

Characterization of pullulans produced from agro-industrial wastes

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Pullulans produced from the *Aureobasidium pullulans* fermentation of various agro-industrial wastes were characterised in terms of carbohydrate analysis, heterogeneity and molecular weights. Grape skin pulp extract (GSPE), starch waste (SW), olive oil waste effluents (OW) and molasses (M) were used as substrates for the production of pullulan. A glucose based defined medium (D) was used for comparison purposes. Weight average molecular weights were determined using size exclusion chromatography/multiangle laser light scattering. In each of the ethanol precipitated substances the molecular weights were higher than that of the reference pullulan (Sigma), and the elution profiles indicated the polydisperse nature of the pullulans. When using D, the molecular weight of pullulan produced decreased with increasing fermentation time. Different substrates produced pullulans with different molecular weights. Of the substrates studied, D, GSPE and SW produced pullulans in high yields, which were relatively homogeneous, while OW alone or with M produced greatly heterogeneous ethanol agglutinating substances.

INTRODUCTION

Extracellular polysaccharide production is frequently encountered in many microorganisms. Many strains of bacteria, yeasts and fungi have been selected and are used commercially because they have been found to produce enough extracellular polysaccharide in broth culture to be of economic interest.

Pullulan is a homopolysaccharide which is of industrial interest and economic importance and is produced by the yeast-like fungus *Aureobasidium pullulans*. It is a glucan composed basically of maltotriose units linked by α -1,6-glycosidic linkages (Cattley, 1970, 1971). The relative molecular mass varies greatly from 5×10^4 up to 10×10^6 Da in some recently described strains (Pollock *et al.*, 1992). The structure and biosynthesis of pullulan have been studied extensively by Cattley (1971). Fermentation parameters such as temperature, pH, dissolved oxygen, agitation, and the influence of carbon and nitrogen sources, etc., have been studied by several investigators (Cattley, 1971; Imshenetsky *et al.*,

1981a,b; McNeil & Kristiansen, 1987, 1990; Wecker & Onken, 1991; West & Reed-Hamer, 1991). A thorough review of the application of *A. pullulans* in industry has been given by Deshpande *et al.* (1992).

Typical industrial uses of pullulan are as food coatings and packaging material; an ingredient of low calorie foods and as a starch substitute; films, with properties similar to poly(vinyl alcohol), but superior in many ways as well as being biodegradable; adhesives in the forms of pastes with water; construction material (after esterification) fibres similar in strength and elasticity to nylon; and a bulking agent and stabiliser for tablets in the pharmaceutical industry (Yen, 1974).

Pullulan can be synthesised from a variety of carbohydrate substrates incorporated into either defined (synthetic) or non-defined media. Within the latter are several agro-industrial wastes which have been shown to be suitable for pullulan production (Zajic *et al.*, 1979; Le Duy & Boa, 1982; Le Duy *et al.*, 1983; Shin *et al.*, 1989; Iniotakis *et al.*, 1991). Utilisation of these substrates would seem to be ecologically sound and

economically advantageous as they have low or even negative costs. In this way the potential of pullulan production from agro-industrial wastes is expected to lower the cost of production and seems to be a very promising ecologically and economically sound way of bioconversion.

However, the pullulan which is produced from different substrates may vary in purity and other physical and chemical characteristics. This is more pronounced when agro-industrial wastes are used as a carbon source for the fermentation. Some of these characteristics like the molecular weight are very important when commercialisation of the production of pullulan from these wastes is under consideration. This paper reports on the characterisation (in terms of heterogeneity and molecular weight) of the various pullulans produced from the fermentation of different agro-industrial wastes.

MATERIALS AND METHODS

Micro-organism

Aureobasidium pullulans, NRRLY-6220, was used for these fermentations. It was maintained on potato dextrose agar (Oxoid) at 4°C.

