Rheological characterization under shear of a fraction of polymer produced via fermentation of whey-related media by *Rahnella aquatilis*[1]

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Abstract

Production of lactan, a polysaccharide composed of mannose, galactose and galacturonic acid (at the ratio of 5:3:2), starting from a semi-defined medium containing lactose via fermentation with *Rahnella aquatilis* was described previously. In this communication, such polysaccharide was produced from five alternative fermentation media: (1) a synthetic (defined) medium, plain whey (under (2) aerobic and (3) anaerobic conditions), (4) whey permeate and (5) whey with 2% NaCl (w/v). The effect of the concentration of polysaccharide, pH and ionic strength at harvest on the rheological properties of the polysaccharide was studied using lactan-enriched fractions recovered from each medium and analysed in solution under steady shear flow. Lactan solutions showed a shear-thinning behaviour in all cases, and increases in viscosity were observed at increasing concentrations of polysaccharide, as expected. The polysaccharide fraction produced from whey with 2% (w/v) NaCl and plain whey under anaerobic conditions exhibited lower viscosity than that produced from the other media, an observation that is associated with the lower concentration of polysaccharide. Post-harvest addition of salts (KCl or $CaCl_2$) and changes in pH (3–11) affected slightly the viscosity of the polysaccharide solutions.

Keywords: Lactan; *Rahnella aquatilis*; Polysaccharide

1. Introduction

Whey and whey permeate, by-products of cheese manufacture, have created a world wide problem of waste disposal owing to their high biological oxygen demand. Whey is composed mainly of lactose (4.9% w/v) and protein (0.8% w/v), but also contains traces of vitamins, minerals and organic acids (Schwartz and Bodie, 1986). Utilization of whey lactose as fermentation feedstock has been attempted widely by the dairy industry and many biopolymers have accordingly been reported in the literature, e.g. dextrans produced by *Leuconostoc mesenteroides*, phosphomannans produced by *Hansenula* spp., a polysaccharide produced by *Arthrobacter* spp. (Smiley, 1966), an orange-pigmented gum produced by *Corynebacterium* spp. (Shams and Jaynes, 1983), xanthan gum produced by *Xanthomonas campestris* (Rocks, 1971; Schwartz and Bodie, 1986), gellan produced by *Pseudomonas elodea* (Dlamini and Peiris, 1997), pululan produced by *Aerobasidium pullulans* (Le Duy et al., 1983), alginic acid produced by *Azobacter vinalandi* (Righelato and Deaven, 1978), indican produced by *Beijericka indica* (Lawson and Symes, 1982), galactoglucan produced by *Zooglea ramigera* (Stauffer et al., 1980), levan produced by *Alcaligenes viscous* (Stauffer and Leeder, 1978) and several heteropolysaccharides produced by *Streptococcus thermophilus*, *S. cremoris*, *S. lactis*, *Lactobacillus bulgaricus*, *L. pastorianus*, *Propionibacterium acidi-propionici*, *P. zeae*, *P. shermanii* and *P. freudenreichii* (Duncan and Seeley, 1965; Reddy et al., 1973; Vedamuthu and Neville, 1986; Cerning et al., 1986; Martino et al., 1991; Racine et al., 1991). These microbial exopolysaccharides are produced in the form of capsules or as amorphous masses excreted to the media and are valuable to industry primarily because of their ability to modify the rheological behaviour of aqueous systems; several commercial applications actually exist both in the food and non-food industries.

Production of lactan, a polysaccharide composed of mannose, galactose and galacturonic acid (at the molar ratios 5:3:2) starting from a semi-defined lactose-rich medium via fermentation brought about by *Rahnella aquatilis* was previously described by Flatt et al. (1992a, b). Although wheys (or whey-derived media) have been claimed (and implicitly assumed) to be good feedstocks

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for such type of fermentation, no data were ever reported on the outcomes of such attempts (if any ever existed). On the other hand, use of lactose as constituent of a natural (unformulated) feedstock is appealing not only because it can provide a partial solution to the global whey disposal problem, but also because it is a nonexpensive fermentation substrate. In this work, the rheological behaviour, under steady shear, of (natural or formulated) solutions of lactan fractions obtained from several fermentation media based on whey and using the aforementioned microorganism is described and discussed.

