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A Comparison of the Poly(hexamethylenebiguanidinium chloride) Assay and a Neutral Equivalent Method for the Determination of Alginates in Industrial Liquors Extracted from Brown Seaweed

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SUMMARY

The poly(hexamethylenebiguanidinium chloride) assay and a neutral equivalent method have been used to estimate the sodium alginate content of industrial liquors extracted from brown seaweed. With the liquor samples examined, agreement to within 5% was achieved with the two methods under defined alginate concentration conditions. Statistical evaluation of the data using a paired comparison t-test has shown that a difference of only 0·10 ± 0·05 can be expected with 95% confidence, between the two methods in the analysis of liquor samples with sodium alginate contents in the range 2·00–3·83 mg ml^{-1}. In the majority of cases the PHMBH$^+$ Cl$^-$ method gave a slightly higher estimate that the neutral equivalent method. Consideration of the practical aspects of the two alternative methods allows this difference to be explained. The simplicity of the PHMBH$^+$ Cl$^-$ assay renders it suitable for the rapid screening of large numbers of alginate samples.

INTRODUCTION

Several algal and microbial polysaccharides, such as alginate and xanthan respectively, are produced on an industrial scale for use in food and non-food applications (Cottrell & Kovacs, 1977). An important part

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of the production process is the estimation of the polysaccharide in liquor solutions, which may arise by extraction of brown seaweed in the case of alginate or by fementation with *Xanthomonas campestris* in the case of xanthan. To date, few reports have been concerned with this analytical task. Precipitation of the polysaccharide from solution followed by quantification of the dry weight can be used but is time-consuming and contamination with other solids present in the liquor is likely. In the specific case of alginate, one traditional procedure (neutral equivalent method) which can be used involves conversion of the polysaccharide to alginic acid, followed by titration of the acidic groups with alkali to allow quantification (Achwal, 1981). Again, this method is time-consuming and further requires a large sample volume for analysis.

The basic chemical structure of alginate consists of varying lengths of three types of (1→4) linear blocks, poly-β-D-mannuronic acid, poly-α-L-guluronic acid and mixed blocks of the two uronic acids (Haug *et al.*, 1967*a, b*; Penman & Sanderson, 1972; Grasdalen *et al.*, 1979). Variations in the carbohydrate composition (D-mannuronic acid/L-guluronic acid ratio) of the polysaccharide can occur with both algal (Haug *et al.*, 1974) and bacterial (Haug & Larsen, 1971) alginates. Unfortunately, many of the methods which can be used for the quantitative determination of alginates in solution show variation in response with different sugars. This includes various colorimetric procedures employing such reagents as carbazole (Bitter & Muir, 1962) and 3-hydroxydiphenyl (Blumenkrantz & Asboe-Hansen, 1973). Unless the hexuronic acid composition of the alginate sample was known, results obtained with such methods would be difficult to interpret.

Recently, we reported (Kennedy & Bradshaw, 1984) a new, rapid method for alginate determination in solution using a polymeric cationic reagent (Fig. 1), poly(hexamethylenebiguanidinium chloride) [$PHMBH^+Cl^-$]. The procedure consists of the addition of a known excess of $PHMBH^+Cl^-$ in acetate buffer to alginate solutions to precipitate the sample quantitatively and thereafter measuring the UV absorption of residual $PHMBH^+Cl^-$ in the supernatant. Due to the simplicity of the procedure it was found to be a quick and convenient method of accurate alginate determination (Kennedy & Bradshaw, 1984). Further development of the $PHMBH^+Cl^-$ method resulted in a procedure suitable for the assay of alginate in liquors extracted from brown seaweed (Kennedy & Bradshaw, 1987). This work also indicated that the response of the method was independent of a hexuronic acid composition to the alginate sample.

