%, w/v), gellan gave very high apparent viscosities. It reached over 2000 cP at 6 s^{-1} compared to 1400 and 1100 cP reached by KOP and xanthan, respectively, at the same shear rate. The results presented on Figures 3 and 4 also have shown that the rheological behavior of the KOP resem-

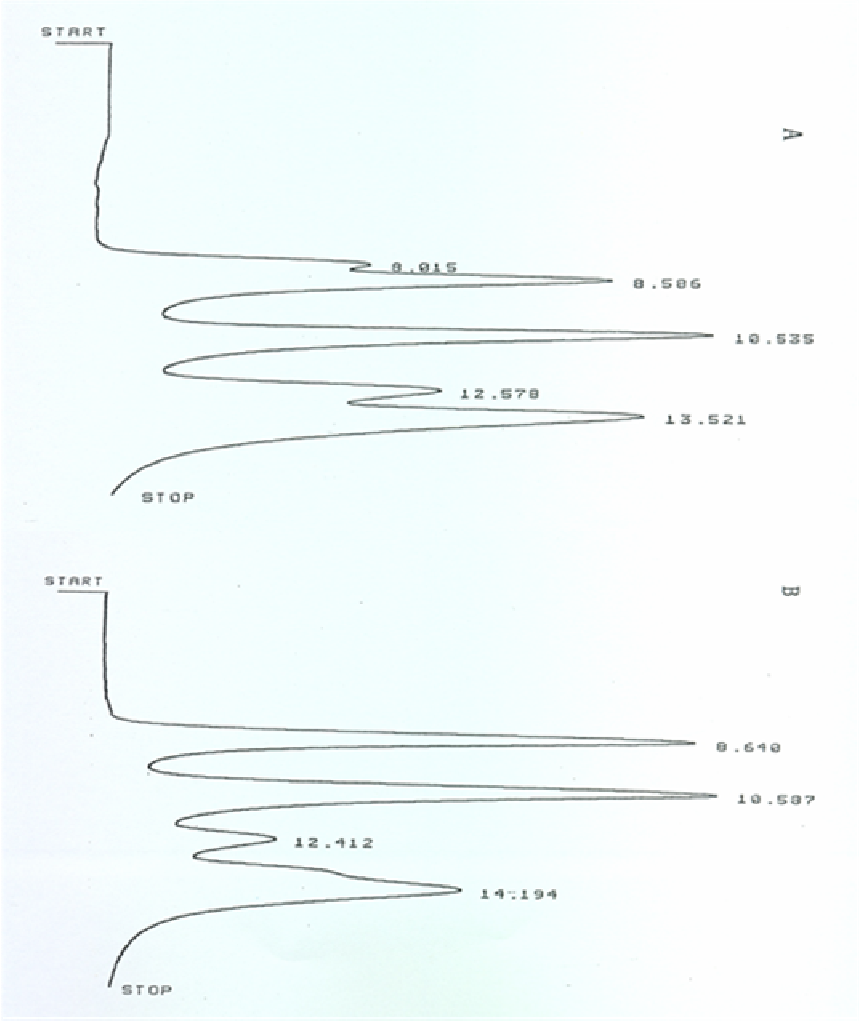

Figure 1. Neutral sugars standards used for HPLS analysis of the *Klebsiella oxytoca* polysaccharide. Retention times in **A** corresponds to: raffinose = 8.15; cellobiose = 8.587; glucose = 10.535; rhamnose = 12.578; and arabinose = 13.521. Retention times in **B** corresponds to: cellobiose = 8.640; glucose = 10.587; galactose = 12.412; and mannose = 14.194

bled that of xanthan more than gellan gum.

The results presented on Figure 5 shows the relationship between apparent viscosity and shear rate in the three gums at different concentrations. The results demonstrated that the KOP formed non newtonian fluids. The viscosity increased as the shear rates were decreased. This indicated that KOP is a pseudoplastic biopolymer and has a shear thinning effect. Although the KOP solutions displayed pseudoplastic behaviour, the results on Figure 6 have shown that increases in shearing time did not result in significant changes on the apparent viscosity of the gum.