Inoculum

The inoculum was grown on the same medium as that used for fermentation. Three- to five-day-old inocula were used to inoculate the fermentation media at a ratio of 5% (v/v). Pullulans (ethanol precipitated substances) were derived from the fermentation of the following substrates. (a) Grape skin pulp extract (GSPE). One part hot water (65–70°C) was added to two parts grape skin pulp and stirred thoroughly. The solids were removed after settling for 30 min. (b) Starch waste (SW). A by-product of the potato crisp industry (supplied by Walker's Crisp Co., Leics., UK). A suspension of 35% dry solids was autoclaved and then partially hydrolysed with α -amylase, pullulanase and glucoamylase (Sigma). (c) Olive oil waste effluents (OW) from the Greek island of Evoia. It was diluted three parts OW to one part water. (d) Molasses (M). Beet molasses was used after dilution 1 : 10 with water. (e) Olive oil waste plus molasses (OW/M). Three parts of olive oil waste to one part of a 20% (w/v) molasses solution. (f) Defined medium (D). This was prepared as follows: (as %, w/v) yeast extract 0.3; malt extract 0.3; peptone 0.5; dextrose 1.0.

For the ethanol precipitated substances from corresponding substrates, abbreviations will be used in the text as in parentheses. All media were adjusted to pH 5.5 with 1.0 M HCl. Fermentations were carried out in 500 ml Erlenmeyer flasks containing 120 ml of medium.

Flasks containing the medium were autoclave sterilised at 121°C for 20 min. The fermentations were carried out at 28°C on a gyratory shaker at 200 rpm for 7 days. In the case of the defined medium there were also runs for 8, 34, 48, 72 and 120 h. In addition, a reference pullulan sample obtained from Sigma Chemical Co. (Poole, UK) was also analysed in the experiments.

Analytical methods

Isolation of crude pullulan samples from the cell-free culture medium was achieved by adding 2 vol of 100% ethanol (Le Duy & Boa, 1982; Iniotakis *et al.*, 1991) with stirring. Samples were then filtered through glass fibre filters and dried. Total sugars were analysed by the method of Dubois *et al.* (1956). Reducing sugars were determined by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952). Glucose was determined enzymatically by the glucose oxidase, peroxidase/guaiacum assay (Morley *et al.*, 1968).

Pullulan-containing crude precipitates (20 mg) were dissolved in 0.03 M citrate/phosphate buffer (4 ml, pH 5.0) and 0.5 ml of this was hydrolysed with 20 nKat of pullulanase (Sigma) (Simon *et al.*, 1993). After incubation at 37°C for 3 h, reducing sugars were determined. Also, the same precipitates were hydrolysed by acid hydrolysis for 3 h as described elsewhere (Israilides *et al.*, 1994).

Molecular weights and molecular weight distributions of the pullulan samples were determined using size exclusion chromatography/multiangle laser light scattering (SEC/MALLS). Molecules are separated according to size by a column matrix of defined pore size, and molecular weights of the eluting fractions are determined by on-line light scattering. The light scattering cell is illuminated by a He–Ne laser (wavelength 633 nm, 5 mW) and the intensity of the scattered light is measured at 15 different angles simultaneously. A concentration detector is connected on-line to the light scattering detector, allowing the concentration of the eluting fractions to be determined (full details of SEC/MALLS may be found in Wyatt, 1993). The weight average molecular weight of a macromolecule from light scattering measurements may be calculated by the following equation:

$$\frac{K_C}{R_\theta} = \frac{1}{M_w(P\theta)} + 2A_2c + \dots, \quad (1)$$

where

K = polymer constant for a particular scattering system:

$$K = \frac{2\pi^2 n_0^2 (dn/dc)^2}{\lambda^4 N_A}, \quad (2)$$

where

c = solute concentration;

R_θ = excess Rayleigh factor, $R_\theta = R_{\theta,\text{solution}} - R_{\theta,\text{solvent}}$;

M_w = weight average molecular weight;

$P(\theta)$ = particle scattering function which will give information regarding radius of gyration (R_g) and under certain conditions the shape of the molecule;

A_2 = second virial coefficient, which is a measure of solvent-solute interactions. Because of the very low concentrations passing through the light scattering cell after fractionation/dilution through the column the term $2A_2c$ in eqn (1) can be taken to a reasonable approximation to be zero

R_g may be found from the initial slope of a plot of K_c/R_θ versus $\sin^2 \theta/2$ and $1/M_w$ from the intercept.