We now report a detailed comparison of the $PHMBH^+Cl^-$ assay and the more traditional neutral equivalent method (Achwal, 1981) for the

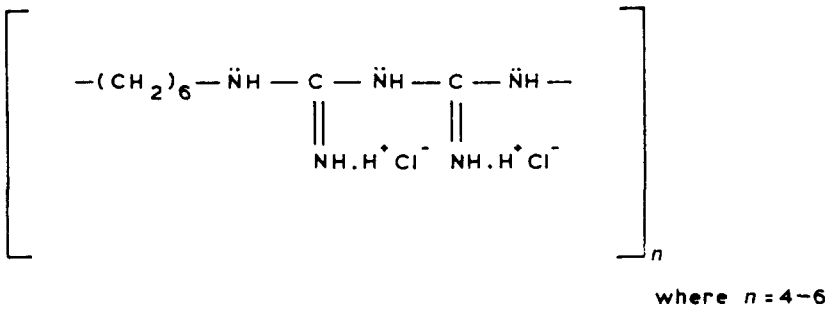

Fig. 1. Structure of poly(hexamethylenebiguanidinium chloride).

determination of sodium alginate in seaweed liquors. A large number of liquor samples have been analysed and statistical analysis performed on the results. The reasons for the observed difference between the two methods in their estimation of sodium alginate content are discussed.

EXPERIMENTAL

Materials and general methods

All powdered alginate samples and alginate liquors were obtained from Alginate Industries Ltd (now Kelco/AIL International Ltd), Girvan, UK. The moisture content of solid samples was assessed by drying at 61°C *in vacuo* over P_2O_5 for 24 h.

The poly(hexamethylenebiguanidinium chloride) was obtained under the trade name Vantocil IB from ICI Ltd, Pollution Control Division, Hyde, Cheshire, UK as a 20% (w/v) solution. Single wavelength UV measurements were performed on a Pye Unicam SP6-550 spectrophotometer.

Standard assay procedure

To duplicate aliquots (5 ml) of sodium alginate solutions (1–5 mg ml^{-1}), aliquots (10 ml) of 0·3% $PHMBH^+Cl^-$ in 1% sodium acetate were added with continuous magnetic stirring. This stage can be performed conveniently in Pyrex test-tubes (150 × 18 mm) using magnetic followers of 12 mm diameter. Agitation of the solutions was continued for a further 5 min to allow formation of the alginate-$PHMBH^+$ precipitates, which were recovered from the supernatant by low speed centrifugation (3000 rev; 5 min). The supernatants were retained and the UV absorb-

ance, after 100-fold dilution, measured at 235 nm against a distilled water blank. The calibration curves were constructed by plotting supernatant absorbance against sodium alginate concentration. The standard solutions were prepared allowing for the non-alginate material known to be present in the sample.

Assay procedure for alginate liquors

Samples of liquors (~ pH 10) were obtained as slurries and prepared for analysis by filtering through a cotton wool bed and then by adding formaldehyde or polyacrylamide, or a combination of these additives. The former additive was used as a preservative and the latter as a settling aid to remove insoluble materials. The liquors were then allowed to settle overnight and the supernatant decanted off, for analysis, from any residue which had been deposited.

Quadruplicate aliquots (5 ml) of alginate liquor were neutralized to pH 6·5 by addition of acetic acid solution (1% w/v; 0·4 ml). The liquor samples were stirred continuously and $PHMBH^+Cl^-$ solution (0·3%; 10 ml) added. The procedure was then continued using the standard assay method outlined above. It was necessary to add distilled water (0·4 ml) to the standard sodium alginate solutions to allow for volume difference.

Determination of alginate in liquors extracted from brown seaweed by the neutral equivalent method

Alginate determination was made by a method based on the work of Achwal (1981).

To 1 litre of alginate liquor pre-filtered with heavyweight nylon cloth, sodium hypochlorite (0·1 M; 10 ml) was added, mixed and the solution allowed to stand for 30 min. To the liquor sample, a mixture of calcium chloride solution (20% w/v; 15 ml)–acetic acid solution (50% w/v; 3 ml) was added slowly, under vigorous agitation. The formed calcium alginate precipitate was allowed to harden for 15 min, filtered through a nylon cloth and finally squeezed as dry as possible.

A 7·5 cm diameter sinter-glass (No. 1 porosity) filter funnel, fitted with a screw-clip, was placed upside down in a beaker of water. Water was sucked into the stem until full and the screw-clip closed. The funnel was then placed over a stand and the calcium alginate precipitate transferred quantitatively to it. Hydrochloric acid (1 M) was added to cover the calcium alginate and the precipitate teased with an ebonite rod. After 5 min contact time, the screw-clip was opened and the liquid allowed to

run off slowly. The leaching process was continued by topping up the funnel with acid and running off the liquid, until a negative calcium test was obtained [to 1 ml of the drainings, an equal volume of ammonium oxalate (saturated) solution was added. The mixture was allowed to stand for 2–3 min; a fine white suspension indicated the presence of calcium]. When the sample was free from calcium, the precipitate was washed with deionized water until free from acid (to 0·5 ml of drainings, 2–3 drops of Tropaeolin 00 indicator was added. A red colour indicated the presence of acid; a yellow colour indicated freedom from acid).