2. Materials and methods

2.1. Feedstock and chemicals

Ethanol was purchased from AGA (Porto, Portugal), whereas NaCl, $CaCl_2$, KCl, H_2SO_4, NaOH, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $MgSO_4$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, $NaMoO_4$, H_2SO_4, HCl, lactose and silicone (all of analytical grade) were purchased from Merck (Darmstadt, Germany). Yeast extract was purchased from Lab M (Bury, UK). Protease A was obtained from Amano Pharmaceutical (Nagoya, Japan). Bovine whey was obtained as by-product of the bulk manufacture of cheese from the milk of a native Portuguese bovine breed (Cachena).

2.2. Equipment

Ultrafiltration of permeate was done using an ultrafiltration system from Romicon (Wooburn, MA, USA). Total nitrogen was determined using the Microkjeltec system from Tecator (Höganäs, Sweden). Lactose was analysed in an HPLC system from Merk using an Aminex HPX-87X column from BioRad (Richmond, CA, USA). Dynamic rheological characterization was performed with a controlled stress rheometer Carri-Med CS-50 (Surrey, UK) fitted with a cone-and-plate device (gap of 118 μm, angle of 4° and cone diameter of 5 cm).

2.3. Microorganism

The strain of *Rahnella aquatilis* (ATCC 55046) employed to bring about the fermentation process was kindly supplied by the American Type Culture Collection (Baltimore, MA, USA).

2.4. Preparation of fermentation media

Plain bovine whey was previously skimmed down to traces of fat. All media prepared with whey were then pasteurized for 15 min at 62°C (to severely reduce the viability of, and hence reduce any effect brought about by enzymes released by, the natural microflora) and hydrolysed with Protease A (at the ratio of 40 g kg^{-1} of protein) for 4 h so as to increase the fraction of nitrogen available for fermentation and avoid precipitation of proteins during posterior whey sterilization. Before inoculation, all media were finally sterilized at 110°C for 20 min. One batch of whey was added with NaCl at the level 2% (w/v) before pasteurization. The synthetic media, composed of lactose, $(NH_4)_2SO_4$, KH_2PO_4, K_2HPO_4, $MgSO_4$, $CaCl_2$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, $CuSO_4$, $NaMoO_4$, yeast extract and NaOH was prepared as described by Flatt et al. (1992a). The permeate was obtained by ultrafiltration of untreated whey using a hollow fiber cartridge characterized by a molecular weight cut-off of 50 000 Da.

2.5. Performance of chemical analyses

The moisture content of whey was determined according to the IDF method (FIL/IDF 4, 1958). The nitrogen contents were determined according to the IDF method (FIL/IDF 20B, 1993) adapted to micro conditions by using one tenth of sample aliquots and reagents. The ash content was determined using the official method (AOAC, 1997). Lactose was quantified by HPLC analysis using an ion exchange column; the separation conditions were a flow-rate of 0.6 ml min^{-1} of 5 mM H_2SO_4 as eluant, 50 μl as injection volume, 60°C as separation temperature and refractive index at 30°C as detection method.

2.6. Performance of fermentation

Fermentations were carried out in a Braun Biostat B (München, Germany), a 2 l fermentor filled with 1.5 l of fermentation broth for each batch. The experiments were carried out at 26°C, a stirring rate of 800 rpm and a pH of 7.0. Sterile air (in the case of aerobic fermentation) or nitrogen (in the case of anaerobic fermentation) was bubbled at a flow-rate of 2 l min^{-1}. Silicone was added to the initial medium as antifoam agent at the concentration of 0.4 g l^{-1}. A 5% (v/v) preparation of standard inoculum (*R. aquatilis* in the late exponential growth phase) was used to start up every fermentation, which took place for 48 h prior to polysaccharide harvest.

2.7. Recovery of polysaccharide

The polysaccharide fraction was recovered from the medium by precipitation with cold absolute ethanol at the ratio 1:1 (v/v). To speed up thermal equilibration, so promoting precipitation, the medium was stored at 4°C for 24 h prior to addition of ethanol. The precipitate was collected by filtration, under a vacuum, through a porous glass filter, washed with ethanol and lyophilized; the clean precipitate was then ground to a fine powder and stored before further physical analysis.