Figure 7 shows log-log plots of shear stress as a function of shear rate for KOP, xanthan and gellan gums' solutions at three concentrations. The results have shown that the consistency behavior of the gums solutions increased as the concentrations were increased from 0.5 to 1% (w/v). Type and concentration of gum also affected the flow behavior index. As the concentrations were increased, flow behavior index also increased. Greatest increases were observed in gellan solutions, where the steepest slope was observed in 1% (w/v) solutions.

The effect of pH was tested in KOP solution of 0.5% (w/v) concentration. The results presented on Figure 8 shows that the biopolymer produced by *K. oxytoca* was fairly stable to pH variation. The apparent viscosity was constant from pH 2 to 8. However, at above pH 8, a drop in broth viscosity was observed. At pH 12, solution's apparent viscosities dropped to 500 cP and this was at least 30% of the viscosity recorded at neutral pH.

The KOP was relatively stable to increases in temperature. Figure 9 shows that the KOP was able to retain its apparent viscosity up-to 100^{o}C heating for 20 min. Figure 10 shows that the heat stability of the KOP was comparable to that of xanthan at temperatures below 100^{o}C.

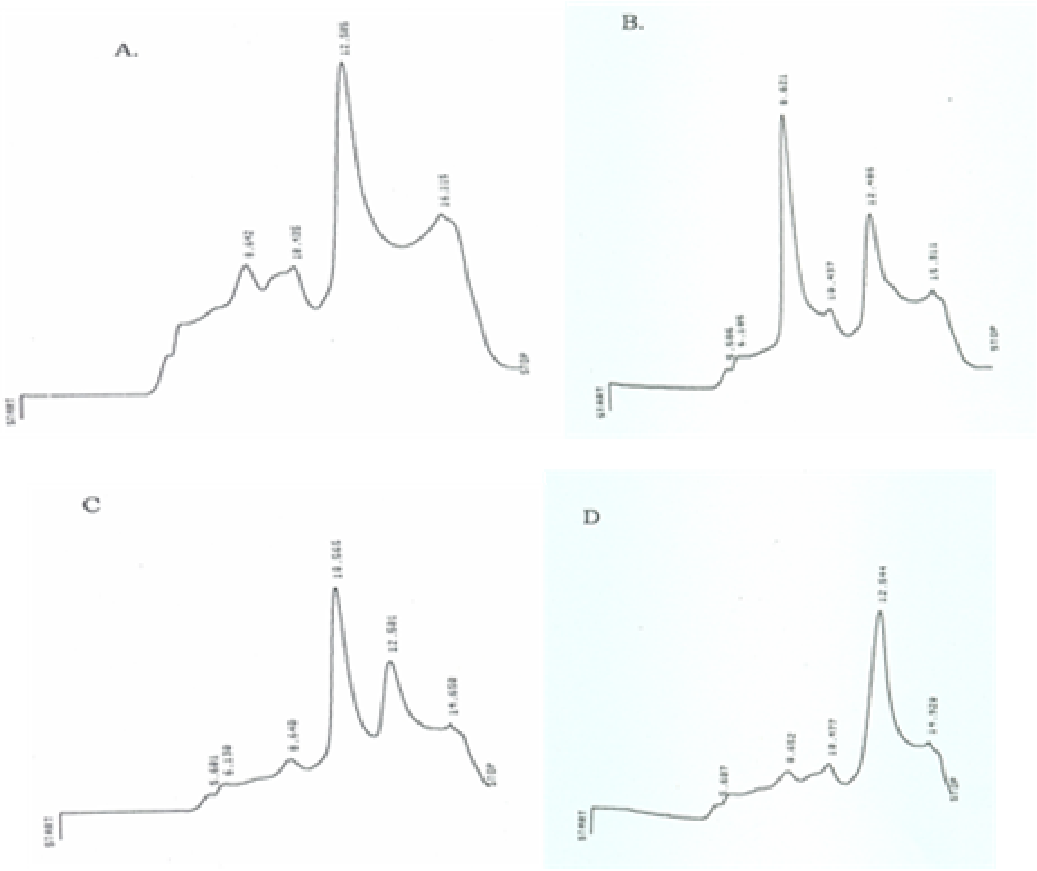

Figure 2. High performance liquid chromatography chromatograms of the *Klebsiella oxytoca* polysaccharide (KOP) hydrolysate alone and spiked with standard sugar solutions. **A** = KOP hydrolysate alone; **B** = KOP hydrolysate + cellobiose; **C** = KOP hydrolysate + Glucose; and **D** = KOP hydrolysate + Rhamnose.

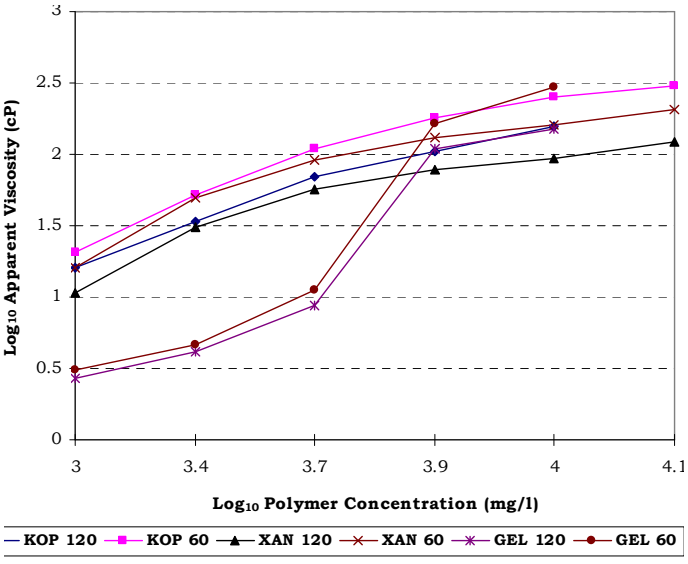

Figure 3. Effect of polymer concentration on apparent viscosity of *Klebsiella oxytoca* polysaccharide (KOP), xanthan gum (XAN) and gellan gum (GEL) at shear rates of 60 and 120 s^{-1}. Results are averages of 3 replicate samples.

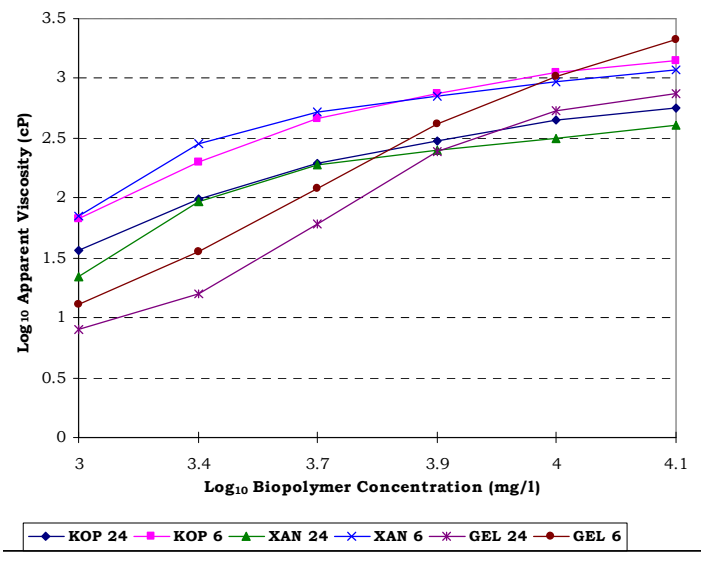

Figure 4. Effect of polymer concentration on apparent viscosity of *Klebsiella oxytoca* polysaccharide (KOP), xanthan gum (XAN) and gellan gum (GEL) at shear rates of 6 and 24 s^{-1}. Results are averages of 3 replicate samples