The sample was then transferred to a beaker by washing with a small amount of deionized water. After addition of a few drops of phenolphthalein indicator, the mixture was stirred mechanically with the aid of a glass stirrer and titrated with sodium hydroxide solution (0·5 M) until a permanent pink colour was achieved.

Using the assumption that the equivalent weight of sodium alginate is 216, the polysaccharide content of the liquor is calculated as follows:

$$\text{sodium alginate content} = \frac{T \times 0{\cdot}216 \times M}{10}\% \text{ (w/v)}$$

Where T = titre volume (ml) and M = molarity of NaOH solution.

RESULTS AND DISCUSSION

Recently, we reported on the initial development of the $PHMBH^+Cl^-$ assay for the estimation of alginate in liquors extracted from brown seaweed (Kennedy & Bradshaw, 1987). The assay has many useful features for alginate determination. In particular, assay response is easily reproducible and insensitive to variation of salt concentration, pH and temperature within defined limits (Kennedy & Bradshaw, 1984). Further, the assay response is independent of the hexuronic acid composition of the alginate sample (Kennedy & Bradshaw, 1987). Also, the presence of settling aids and preservatives added to the liquors does not cause interference to the assay, and the short time (15 min) required to perform the $PHMBH^+Cl^-$ assay renders it suitable for the screening of large numbers of samples.

In this present study we have analysed a large number of alginate liquor samples by the $PHMBH^+Cl^-$[1] assay and neutral equivalent method to compare the accuracy of the two methods and ascertain what difference, if any, in results can be expected. The latter method was chosen because it gives an absolute value and further, it will give the

same response with alginates of different D-mannuronic acid/L-guluronic acid ratio. The principle of the method involves precipitation of alginate as the calcium salt, conversion to the H^+ form (alginic acid) with dilute acid and finally titration of the newly formed acidic groups with alkali (Achwal, 1981). From the quantity of alkali required, the equivalence of acid and hence the amount of alginate can be calculated.

The standard $PHMBH^+Cl^-$ procedure developed previously was used for the assay of the sodium alginate content of liquor samples (Kennedy & Bradshaw, 1984, 1987). This involved neutralization of the liquor sample with acetic acid followed by addition of a known excess amount of $PHMBH^+Cl^-$ to precipitate the alginate quantitatively. The precipitation step is carried out in the presence of acetate buffer to facilitate the complexation reaction and avoid any formation of turbidity in solution (Kennedy & Bradshaw, 1984). Since $PHMBH^+Cl^-$ has a UV absorption at 235 nm, the residual reagent in the supernatant can be used for the quantitative assessment of alginate. A typical calibration curve for 1–5 mg ml^{-1} sodium alginate using the $PHMBH^+Cl^-$ assay is shown in Fig. 2. A standard sodium alginate of known purity was used in the calibration of the assay, which can be readily reproduced (Kennedy & Bradshaw, 1984, 1986, 1987).

The results from the analysis of the alginate liquors by the standard $PHMBH^+Cl^-$ procedure for sodium alginate content are shown in Table 1. Each analysis was performed in quadruplicate, but only the average of the four estimations is shown in Table 1. For comparison, the estimation (average of triplicate analyses) of the sodium alginate content by the neutral equivalent method (performed by an independent laboratory) is also shown. All sodium alginate concentrations of samples are quoted as mg ml^{-1} on a dry weight basis. The results (Table 1) show that agreement to within 5% was reached in the analysis of the majority of the liquor samples. Further, with some liquor samples the agreement was considerably closer than 5%. These results confirm our earlier observations that good agreement can be reached between the $PHMBH^+Cl^-$ and neutral equivalent methods for the analysis of algal alginate production liquors (Kennedy & Bradshaw, 1987).