2.8. Performance of rheological analyses

The polysaccharide fraction recovered from each fermentation batch was solubilized in deionized water at the level

1.5% (w/w) so as to form a stock solution; this solution was then diluted to several concentrations, viz. 1.0%, 0.75%, 0.5%, 0.25% and 0.10% (w/w), and pH was in each case adjusted to 7.0 with NaOH. Solutions containing 1% (w/w) polysaccharide fraction were also adjusted to several pH values (3.0, 5.0, 9.0 and 11.0) with either NaOH or HCl as appropriate. Solutions containing 1% (w/w) polysaccharide fraction were also mixed with solutions of different molarities of KCl or $CaCl_2$ in order to obtain solutions of 0.5% (w/w) polysaccharide fraction with several ionic strengths (associated with set concentrations of 0.02, 0.1, 0.5 and 1.0 M). All samples were analysed under steady shear flow using a controlled stress rheometer with cone-and-plate geometry. Steady shear rates were applied in a continous manner either at increasing or decreasing torque.

3. Results and discussion

The plain bovine whey, the whey permeate and the synthetic medium used in the experiments possessed, respectively, 4.34%, 4.09% and 4.49% (w/v) of lactose, 0.85%, 0.24% and 0.36% (w/v) of protein, 93.89%, 94.98% and 95.37% (w/v) of moisture and 0.56%, 0.46% and 0.53% (w/v) of minerals. These values are within those usually found for whey of similar origins (Schwartz and Bodie, 1986). It is important to point out the significant difference between these three feedstocks in what concerns the carbon:nitrogen molar ratio (ca. 14:1, 46:1 and 34:1, respectively). The polysaccharide was recovered from the fermention media without extensive purification in order to more closely mimick a technological process with potential commercial applicability, following the same rationale that led to decision on use of actual whey rather than a synthetic medium only. The cells could not be extracted from the polysaccharide fraction harvested due to the high viscosity of the final media; if separation of cells were attempted by centrifugation, destabilization of the polysaccharide network would likely occur (Martino et al., 1991) and would certainly jeopardize significance of the experimental results. However, the precipitation steps of polysaccharide brought about by alcohol were expected to eventually eliminate most viability of said cells (Smiley, 1966; Rocks, 1971), so their interference in the parameters analysed was virtually negligible. The composition of the polysaccharide fractions recovered from the fermented media is listed in Table 1. Lactose was not detected in the samples, as expected, because only traces of lactose could already be observed for the broth fermented for 48 h. The high amounts of protein measured in the precipitates obtained from plain whey is due to its original high content, part of which was eventually converted into biomass entrapped in the gum (Brown and Lester, 1980); the lowest protein contents were obviously those of the whey permeate. The ash content of the precipitates, which is of the same order of magnitude of that obtained by Shams and Jaynes (1983), was also highest for the whey-based media because the organic solvent used to selectively precipitate the polysaccharide concomitantly precipitated the entrapped proteins and minerals (mostly in salt form) (Martino et al., 1991). The rheological properties of aqueous solutions prepared a posteriori using the crude polysaccharide recovered from each fermentation medium were measured in order to assess to what extent the various nutrient compositions prevailing during fermentation can alter the metabolism of the selected bacterial strain. In spite of several compounds present together with polysaccharide in the precipitates recovered from all media, it is possible to closely associate the rheological characteristics measured to the presence of polysaccharide because, as will be seen below, samples with different concentrations of proteins and minerals originate solutions that display close rheological properties. All polymer solutions analysed showed a shear-thinning behaviour (as apparent in Fig. 1), i.e. the viscosity decreases when the shear rate increases, in a way similar to what happens with several other microbial polysaccharides (Smiley, 1966). One consequence of this property is that lactan will not exhibit a slime mouth feel; such rheological properties thus potentiate several industrial applications, e.g. as thickening, suspending, emulsifying or stabilizing agent (Smiley, 1966; Rocks, 1971). As was observed with the fermention media themselves, the solutions of the polysaccharide fraction recovered from fermented whey containing 2% salt and plain whey fermented under anaerobic conditions exhibited lower viscosities than those measured for the other media. For all solutions, as expected, the viscosity increased with increasing polysaccharide concentration, which confirms that the polysaccharide itself present in the precipitates is a major player with respect to the rheological properties measured; a typical example of this trend is depicted in Fig. 2 for the

Table 1
Average composition of crude material recovered as precipitate from the various fermentation media

	Content			
Fermentation medium	Protein % (w/w)	Moisture % (w/w)	Ash % (w/w)	Polysaccharide% (w/w)
Synthetic medium	10.94	7.56	20.22	61.28
Plain whey under aerobiosis	17.93	10.06	12.42	59.59
Plain whey under anaerobiosis	28.19	6.24	21.94	43.63
Whey with 2% NaCl	25.40	7.51	21.49	45.60
Whey permeate	8.42	10.96	10.91	69.71

