European J Appl Microbiol Biotechnol (1982) 16:23–27

European Journal of Applied Microbiology and Biotechnology

The Use of Extraction Methods for the Quantification of Extracellular Polymer Production by *Klebsiella aerogenes* Under Varying Cultural Conditions

Thomasine Rudd, Robert M. Sterritt, and John N. Lester

Public Health Engineering Laboratory, Imperial College, London, SW 7 2BU, United Kingdom

Summary. A combination of ultrasonication at 18 W for 10 min and centrifugation at 33,000 g for 60 min was found to achieve the highest recovery of extracellular polymer from *Klebsiella aerogenes*, NCTC 8172, allowing both soluble and colloidal phases to be separated. Estimates of the total polymer present, made by gravimetry and by acid hydrolysis, indicated that polymer production was greater in continuous culture than in batch culture, and increased in continuous culture as the dilution rate decreased from 0.5 h^{-1} to 0.01 h^{-1}. Extraction of polymer from these cultures varied in efficiency; up to 73% of the total polymer was extracted at low dilution rates where the colloidal form of polymer was predominant, but only 22% was extracted at high dilution rates where the majority of the polymer was in the soluble phase. Consequently, this extraction method was considered to be unsuitable as a quantitative measure of polymer production.

Introduction

Bacterial extracellular polymers have been implicated in the removal of heavy metals from settled sewage in the activated sludge process (Dugan and Pickrum 1972; Brown and Lester 1979; Brown and Lester 1982) which is important in maintaining the quality of receiving waters and preventing the infringement of standards set for surface water which is to be abstracted for drinking water (European Economic Community 1975) and for potable supplies (World Health Organisation 1971; European Economic Community 1980). The role of extracellular polymers may be investigated using continuous cultures of sewage microorganisms grown at low dilution rates in a chemostat to simulate conditions in an activated sludge plant (Postgate 1973).

The extraction methods most frequently used in the study of polymers have been summarised by Novak and Haugan (1981) and their efficiencies in producing extracellular polymer uncontaminated by intracellular material have been compared by Brown and Lester (1980). Few authors, however, have attempted to determine the total quantity of polymer present and subsequently the percentage extraction efficiency of the methods employed, hence the present study was undertaken in order to assess the efficiency of extraction based on the method recommended for pure cultures by Brown and Lester (1980).

Materials and Methods

Bacterial Cultures. Two pure cultures of *K. aerogenes*, a polymer-forming capsulated strain, NCTC 8172, and a non-capsulated strain, NCTC 9528, were grown in batch culture in a shaking incubator at 70 rev/min, at a temperature of 25 °C ± 2 °C maintained by a thermostat and 500 W fan heater. Two successive subcultures of 125 ml in 2 l conical flasks were made before inoculation of triplicate 200 ml cultures which were harvested after 5 d.

Continuous culture of both strains was carried out using an LKB 1601 Ultroferm fermentation system (LKB Instruments Ltd., Selsdon, Surrey) operated at 25° ± 0.2 °C, pH 6.8 and a dissolved oxygen concentration of approximately 6.3 mg/l. An equilibrium period equivalent to three volume changes was allowed prior to sampling at each dilution rate. The medium contained the following A.R. components (g/l): sucrose, 10; $(NH_4)_2SO_4$, 0.3; $NaH_2PO_4 \cdot 2H_2O$, 2; $K_2HPO_4 \cdot 3H_2O$, 2; K_2SO_4, 1; NaCl, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 6H_2O$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.001.

Culture samples for viable count were serially diluted in 0.1 M phosphate buffer, plated onto Casitone-glycerol-yeast

Offprint requests to: J. N. Lester

0171-1741/82/0016/0023/$01.00

Table 1. Total and extractable extracellular polymer in batch and continuous cultures of *Klebsiella aerogenes* NCTC 8172

Fraction determined	Method	Batch culture		Continuous culture[a]	
		Polymer (mg/l)	Protein	Polymer	Protein
Total	Cell dry weight	728.8 (7.5)[b]		2,628.9 (4.3)	
Total	Hydrolysis	323.2 (8.1)		1,966.5 (4.3)	
Residual	Filtration	25 (9.6)	16.85 (4.2)	18 (2.3)	100 (1.4)
Extracted soluble	18 W ultrasonication 10 min centrifugation	24 (5.4)	15.17 (3.4)	27.4 (16.6)	111 (13.1)
Extracted soluble	80 W ultrasonication 10 min centrifugation	25.8 (5.6)	12.5 (3.1)	135.5 (69)	160 (29.7)
Extracted soluble	18 W ultrasonication 60 min centrifugation	44.8 (7.0)	21.6 (1.2)	57.4 (12.4)	84.3 (8.6)
Colloidal		245.9 (0.6)	–[c]	713 (5.8)	–
Total		322 (6.9)	–	751.7 (3.2)	–