Chromatograms obtained from both detectors are overlaid and divided into a number of slices. Although the M_w at each slice is, according to eqn (1), the weight average, if the slices are assumed to be approximately monodisperse, then the number and z -averages over the whole distribution can be found from the simple formulae (Tanford, 1961):

$$M_n = \frac{\sum c_i}{\sum (c_i/M_i)}$$

$$M_w = \frac{\sum c_i M_i}{\sum c_i}$$

$$M_z = \frac{\sum c_i M_i^2}{\sum c_i M_i},$$

with the weight average being the primary parameter. Alternatively, a SEC calibration plot of M_i versus elution volume can be given and from this a molecular weight distribution (M_i versus c_i) may be obtained.

Chromatographic conditions

The eluent used was a phosphate/chloride buffer of ionic strength 0.1 and pH 6.8. The chromatographic

system consisted of a Waters 590 Solvent Delivery module (Waters, Millipore, Watford, UK), a Rheodyne injection valve (Model 7125) fitted with a 100 μ l loop (Rheodyne, St Louis, MO, USA), a guard column and two analytical columns (Hema Bio linear and Hema Bio 40, PSS GmbH, Mainz, Germany), the latter consisting of a cross-linked hydroxyethylmethacrylate porous packing material, the first column having a linear separation range for dextrans of 7 million to <2000, and the second column having a separation range below 40 000. The scattered light intensities were measured using a Dawn F multiangle laser light scattering photometer (Wyatt, Santa Barbara, CA, USA) and concentrations were measured using an Optilab 903 (Wyatt) interferometric refractometer. The eluent was pumped at a flow rate of 0.8 ml/min at ambient temperature, and full loop injections of samples (concentration 5 mg/ml) were performed.

RESULTS AND DISCUSSION

Table 1 shows the chemical analysis of the various ethanol agglutinating substances from the fermentation of the respective agro-industrial wastes. The ethanol precipitate from defined media followed by SW and GSPE resemble the reference pullulan in as much as they contain mostly sugars and very little protein. The OW precipitate alone or with molasses consisted mostly of protein and the rest was carbohydrate in nature. In the case of molasses as a substrate, total sugars were of the same order as OW media, but the protein level was lower. Free glucose concentration was very low in all the substrates used for these fermentations.

When the samples were completely hydrolysed (3 h with 3 M H_2SO_4), the defined medium, SW and GSPE

Table 1. Composition of various ethanol precipitated substances from the fermentation of agro-industrial wastes

Ethanol precipitate from fermentation of the following substrates	Protein (% dry wt) (Bradford, 1976)	Sugars (% dry wt)			Sugars (% dry wt) in H_2SO_4 hydrolysate			Reducing sugar (% dry wt) ^c	Ratio ^f of glucose ^d to red. sugar ^e (%)
		Total sugar ^a	Reducing sugars (as glucose) ^b	Glucose ^c	Total sugars ^a	Reducing sugar (as glucose) ^b	Glucose		
GSPE	7.8	85.2	3.4	1.280	79.0	69.5	69.3	22.4	3.1
SW	2.5	82.5	2.6	0.665	96.8	86.0	83.6	9.3	8.9
OW	10.3	29.7	10.6	0.854	16.8	16.8	5.3	8.7	0.6
M	8.3	27.1	1.8	0.124	30.7	28.7	7.7	1.9	4.1
OW/M	7.3	30.2	10.5	0.911	21.5	21.0	8.2	6.8	1.2
D	2.9	95.1	10.0	0.459	76.3	70.5	74.4	18.9	3.9
Ref. Pullulan	0.5	93.9	0.1	0.881	102.6	95.7	103.0	32.3	3.2

^aEstimated by the Dubois method.

^bEstimated by the Somogyi-Nelson method.

^cEstimated by the glucose oxidase method.

^dGlucose estimated by the glucose oxidase method after H_2SO_4 hydrolysis.

^eReducing sugar estimated as glucose from Nelson's method after pullulanase hydrolysis.

^fApparent number of residues in repeating unit.