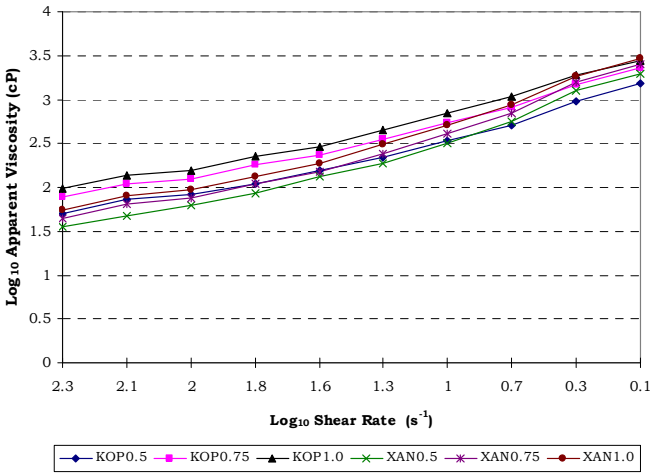

Figure 5. Effect of shear rate on the apparent viscosity of the *Klebsiella oxytoca* Polysaccharide (KOP) and xanthan gum (XAN) at 0.5, 0.75 and 1.0% (w/v) concentrations. Results are averages of three replicate samples

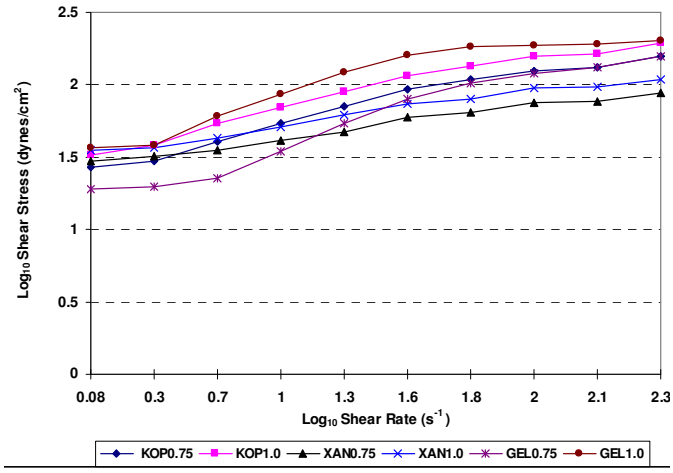

Figure 7. Effect of shear rate on the shear stress of *Klebsiella oxytoca* polysaccharide (KOP), xanthan (XAN) and gellan (GEL) gums at 0.75 and 1.0% (w/v) concentrations. Results are averages of 3 replicate samples

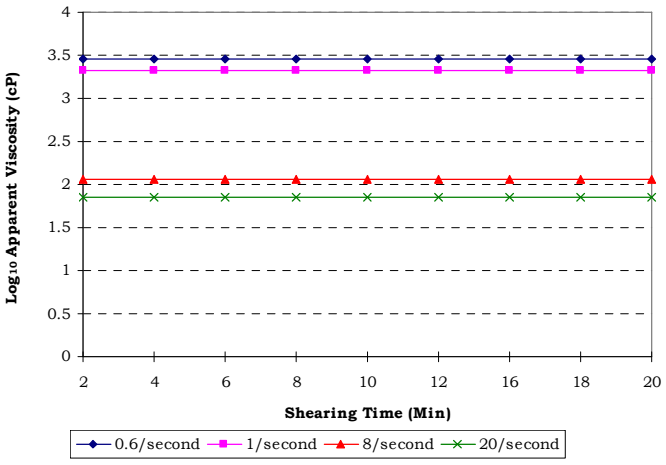

Figure 6. Effect of shearing time on the apparent viscosity of 0.5 % (w/v) *Klebsiella oxytoca* polysaccharide (KOP) solution at different share rates (s^{-1}). Results are averages of three replicate samples