[a] dilution rate 0.05 h^{-1}
[b] mean and relative standard deviation of nine replicates
[c] not determined; interference in u.v. absorption due to presence of colloid

extract agar (Pike and Carrington 1971) and incubated at 25 °C for 24 h. Cell dry weight was determined by filtering samples through a 0.2 μm micropore cellulose acetate filter (Amicon, Woking, Surrey) and weighing the residue after drying to constant weight at 105 °C.

Polymer Extraction. Samples were ultrasonicated for 10 min in a Kerry 125 ultrasonic bath (Kerry Ultrasonic Ltd., Hitchin, Herts.) regulated to produce a power output of 18 or 80 ± 5 W. An MSE Hi-spin 21 centrifuge (MSE, Crawley, Sussex) was used to centrifuge samples at 33,000 g for 10 min. The pellets were resuspended and centrifuged for a further 10 or 60 min to increase the shear force exerted on the cells. Supernatants were filtered through a 0.2 μm micropore filter to remove residual cells.

Polymer Recovery. Extracted samples were purified either by gel filtration using a 154 ml column of Sephadex G50 (Pharmacia Fine Chemicals, Hounslow, Middlesex) using 0.02 M NaCl as eluent and a flow rate of 19.8 ml/cm^2/h at room temperature, or by dialysis in visking tubing size 3-20/32 (Medicell International, London) against running tap water for 12 h and two changes of distilled water for 36 h at 8 °C. Dialysis was found to remove > 99.4% of residual sucrose.

Polymer Assay. Extracted extracellular polymer was estimated as glucose equivalents (Brown and Lester 1980) using the phenol/sulphuric acid method of Dubois et al. (1956). Protein concentrations were determined as a measure of cell lysis caused by extraction using the method of Lowry et al. (1951). Total polymer was estimated by hydrolysing dialysed culture samples with an equal volume of 6 M HCl for 5 h in a boiling water bath. The hydrolysates were cooled, neutralised with 6 M NaOH and filtered. Polymer determination after hydrolysis of the capsulated culture was corrected with that of the non-capsulated strain in order to account for contamination by intracellular polysaccharide.

Results

Evaluation of Extraction Techniques

Various combinations of centrifugation and ultrasonication were investigated. Ultrasonication at 18 W followed by 10 min centrifugation at 33,000 g and filtration extracted very little polymer from either culture, the majority of the yield being residual. Increasing the ultrasonication to 80 W had little effect on the batch culture, but caused considerable disruption of the continuous culture with an increase in both polymer and protein yield. These increases were not proportional; more polymer was released in relation to residual values than protein. The large relative standard deviation however, indicates that the method was inconsistent, yields from triplicate samples varying from 30-691 mg/l.

During the course of these extractions, it was observed that after filtration of the supernatants, a clear gel layer remained on the filter paper. This layer could be removed and was resuspended in a volume of distilled water equal to that of the filtrate, to ascertain its concentration in the original sample. The suspension gave a positive reaction to the phenol/sulphuric acid assay, and on passage through a Sephadex G50 column was eluted with 95% recovery at the void volume suggesting a relative molecular mass exceeding 10^4. It could be refiltered and resuspended with a recovery of 80%, and was considered a colloidal form of bacterial extracellular polymer. As an alternative to filtration, which retained this fraction of the extracted

Table 2. Total, soluble and colloidal extracellular polymer extracted from continuous culture of *Klebsiella aerogenes* NCTC 8172 over a range of dilution rates

Dilution rate (h^{-1})	Polymer extracted (mg/l)	% Polymer extracted as:		% Extracted polymer recovered after separation of two fractions
		Soluble	Colloidal	
0.01	2,580	7	75	82
0.05	647.8	9	77	86
0.10	219.3	17	62	79
0.25	128.4	91	23	114
0.50	57	95	14	109