The difference between the sodium alginate value produced by the $PHMBH^+Cl^-$ method and the neutral equivalent method was examined statistically by a paired comparison *t*-test (Campbell, 1974). An estimate of 0·16 was obtained for the standard deviation(s) of difference between the two methods. Further, the average deviation ($\bar{z}$) between the two methods was calculated to be 0·10. From the values of s and $\bar{z}$, it was derived that a value of $0{\cdot}10 \pm 0{\cdot}05$ could be expected, with 95% confidence, for the deviation between the $PHMBH^+Cl^-$ and neutral equiva-

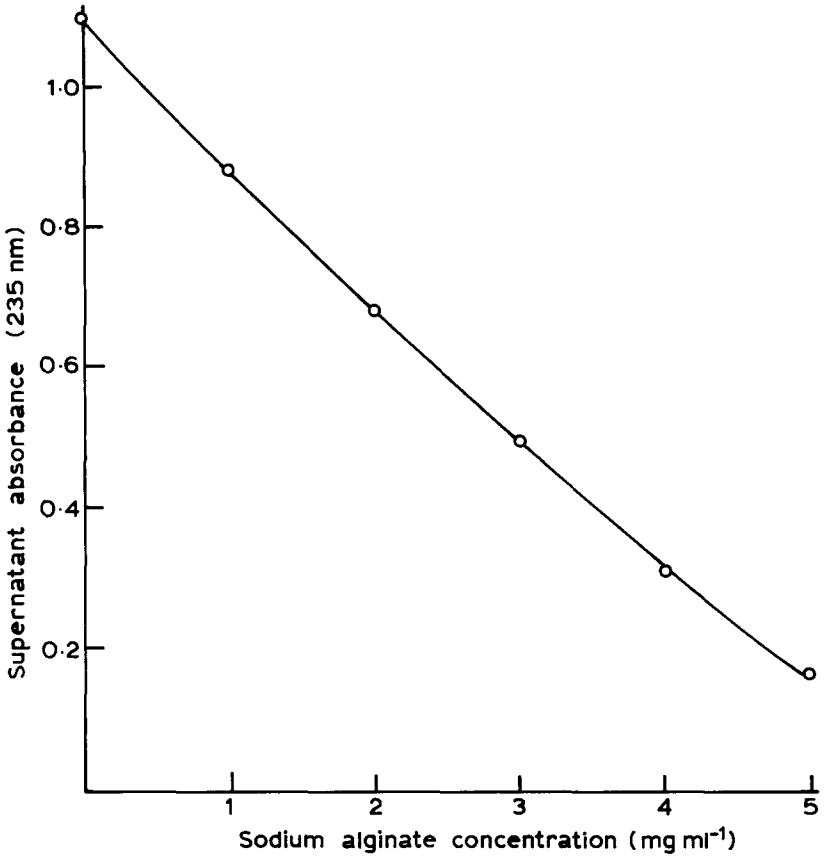

Fig. 2. Typical calibration curve for the response of sodium alginate (1–5 mg ml^{-1}) to the standard $PHMBH^+Cl^-$ assay. Values shown are for absorbance of supernatant (diluted 100-fold) with variation of sodium alginate (5–25 mg) precipitated by $PHMBH^+Cl^-$ (30 mg).

lent methods for the sodium alginate content of a liquor, whose concentration of sodium alginate lay between 2·00 and 3·83 mg ml^{-1}. The aforementioned limits of concentration cover the range of alginate liquors which were analysed. The results of the statistical analysis confirmed that the agreement between the two methods for the assay of alginate liquors is good.

The result for the estimation of sodium alginate content by the $PHMBH^+Cl^-$ assay was plotted against the result obtained by the neutral equivalent method for each liquor sample (Fig. 3). Thus, the line $y = x$ shown in the figure corresponds to the occasions where the estimates by both methods were identical. Figure 3 highlights that for the

TABLE 1
Analysis of Algal Alginate Liquors for Sodium Alginate Content by $PHMBH^+Cl^-$ and Neutral Equivalent Methods