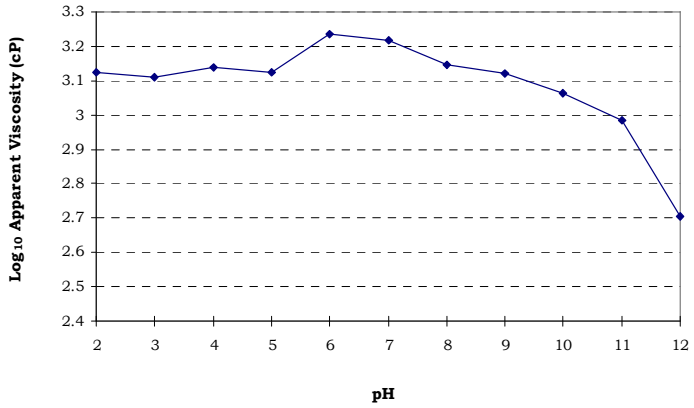

Figure 8. Effect of pH on apparent viscosity of 0.5% (w/v) *Klebsiella oxytoca* polysaccharide (KOP) at 0.6 s^{-1}. Results are averages of 2 replicate samples.

The KOP and xanthan had identical shear thinning behavior from 25 to 85°C temperature treatments. When the gums were heated at 110 and 121°C for 15 min KOP, however, lost up-to 60 and 90% respectively of its initial apparent viscosities (Figure 11).

The effects of monovalent (sodium and potassium chlorides) and divalent (calcium and magnesium chlorides) salts on the rheology of the KOP were tested. Salt concentrations of 0.1 to 5 % (w/v) were used. Salt concentration did not affect the solubility of KOP but decreased viscosity. The results presented on Figures 12 and 13 have shown that increases in salt concentrations resulted in decrease of apparent solution viscosities. This reduction was more noticeable in divalent salt treatments, particularly at lower shear rates.

DISCUSSION

Results from this work have shown that the exopolysaccharide produced by *K. oxytoca* is a neutral water soluble polysaccharide. This agrees with earlier reports (Peiris et al., 1998) where it was shown that at the end of the fermentation, pH remained at 7 despite that no pH control was carried out. The polysaccharide gum produces high solution viscosity at low concentrations. This property is comparable to well established industrial gums such as gellan or xanthan gums (Johns and Noor, 1991). It was, however, observed that the KOP could not form gels under conditions of this study hence it can not be recommended for use as a gelling agent.

Based on the result of the HPLC analysis, it was concluded that the major monosaccharide for this KOP are rhamnose and glucose. The remainder of the peaks

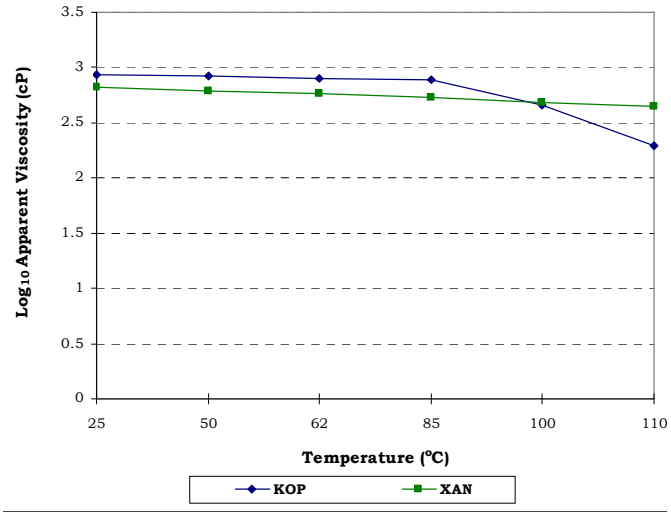

Figure 9. Effect of temperature on the apparent viscosity of 1% (w/v) *Klebsiella oxytoca* polysaccharide (KOP) and xanthan gum (XAN) at 12 s^{-1}. Results are averages of 3 replicate samples.