Table 2. Weight average molecular weights and polydispersities (M_w/M_n) for pullulans from defined medium after different fermentation times and Sigma pullulan

Sample (length of fermentation)	$10^{-6} \times M_w^a$	M_w/M_n
8 h	1.38	2.7
1 day	3.39	1.2
2 days	1.59	1.6
3 days	0.65	1.3
5 days	0.52	1.3
7 days	0.58	1.5
Sigma pullulan	0.24	2.4

^aEstimated errors less than $\pm 10\%$.

showed that almost all of the total sugar was glucose, indicating the presence of a homopolysaccharide. In the case of the OW media, about 33% of the total sugar was glucose, while in the case of molasses it was even less (about 25%).

When the ethanol precipitated substances were subjected to enzymic hydrolysis with pullulanase the reference pullulan gave 32.3% (dry wt) reducing sugars, indicating complete hydrolysis of the α -1,6-glycosidic linkage to give maltotriose. The percentage ratio of glucose after acid hydrolysis to reducing sugar after enzymic hydrolysis with pullulanase may serve as an indication of the presence of pullulan. According to some authors (Simon *et al.*, 1993), ratios between 3.2 and 4.2 are indicative of pullulan while below 3.2 or above 4.2 they are indicative of polysaccharides other than pullulan.

Weight average molecular weights and polydispersity indices (M_w/M_n) for the pullulans obtained from defined medium and the Sigma pullulan are shown in Table 2, and a graphical description of their variation with fermentation time is given in Fig. 1. The pullulan from Sigma is chemically pure, but it does not have a defined molecular weight range. This is borne out by the chromatographic traces shown in Fig. 2. The light scattering trace has a shoulder on the high molecular weight side, which is barely visible on the concentration detector. Such a situation is indicative of small amounts of high molecular weight material. This is confirmed in the molecular weight versus elution volume plot (see Fig. 3), which shows good separation over the whole peak area. As expected, the polydispersity of this sample is high (i.e. 2.4, see Table 2). A Debye plot (R_θ/K_c versus $\sin^2 \theta/2$) of a slice at the peak of the light scattering trace, demonstrating the angular dependence of the scattering intensity, is shown in Fig. 4. The linearity of the plot confirms that the individual slices are quasi-monodisperse and free from any large aggregates.

The molecular weights of the pullulans obtained from the defined medium from the different fermentation times shown in Table 2 and Fig. 1 follow the expected pattern (see, e.g. Tsujisaka & Mitsuhashi, 1993) in that the molecular weight initially increases with fermenta-

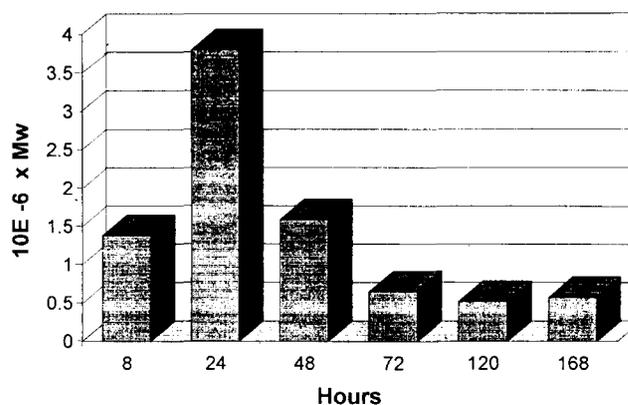


Fig. 1. Molecular weights of pullulans obtained from defined medium with increasing time.

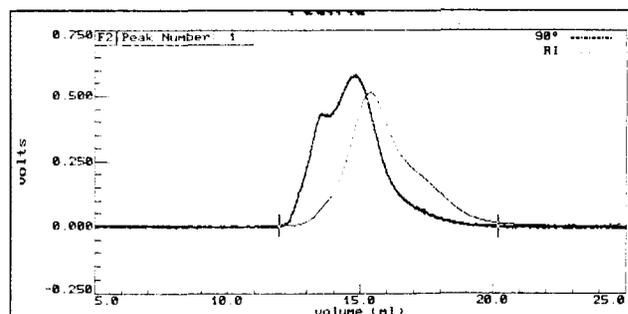


Fig. 2. Chromatographic traces from light scattering (90°) and refractive index detectors from Sigma pullulan.