Sodium alginate content (mg ml^{-1})			
$PHMBH^+Cl^-$ [a]	*NEM [b,c]*	*$PHMBH^+Cl^-$ [a]*	*NEM [b,c]*
2·32	2·35	3·00	2·92
2·34	2·50	3·31	3·26
2·65	2·66	2·98	2·88
3·12	3·21	2·68	2·65
3·02	3·29	3·21	3·12
3·09	3·21	3·72	3·42
2·48	2·18	2·75	2·69
3·74	3·54	2·90	2·81
3·70	3·57	2·10	2·00
2·16	1·98	2·11	2·00
2·31	2·04	2·00	1·98
2·34	2·07	2·64	2·52
2·52	2·47	2·73	2·39
2·16	2·20	2·72	2·63
3·23	3·48	2·82	2·42
2·42	2·18	2·59	2·47
3·06	2·97		

[a] Average of four estimates.
[b] Average of three estimates.
[c] Neutral Equivalent Method.

majority of results (80%), the $PHMBH^+Cl^-$ method produced a higher estimate than the neutral equivalent method. A plot (not included here) of the difference between the two methods against the average value indicated that variation was random. However, it is noticeable (Fig. 3) that agreement between the two methods is better at higher concentrations (2·50–3·83 mg ml^{-1}) of sodium alginate than at lower concentrations (2·00–2·50 mg ml^{-1}). The trend of results for the analysis of alginate liquors showed that the $PHMBH^+Cl^-$ method, in general, gave a slightly higher value for the sodium alginate concentration than the neutral equivalent method. It was found that a critical examination of the practical steps in both methods helps to explain this deviation. The neutral equivalent method involves an initial 'cleaning up' of the liquor with filtration and NaOCl addition, followed by precipitation of the alginate as the calcium salt. The precipitation step is a critical stage, since if any alginate is left in solution it will lead to an underestimation of poly-

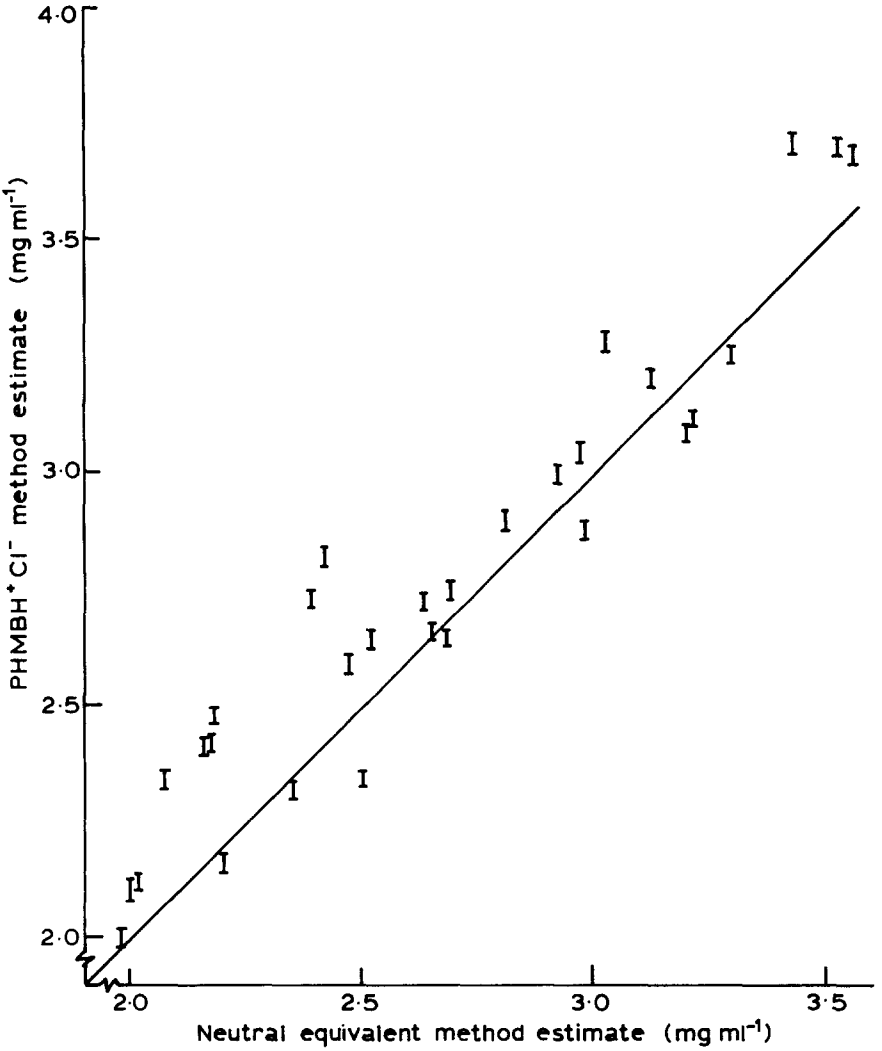

Fig. 3. A plot of $PHMBH^+Cl^-$ method estimation (y) against neutral equivalent method estimation (x) for the sodium alginate content of liquors extracted from brown seaweed. The line $y = x$ is included in the figure. Standard error ($\pm 0{\cdot}02$) about the mean is shown for each point.