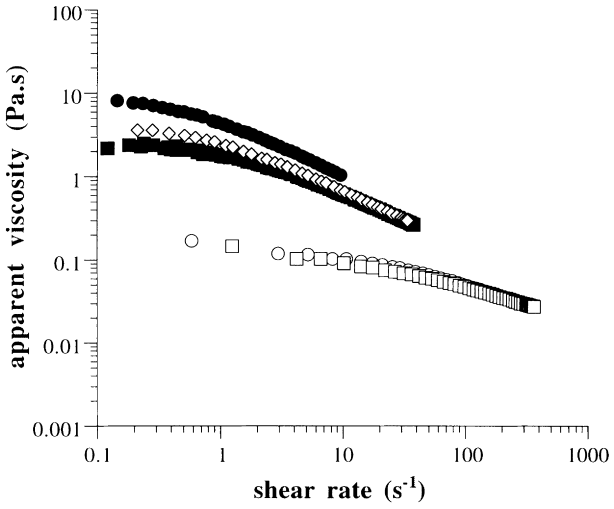

Fig. 1. Flow curves, as viscosity versus shear rate at 20°C of 1% (w/w) aqueous solution of polysaccharide obtained from: synthetic medium (■); whey permeate (●); plain whey under aerobiosis (◇); plain whey under anaerobiosis (○); and whey with 2% NaCl (w/v) (□).

gum fraction obtained from plain whey fermented under aerobic conditions. However, the differences in viscosity cannot be explained simply by differences in polysaccharide concentration. The lowest polysaccharide content in Table 1 (for culture in plain whey under anaerobiosis) is about 60% of the highest value (for whey permeate), but the viscosities at low shear rate differ by almost two orders of magnitude. By comparison, the flow curves in Fig. 2 indicate that a similar reduction in viscosity for a single preparation would require the concentration to be reduced by more than a factor of 3 [from 1.5% (w/w) to somewhere between 0.5% and 0.25% (w/w)]. The hypothesis that different compositions of the fermentation media may differently influence polymerization of polysaccharide is supported by the observed behaviour of the zero-shear rate viscosity as function of the polymer concentration (see Fig. 3). Although the

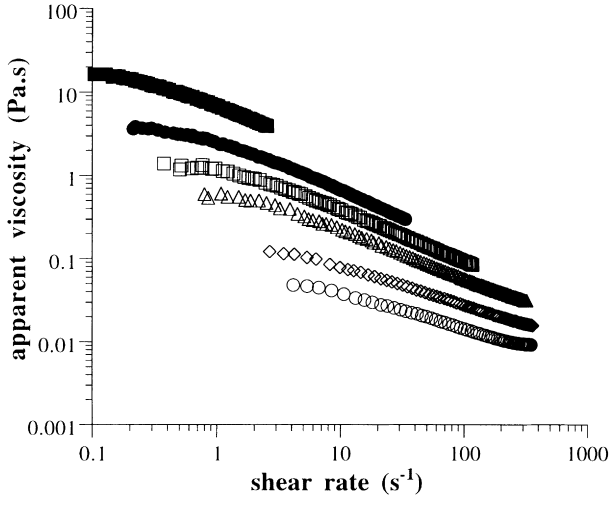

Fig. 2. Flow curves, as viscosity versus shear rate at 20°C of aqueous solutions of polysaccharide obtained from plain whey under aerobiosis: at 0.1% (○); 0.25% (◇); 0.5% (△); 0.75% (□); 1.0% (●); and 1.5% (w/w) (■).

observed behaviours for the polysaccharide fractions obtained from synthetic medium, whey permeate and plain whey under aerobiosis are very close to those observed for other disordered polysaccharides (Morris et al., 1981), the same does not strictly hold for those fractions obtained from whey added with NaCl and from plain whey under anaerobiosis. However, the limit concentration (c^*) seems to be similar in all cases. Other components entrapped in the polymer fraction, e.g. polypeptides, may also play a role on the rheological behaviour of these polymer factions.