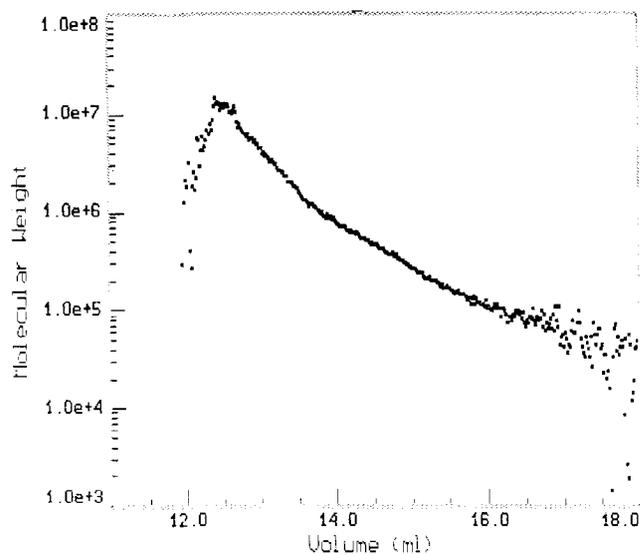


Fig. 3. Molecular weight versus elution volume plot from Sigma pullulan.

tion time, but then decreases. The latter effect may be a result of pullulan degrading enzymes which are also formed during the cultivation process. The polydispersity of the material with the highest molecular weight (i.e. 1 day fermentation) appears to be very low

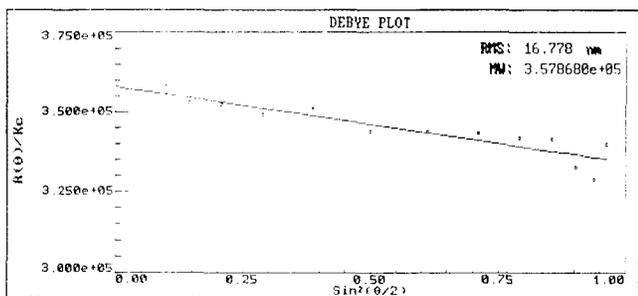


Fig. 4. Debye plot of peak fraction (elution volume 14.8 ml) from Sigma pullulan.

(i.e. 1:2, see Table 2). This is most likely a result of lack of resolution of the column system in the high molecular weight region and not a true reflection of the polydispersity of the sample.

Elution profiles of the materials produced from fermentation of agro-industrial wastes are shown in Fig. 5(a)–(e). These wastes had not been purified prior to fermentation; thus the elution profiles reflect the number of different materials in the ethanol precipitate of the cell free supernatant after fermentation. The weight average molecular weights of the high molecular weight peaks obtained by SEC/MALLS are given in Table 3 and compared graphically with those obtained from Sigma pullulan (P) and the pullulan from the defined medium in Fig. 6. Due to the lack of resolution of the different molecular weight components, peak integrations were fairly wide and the molecular weight values obtained may include a number of different components. There is, therefore, no guarantee that the molecular weight values quoted refer to pullulan alone. Also, the light scattering intensities for the peaks at