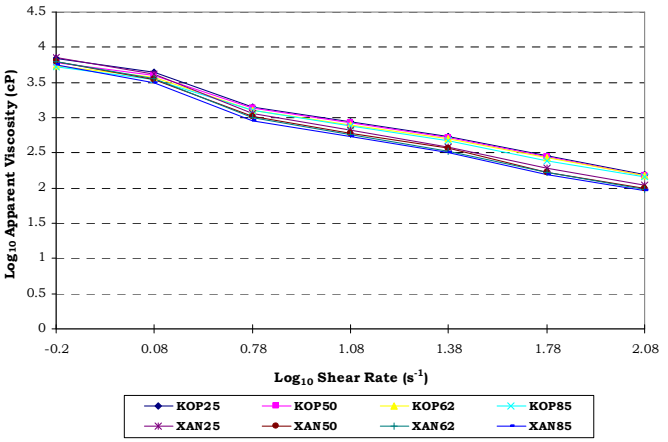

Figure 10. Shear thinning behavior of *Klebsiella oxytoca* polysaccharide (KOP) and xanthan gum (XAN) at 25, 50, 62 and 85°C. Results are averages of 3 replicates.

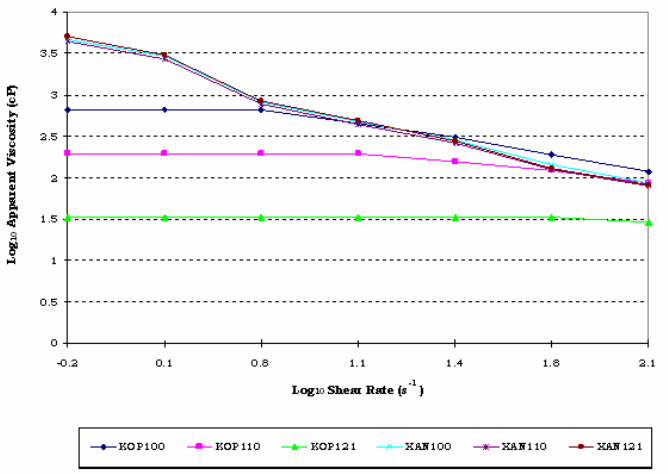

Figure 11. Shear thinning behavior of *Klebsiella oxytoca* polysaccharide (KOP) and xanthan gum (XAN) at 100, 110 and 121°C. Results are averages of three replicates.

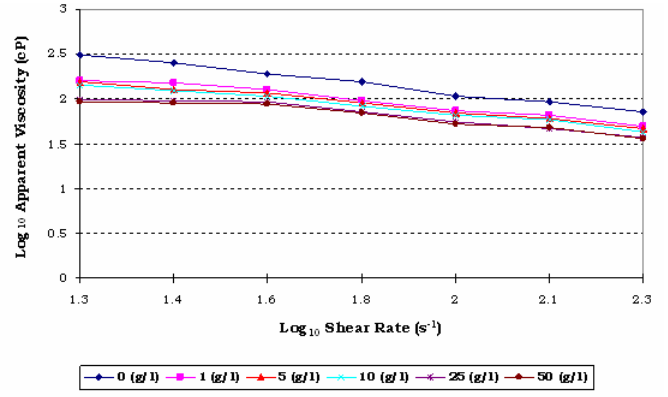

Figure 12. Effect of sodium chloride concentration (g/l) on the rheology of *Klebsiella oxytoca* polysaccharide (KOP). Results are averages of 3 replicates.