Addition a posteriori of chloride salts (e.g. KCl and $CaCl_2$) to the aqueous solutions of polysaccharide led to decreases in viscosity, but those decreases were virtually identical irrespective of salt concentration (see Fig. 4); these findings are in agreement with those reported by Flatt et al. (1992a). This behaviour could probably be accounted for by contraction of the polysaccharide chain triggered by electrolyte-induced charge shielding, which is known to reduce repulsion between anionic moieties (e.g. galacturonic acid) within the polysaccharide chain (Flatt et al., 1992a). As observed in Fig. 4, at lower shear rates the initial (or newtonian) plateau was observed only for those samples with higher polysaccharide contents (i.e. those obtained from plain whey fermented under aerobic conditions, from synthetic medium and from whey permeate), which is a consequence of the stronger intermolecular interaction observed when the polysaccharide concentration increases.

Changes of pH in the range 3–11 affected slightly the viscosity of the polysaccharide solutions, as shown in Fig. 5; however, the highest viscosities were obtained at pH 7 and the lowest at pH 3. Identical effects of pH were reported for other microbial polysaccharides (Smiley, 1966; Rocks, 1971) and might provide an indication of the usefulness of lactan if formulation of foods with stable characteristics over a wide range of pH is sought. The slight effect of pH can be somewhat implicated with the galacturonic acid residues present in the polymer chain (Flatt et al., 1992a) because the highest viscosities are observed at pH 7 at which the highest ionization of galacturonic acid residues is also observed.

4. Conclusions

Lack of molecular oxygen and presence of salt inhibit prodution of polysaccharide by *Rahnella aquatilis* from whey media. The main rheological characteristics observed for all solutions can, at least partially, be attributed to the polysaccharide itself, although other compounds that coprecipitate may also play a role in this respect. Solutions poorer in polysaccharide also exhibit lower viscosity, and a shear-thinning behaviour was observed in the whole concentration range tested. The ionic strength and pH have slight effects on the rheological behaviour of aqueous solutions of polysaccharide probably owing to the galacturonic acid residue-rich nature of said polymer.

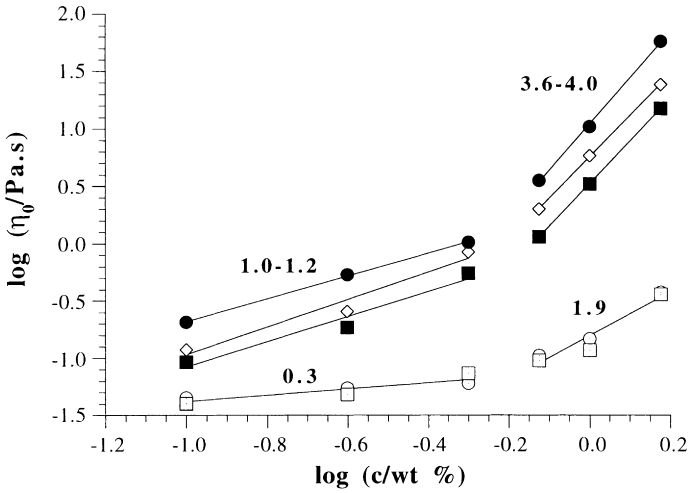

Fig. 3. Concentration dependence of 'zero shear' viscosity for: synthetic medium (■); whey permeate (●); plain whey under aerobiosis (◇); plain whey under anaerobiosis (○); and whey with 2% NaCl (w/v) (□). The slopes of the best linear fits are denoted in the plot.