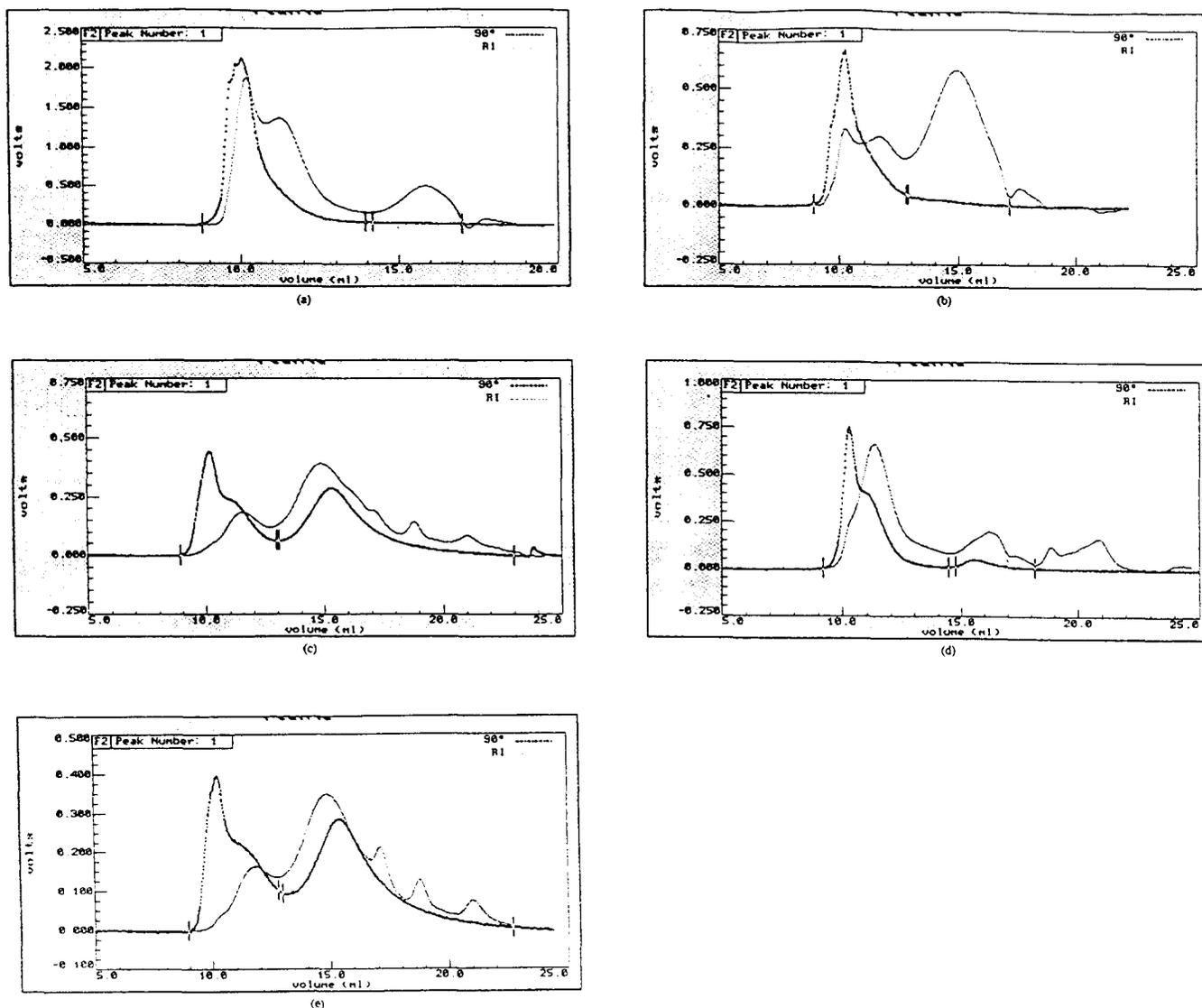


Fig. 5. Chromatographic traces from light scattering (90°) and refractive index detectors of pullulans from agro-industrial wastes: (a) GSPE; (b) SW; (c) OW; (d) M; (e) OW/M.

Table 3. Weight average molecular weight values of fermentation products using agro-industrial wastes

Substrate	$10^{-6} \times M_w^a$ (Peak 1)
GSPE	4.22
SW	3.29
M	0.55
OW	1.28
OW/M	1.13

^aEstimated errors less than $\pm 10\%$.

higher elution volumes were very low; the resulting molecular weight values are, therefore, meaningless and not quoted here. Discussion of molecular weight values has, therefore, been limited to the high molecular weight peaks. As these peaks include shoulders, polydispersity indices are not very meaningful and have not been included in either Table 3 or the discussion.

Fermentation using the GSPE and defined medium produced the pullulan with the highest molecular weight and at the highest concentration as assessed by the gel elution profile, glucose content and the apparent number of residues in repeating units (see Table 1). The elution profile from the product of the fermentation, using starch waste as a substrate, was very similar to that of GSPE. However, the amount of high molecular weight material was much lower—most of the product was found to be of relatively low molecular weight; this was probably derived from incomplete hydrolysis of the starch substrate.