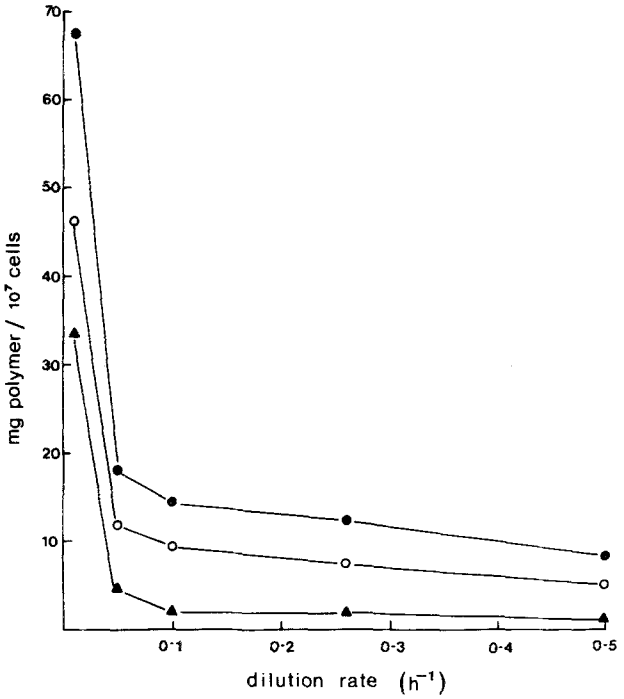

Fig. 1. The influence of dilution rate on total and extractable extracellular polymer from continuous culture of *Klebsiella aerogenes* NCTC 8172. ▲, quantity of polymer extracted; ●, total polymer estimated by cell dry weight; ○, total polymer estimated after acid hydrolysis

polymer, prolonged centrifugation was used to produce a cell free supernatant from which the fraction could be isolated. This method proved satisfactory for continuous cultures at all dilution rates except 0.01 h^{-1}, where due to the high viscosity of the culture the small volume of supernatant produced was difficult to separate by decantation.

Compared to the method incorporating filtration, a combination of 18 W ultrasonication and 60 min centrifugation produced 13-fold and 27-fold increases in the final concentration of extracellular polymer extracted from batch and continuous cultures respectively, consequently this method was employed for all subsequent extractions.

The Effect of Dilution Rate on Polymer Production and Extraction Efficiency

The absolute quantity of extracellular polymer extracted from continuous culture at five dilution rates (Table 2) increased as the dilution rate was decreased. Figure 1, however, indicates that the extraction per viable cell was relatively constant at the three higher dilution rates, and only began to increase at 0.05 h^{-1} and 0.01 h^{-1}. The percentage of the extracted fraction which was soluble was highest at the higher dilution rates, with a predominance of colloidal polymer at the lower dilution rates. The recovery of extracted polymer after separation of the soluble and colloidal components varied by ± 20%, the lower percentages occurring when the colloidal form predominated due to incomplete recovery of that fraction of the gel held within the filter paper.

Attempts to quantify total extracellular polymer were made by a gravimetric determination; the difference in dry weight per viable cell between the capsulated strain (growth rate = 1.1 generations/h) and the non-capsulated strain (growth rate = 1.15 generations/h) being assumed due to extracellular polymer, and after acid hydrolysis (Fig. 1). Although these two empirical methods did not give identical results, polymer determination after acid hydrolysis gave a consistent percentage of 65 ± 4% of that determined by cell dry weight. The fraction extracted at each dilution rate, however, was not a consistent proportion of the total polymer present, the extraction efficiency increasing as the dilution rate was reduced (Table 3).

Discussion

Dugan and Pickrum (1972) have emphasized that purification of polymer may alter its physical properties and thus its ability to adsorb metals. Non-disruptive methods such as those used here are likely to yield mostly slime polymers, as distinct from adherent capsular material (Wilkinson 1958). Slime polymers exist as colloids or in

Table 3. Percentage total polymer (determined by cell dry weight and after acid hydrolysis) extracted at varying dilution rates from continuous culture of *Klebsiella aerogenes* NCTC 8172

Dilution rate (h^{-1})	% Total polymer extracted	
	Cell dry weight	Hydrolysis
0.01	49.5	72.5
0.05	26.0	39.2
0.10	13.0	20.1
0.35	14.6	24.1
0.50	15.0	21.8

solution (Dugan 1975), yet are frequently extracted only as soluble forms, thus limiting further study to the investigation of soluble phase interactions only. The colloidal phase extracted in this study however, may bear a closer resemblance than the soluble fraction to the capsular polymer produced by bacteria in activated sludge.

It has been suggested that cation-anionic polysaccharide interactions are stronger in gels than in solution (Rendelman 1978a/b) thus the colloidal form of polymer, which has been shown to be predominant at low dilution rates, may be partly responsible for increased metal binding such as that noted by Brown and Lester (1982) for cadmium uptake by *Klebsiella aerogenes* grown in a chemostat at the lowest dilution rate studied ($0.06\ h^{-1}$).