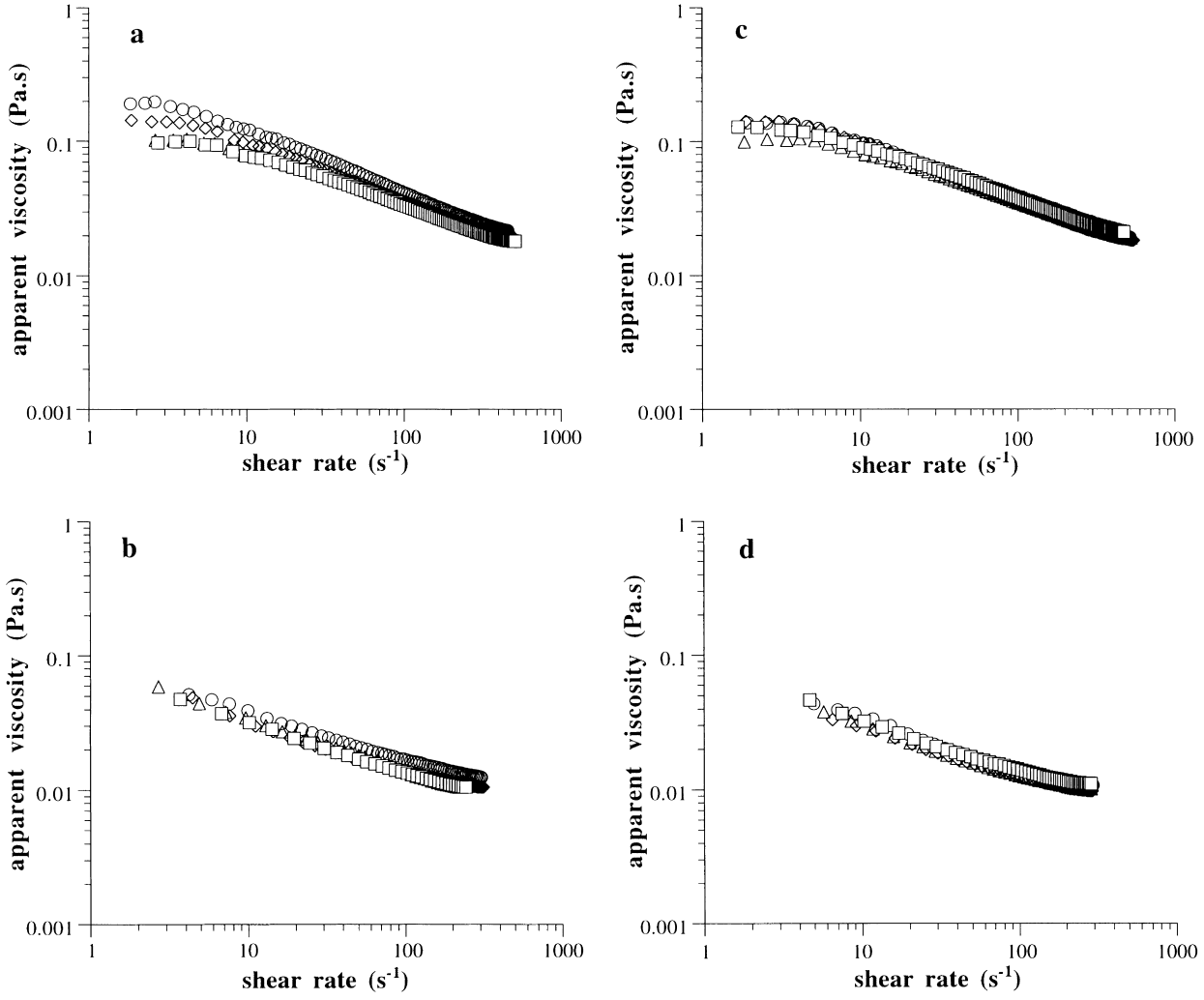

Fig. 4. Flow curves, as viscosity versus shear rate at 20°C of 0.5% (w/w) aqueous solutions of polysaccharide obtained from plain whey: (a) under aerobiosis and (b) under anaerobiosis, with 1.0% (w/w) KCl at 0.02 (○), 0.1 (◇), 0.5 (△) and 1.0 M (□); and from plain whey (c) under aerobiosis and (d) anaerobiosis, with 1.0% (w/w) $CaCl_2$ at 0.02 (○), 0.1 (◇), 0.5 (△) and 1.0 M (□).