saccharide concentration. In particular, it is known that the neutral equivalent procedure gives low readings for low molecular weight alginates (Bradshaw, 1982) due to incomplete precipitation and a certain amount of such material would be expected in the liquors.

The conversion stage of calcium alginate to alginic acid would also tend to produce underestimations rather than overestimations in the

assay. In particular, low molecular weight alginate material is known to be lost during the leaching stage of the polysaccharide (Bradshaw, 1982). Tests are used in the neutral equivalent assay to ensure that complete conversion of calcium alginate to alginic acid takes place and also that the alginic acid precipitate is washed free of excess acid and therefore those possible sources of error have to be accepted as minimal. The conversion stage of this method is partly a purification step and should remove impurities for the final titration stage. However, if any protein material was present in the precipitate, interference to the titration could occur, since it would be capable of forming carboxylic acid groups in the H^+ form.

The final step of the neutral equivalent assay involves titration of the carboxylic acid groups (H^+ form) in the alginic acid with alkali. Some error may occur with the titration procedure using phenolphthalein as it does not give a permanent end-point. However, from the above, it would be expected that there was an overall tendency for the neutral equivalent method to lead to an underestimation of the sodium alginate content of alginate liquors.

Since the $PHMBH^+Cl^-$ assay involves a simple procedure, there is less scope for errors to arise from practical aspects of the method. One possible source of error could be in the precipitation stage. In particular, if the $PHMBH^+Cl^-$ formed an insoluble complex with acidic material present in the liquors, other than alginate, such as protein or non-alginate polysaccharide this could lead to an overestimation of the sodium alginate content. However, our previous evidence suggests that this type of interference was minimal (Kennedy & Bradshaw, 1987). Also, the $PHMBH^+Cl^-$ method has previously been found to be suitable for low molecular weight alginate samples (Kennedy & Bradshaw, 1986). It is relevant to note that the final result produced by the $PHMBH^+Cl^-$ assay is always dependent upon the calibration curve. Since the purity of sodium alginate standards was assessed by the neutral equivalent method, this source of error can be discounted for causing deviations between the two methods.

In terms of practical aspects, the $PHMBH^+Cl^-$ method is more convenient than the neutral equivalent method for the analysis of alginate liquors. However, equally important, the $PHMBH^+Cl^-$ method shows reasonable agreement with the established neutral equivalent procedure for the sodium alginate content of production liquors. In general, the $PHMBH^+Cl^-$ assay produces slightly higher results than the neutral equivalent method. Sufficient liquor samples have been analysed in this detailed study to suggest that this is due to an inherent difference between the two methods. Certainly, it would have been surprising if two

such different procedures produced more or less identical values for the sodium alginate content of alginate liquors. Nevertheless, the reasons for the deviation between the two methods are evident from consideration of the practical aspects.

The proven reliability of the $PHMBH^+Cl^-$ assay reported here is sufficient to warrant its use in a large number of applications for acidic polysaccharides. This report has been concerned with alginate liquors from a mixed *A. nodosum–D. potatorum* seaweed origin, but application to alginate liquors produced from other seaweeds would be of interest. The rapid assessment of liquors for alginate content by the $PHMBH^+Cl^-$ method would be of considerable use for the monitoring of the production process and prediction of the amount of polysaccharide product to be expected. Using the $PHMBH^+Cl^-$ method, it is also possible to quantify the low amounts of alginate in waste liquors, a task which the neutral equivalent method is unable to perform (Bradshaw, 1982). The $PHMBH^+Cl^-$ assay is also of use for monitoring the production of microbial acidic polysaccharides and has been applied to *Azotobacter vinelandii* fermentation broths containing microbial alginate (Brivonese, 1985).

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