The effect of dilution rate on the extractability of polymers suggests that its nature or resistance to degradation may vary with cultural conditions. Wilkinson (1958) reported that slime polymers cause increased viscosity in aqueous media, suggesting that in this case, where the culture viscosity increased markedly with decreasing dilution rate, there may have been a preponderance of capsular material at high dilution rates, and greater production of slime at lower dilution rates. It has been shown that some bacteria e.g. *Aerobacter cloacae* produce poorly defined capsules which slowly disintegrate and disperse (Wilkinson et al. 1954). The longer retention time at low dilution rates may allow the disintegration of capsules with release of detached polymer into the culture.

The sugars making up the polysaccharide have been identified for this strain of *K. aerogenes*; serotype K64, NCTC 8172 as mannose, glucose and rhamnose in a ratio of 2:1:1.5, with acetate, pyruvate and uronic acid residues (Churms and Stephen 1974). The phenol/sulphuric acid assay has differing sensitivities for different monosaccharides (Dubois et al. 1956), thus the estimation of polymer as glucose equivalents does not reflect the absolute quantity of polymer present. Applying a correction factor gives an increase of up to 10% in the absolute value of polymer determined after acid hydrolysis, although this figure is tentative since Churms and Stephen (1974), have suggested that the true ratio of glucose to mannose in the polysaccharide may be 2:3 or 2:4 due to the incomplete hydrolysis of aldobiouronic acid linkages involving mannose residues.

Acid hydrolysis did not give a total polymer value identical to the cell dry weight determination but it produced a consistent proportion, based on corrected values, of 71 ± 4% throughout the range of dilution rates studied, suggesting that it may prove a more suitable method for the quantification of total polymer than certain extraction methods, the efficiency of which varies depending on the cultural conditions.

Acknowledgement. Grateful acknowledgement is made to the Science and Engineering Research Council for receipt of financial support for this work.

References

Brown MJ, Lester JN (1979) Metal removal in activated sludge: the role of bacterial extracellular polymers. Water Res 13:817–837

Brown MJ, Lester JN (1980) Comparison of bacterial extracellular polymer extraction methods. Appl Environ Microbiol 40:179–185

Brown MJ, Lester JN (1982) Role of bacterial extracellular polymers in metal uptake in pure bacterial culture and activated sludge: II. Effects of mean cell retention time. Water Res (in press)

Churms SC, Stephen AM (1974) Studies of molecular-weight distribution for hydrolysis products from some *Klebsiella* capsular polysaccharides. Carbohydrate Res 35:73–86

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric methods for determination of sugars and related substances. Anal Chem 28:350–356

Dugan PR (1975) Bioflocculation and the accumulation of chemicals by floc-forming organisms. Report EPA600/275–032, Washington, D.C.

Dugan PR, Pickrum HM (1972) Removal of minerals ions from water by microbially produced polymers. Proc 27th Ind Waste Water Conf, Purdue University, Eng Ext Ser No 141:1019–1038

European Economic Community (1975) Council directive concerning the quality of water intended for the abstraction of drinking water in the member states. (75/440/EEC) Off J European Communities L194/26-L194/31

European Economic Community (1980) Council directive relating to the quality of water intended for human consumption. (80/778/EEC) Off J European Communities L229/11-L229/29

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275

Novak JT, Haugan B-E (1981) Polymer extraction from activated sludge. J Water Pollut Control Fed 53:1420–1424

Pike EB, Carrington EG (1972) Recent developments in the study of bacteria in the activated sludge process. Water Pollut Control 71:583–604

Postgate JR (1973) The viability of very slow-growing populations: a model for the natural ecosystem. Bull Ecol Res Committee (Stockholm) 17:287–292

Rendelman JA (1978a) Metal-polysaccharide complexes, Part I. Food Chem 3:47–79

Rendelman JA (1978b) Metal-polysaccharide complexes, Part II. Food Chem 3:127–162

World Health Organisation (1971) International Standards for Drinking Water, 3rd edn, Geneva

Wilkinson JF (1958) The extracellular polysaccharides of bacteria. Bacteriol Rev 22:46–73

Wilkinson JF, Duguid JP, Edmunds PN (1954) The distribution of polysaccharide production in Aerobacter and Escherichia strains and its relation to antigenic character. J Gen Microbiol 11:59–72

Received April 5, 1982