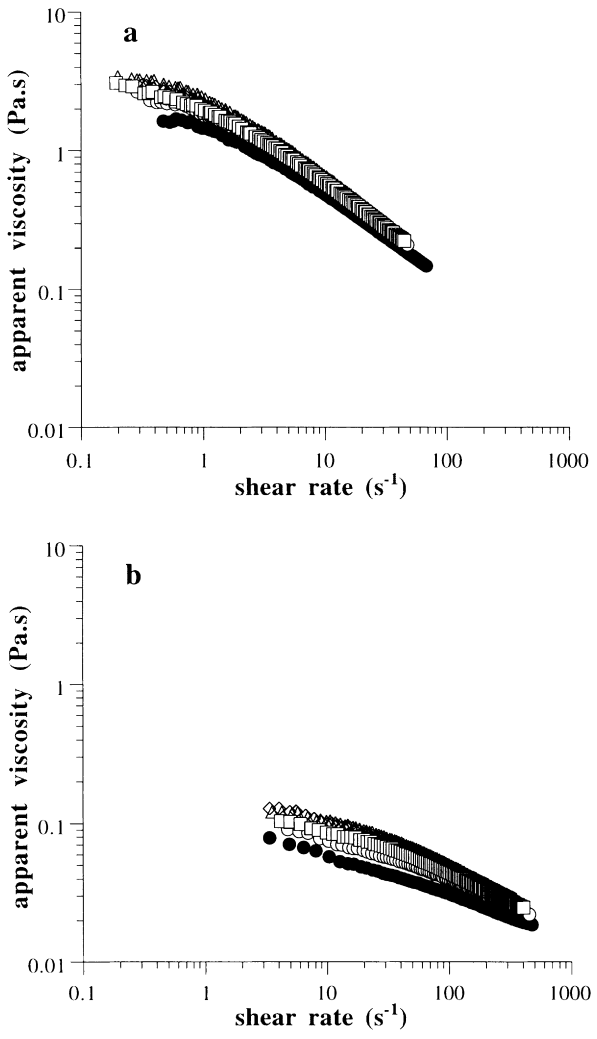

Fig. 5. Flow curves, as viscosity versus shear rate at 20°C of 0.5% (w/w) aqueous solutions of polysaccharide obtained from plain whey: (a) under aerobiosis and (b) under anaerobiosis, at pH 3 (●), 5 (○), 7 (△), 9 (◇) and 11 (□).

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References

AOAC. (1997). *Official Methods of Analysis*. Maryland: Association of Offical Analytical Chemists.

Brown, M. J., & Lester, J. N. (1980). *Appl. Environ. Microbiol., 40*, 179.

Cerning, J., Bouillanne, C., & Desmazeaud, M. J. (1986). *Biotechnol. Lett., 8/9*, 625.

Dlamini, A. M., & Peiris, P. S. (1997). *Appl. Microbiol. Biotechnol., 47*, 52.

Duncan, L. K., & Seeley, H. W. (1965). *J. Gen. Microbiol., 40*, 297.

Flatt, J. H., Hardin, R. S., Gonzalez, J. M., Dogger, D. E., Lightfoot, E. N., & Cameron, D. C. (1992). *Biotechnol. Prog., 8*, 327.

Flatt, J. H., Cooper, T. A., Gonzalez, J. M., Dogger, D. E., Lightfoot, E. N., & Cameron, D. C. (1992). *Biotechnol. Prog., 8*, 335.

IDF. (1958). *Determination of Dry Matter in Cheese and Processed Cheese: Standard 4*. Brussels: International Dairy Federation.

IDF. (1993). *Determination of Nitrogen Content: Standard 20 B*. Brussels: International Dairy Federation.

Lawson, C. J. & Symes, K. C. (1982). US Patent 4,338,432.

Le Duy, A., Yarmoff, J. J., & Chagraouj, A. (1983). *Biotechnol. Lett., 5*, 49.

Martino, M., Youssef-Hakimi, N., Daeschel, M. A., & Torres, J. A. (1991). *Rev. Agroquím. Tecnol. Aliment., 31/3*, 379.

Morris, E. R., Cutler, A. N., Ross-Murphy, S. B., & Price, J. (1981). *Carbo. Polym., 1*, 5.

Racine, M., Dumont, J., Champagne, C. P., & Morin, A. (1991). *J. Appl. Environ. Bacteriol., 71*, 233.

Reddy, M. S., Washam, C. J., Reinbold, G. W., & Vedamuthu, E. R. (1973). *J. Milk Food Technol., 36*, 200.

Righelato, R. C. & Deaven, L. (1978). US Patent 4,130,461.

Rocks, J. K. (1971). Xanthan gum. *Food Technol., 25*, 476.

Shams, M. A., & Jaynes, H. O. (1983). *J. Food Sci., 48*, 208.

Schwartz, R. D., & Bodie, E. (1986). *Appl. Environ. Microbiol., 49*, 203.

Smiley, K. L. (1966). *Food Technol., 20*, 112.

Stauffer, K. R., Leeder, J. G., & Wang, S. S. (1980). *J. Food Sci., 45*, 946.

Stauffer, K. R., & Leeder, J. G. (1978). *J. Food Sci., 43*, 756.

Vedamuthu, E. R., & Neville, J. M. (1986). *Appl. Environ. Microbiol., 51*, 677.