could probably be fragments of partially hydrolyzed gum or by-product of hydrolysis reaction. As reported elsewhere (Flatt et al., 1992), it could be fragments of partially hydrolysed polysaccharide or oligosaccharide. The results have shown that one of the residues was cellobiose, a β-D-glucose-(1-4)-disaccharide. This may mean that the repeat units of the polysaccharide may contain β-D-glucose or the disaccharide cellobiose or a residue of cellulose suggesting that the polysaccharide may have a cellulose backbone. A limited number of bacteria have been reported to produce polysaccharides that yield rhamnose upon hydrolysis. *Pseudomonas elodea* produces gellan which has a repeat unit containing rhamnose and β-(1-3)-D-glucose (O'Neil et al., 1983). *Acinetobacter calcoaceticus* produced a polysaccharide containing rhamnose, glucose and mannose (Kaplan and Rosenberg, 1982; Morin et al., 1987). It has also been reported that a *Klebsiella* sp. produces polysaccharide that contains rhamnose, galactose and glucuronic acid (Morin and Monsan, 1990). The tentative analysis of the KOP has indicated that it has a unique composition from that of previously reported gums. However, further work is required to confirm the primary structure and to elucidate the secondary and tertiary structures of this gum.

The results demonstrate that the KOP forms non newtonian fluids. The viscosity increased as the shear rate was decreased. This indicated that KOP is a pseudoplastic biopolymer and has a shear thinning effect. The practical implication of this characteristic is that upon shaking or stirring or pouring, the viscosity of the gum

solutions decreases. However, as the shear is removed, the viscosity recovers. Although the KOP solutions displayed pseudoplastic behavior, results have also shown that increases in shearing time did not result in significant changes on the apparent viscosity of the gum. This indicated that the gum is neither thixotropic nor rheopectic. The conclusion reached about the application of the gum was that it could be suitable for use as a stabilizing or suspending agent (Morris, 1990).

Shear thinning behavior is also useful in the oil industry for oil recovery (Sutherland, 1992). As reported by Schelhaas and Morris (1985), greater flow index values indicate lower pseudoplasticity of biopolymers. These results have thus denoted that among the three gums tested, xanthan is the most pseudoplastic biopolymer, followed by the KOP and gellan has the least pseudoplastic behavior. The pH of the KOP indicated that it may be more suitable for use in products that requires acidic or near neutral pH processing conditions. These results have also shown that KOP completely lost its shear thinning behavior at higher temperatures, indicating that the polysaccharide gum could be useful in product preparations that require up to pasteurization temperatures.

At all the salt concentrations, apparent viscosity decreased to at least 50% of solutions with no salts. A possible reason for this behavior as suggested by Flatt et al. (1992) could be attributed to the contraction of the polysaccharide chain as a result of electrolyte-induced charge shielding. This reduces the repulsion between anionic substituents within the chain hence a reduced apparent viscosity. The decrease in apparent viscosity as a result of increases in salt concentrations is consistent with the results about the effect of pH on viscosity reported above. As reported before, these results suggest the instability of the KOP viscosities to alkaline or saline conditions. This factor may indicate that the application of the KOP is less efficient under saline conditions.

Conclusions

High performance liquid chromatography analysis revealed that the exopolysaccharide produced by *K. oxytoca* is composed of rhamnose, glucose and cellobiose, a combination that is unique to this biopolymer. Results from this work have shown that the KOP has shear thinning rheological characteristics. This behavior is exhibited by pseudoplastics such as paints, emulsions and dispersions (Morris, 1990). The apparent viscosity of the biopolymer is fairly stable over a wide range of temperatures (25 to 100^{o}C) and pH (2 to 9). Salt solutions decreased the viscosity of the KOP solutions to at least 50% of that for control solutions. The rheological properties of the KOP differed greatly from that of gellan, but closely resembled that of xanthan. This suggests that similar applications to that of xanthan are possible. The results have also shown that the KOP can not be used as a gelling agent but probable as a stabilizer, thickener, suspending or binding agent. Further work may involve the identification of specific exopolysaccharide components responsible for rheological variations in the KOP and determination of the practical application of the polysaccharide gum in foods.

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