Fermentation using molasses as a substrate produced mainly low molecular weight material, although the large light scattering peak associated with the shoulder on the high molecular weight side of the concentration peak indicated the presence of some high molecular weight material. The fermentation product from OW was found to be of slightly higher molecular weight than that from molasses. However, this material was only present in small amounts, whereas a large proportion of the total material was present in the low molecular weight peak (see Fig. 5(c)). Addition of molasses to the OW substrate appears to have had very little effect on the fermentation products. The molecular weight of the first peak decreased slightly as would be expected in the light of the above results from a mixture of the two individual substrates, but the elution profile was very similar to that of the product from OW alone (compare Fig. 5(c) and (d)) and the molecular weight versus elution volume plots were found to be superimposable (see Fig. 7). Molasses produce pullulan as assessed by the apparent number of residues in repeating units, but only in limited amounts as indicated by the glucose content. Finally, OW and OW/M media produced little pullulan and the apparent number of residues in repeating units suggests it is contaminated with other polysaccharides.

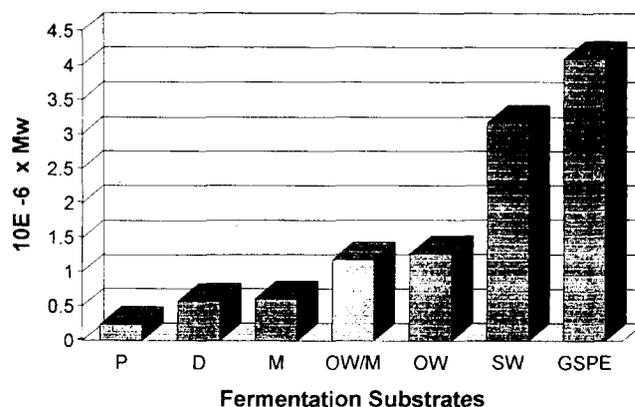


Fig. 6. Molecular weights of pullulans obtained from various agro-industrial wastes.

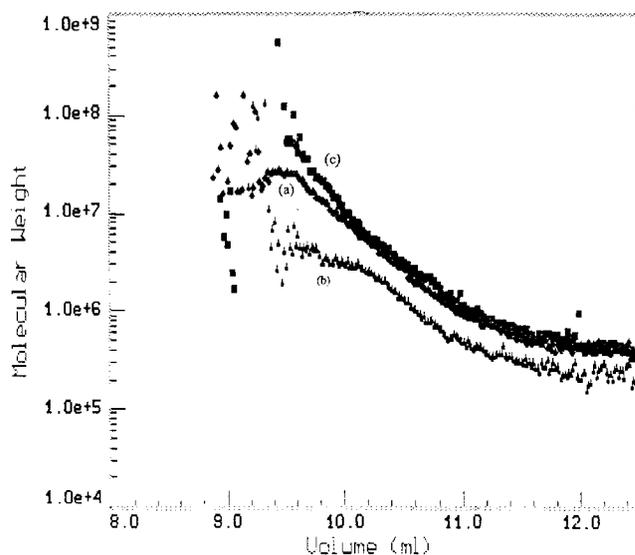


Fig. 7. Overlaid molecular weight versus elution volume plots from (a) OW, (b) M, and (c) OW/M.

The production of pullulan by fermentation is influenced by a number of factors such as the carbon source and its amount present in the fermentation medium, as well as the strain of *Aureobasidium* used for the fermentation (Tsujiisaka & Mitsuhashi, 1993). One substrate used for the production of pullulan on an industrial scale is starch hydrolysate. The fermentation with starch waste would, therefore, have been expected to give good results. However, the dextrose equivalent of the starch syrup is an important factor for the pullulan yield and although measured, this was not controlled in the present experiment.

The results in Table 1 can now be better interpreted in the light of the molecular weight determinations. Thus, we can conclude that (1) the defined medium is producing a highly pure pullulan, while the rest of the substrates contain a mixture of other materials in the agglutinating substances. (2) Although GSPE and SW are producing similar molecular weight pullulans, the

SW in the most part consists of a polysaccharide other than pullulan. (3) In M and OW the first peak which is most likely to be the pullulan represents about 40 and 30%, respectively, of the total mass. Given that these materials are quite heterogeneous, the separation and purification of any pullulan present in them becomes questionable. (4) Different substrates when fermented are producing pullulans with different molecular weights. (5) The molecular weight decreases during the course of the fermentation.

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