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Synthesis of two distinct exopolysaccharide fractions by cultures of the polymorphic fungus *Aureobasidium pullulans*

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Two physically distinct exopolysaccharide fractions were extracted from cultures of the polymorphic fungus *Aureobasidium pullulans* grown in a stirred tank bioreactor and compared with a commercial sample of pullulan. The two fractions were characterised using elemental analysis, infrared spectroscopy, GC-MS and gel permeation chromatography. Both fractions were found to be structurally and compositionally identical, predominantly linear glucans containing α-1,4- and α-1,6-linkages. From these results, it is clear that the differing physical behaviour and appearance of the two fractions can be attributed solely to differences in molecular mass. Possible mechanisms by which the two fractions may have occurred are discussed in the context of other reports concerning exopolysaccharide synthesis by this fungus. © 1997 Elsevier Science Ltd

INTRODUCTION

The polymorphic fungus *Aureobasidium pullulans* has been the subject of intense academic and industrial interest over the past three decades (Deshpande *et al.*, 1992; Harvey, 1984; Yuen, 1974). Research has centred on two main areas, the first relating to the nature of the life cycle and the factors influencing the interconversion of the different morphological forms of the fungus, the second concerned with the factors influencing the synthesis of the exopolysaccharide, pullulan (McNeil and Kristiansen, 1990; McNeil *et al.*, 1989; Ramos and Garcia-Acha, 1975).

There is a large measure of overlap between the two research directions, since it has been shown that pullulan production is either largely or exclusively a function of the yeast-like cells (blastospores), with little or no contribution being made by the hyphal form of the microorganism (Catley, 1980; Reeslev *et al.*, 1991). Thus, any factor influencing the morphology of the fungus, also inevitably influences exopolysaccharide production.

Pullulan has been the subject of many patents (LeDuy *et al.*, 1988), initially concerned with industrial production, then increasingly with specific applications (Tsujisaka and Mitsuhashi, 1993). Pullulan has been said to have particular promise in a number of areas, including the formation of biodegradable moulded items, fibres and films. General usage as a thickener or extender can probably be ruled out, since fungal exopolysaccharides produced by fermentation are likely to be much more expensive than modified starches and cellulosics (McNeil and Harvey, 1993; Wang and McNeil, 1995).

Product purity and molecular mass distribution are vitally important in determining the suitability of pullulan, or any microbial polysaccharide, for certain roles (Kato and Shiosaka, 1975; Sutherland, 1979). In this context, a number of difficulties arise when describing or discussing fermentation processes involving *A. pullulans*, or closely related species said to have the same synthetic capabilities.

A number of reports have described the production by *A. pullulans* of polysaccharides other than pullulan. Some of these have been characterised as β-glucans (Bouveng *et al.*, 1963a; Kikuchi *et al.*, 1973), while others have been described variously as 'acidic' polysaccharides (Bouveng *et al.*, 1963b; Leal-Serrano *et al.*, 1980) or heteropolysaccharides (Bouveng *et al.*, 1963a). Other reports have described the formation of

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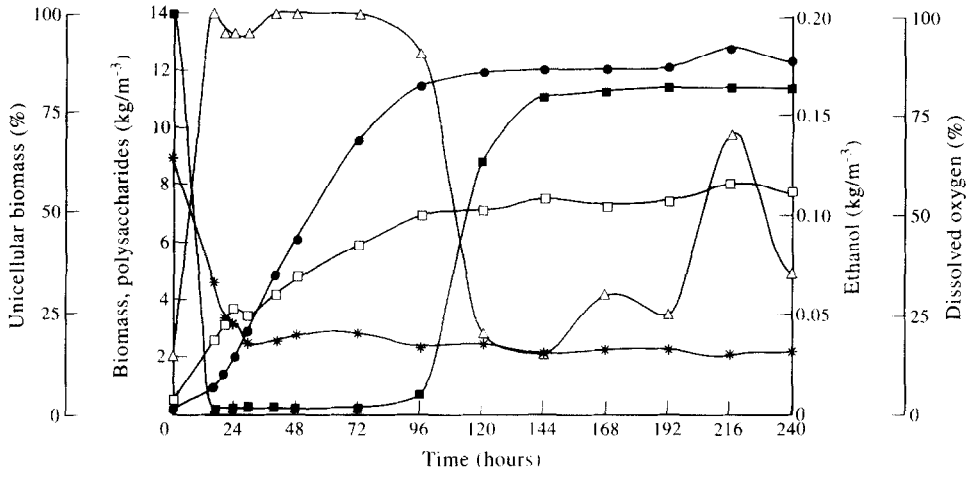

Fig. 1. Total biomass (□), polysaccharides (●), % unicellular biomass (*), ethanol (△) and dissolved oxygen (■) vs time, at pH 4.5 (standard culture).

more than one exopolysaccharide, but have not attempted to characterise the polymers. McNeil and Kristiansen (1987) described the formation of two polysaccharides having distinct molecular weights, while Simon *et al.* (1993) noted, but did not characterise a polysaccharide which became or was converted into pullulan. It is clear from the above that much of the ambiguity surrounding the synthetic capability of *A. pullulans* is attributable to inadequate analysis and characterisation of the polymeric materials produced.

During recovery of polysaccharide by addition of ethanol to cellfree fermentation fluids, we noted the presence of two physically distinct materials. Due to the effect variation in polymer composition and/or molecular mass might have upon the functional properties of the biopolymers, we investigated both materials. The nature of these materials is discussed in the context of the polymers said to be synthesised by *A. pullulans* with a view to offering an explanation of the apparent biosynthetic flexibility of this microorganism when it comes to exopolysaccharide synthesis.

MATERIALS AND METHODS

Microorganism

Aureobasidium pullulans (De Bary) Arnaud (IMI 145194) was the organism employed in this study. The organism was supplied as a lyophilised culture and was resuscitated and cultivated on potato dextrose agar slopes (Oxoid Ltd). Cultures were incubated for 7 days at 28°C.

Shake flask cultures, used for the inocula, were prepared by inoculating 0.5 dm^3 of sterile liquid medium in 3 dm^3 Erlenmeyer flasks with 0.010 dm^3 of cell suspension prepared from two slopes of the organism.

Medium

The composition of the liquid medium employed was as follows: ($kg\,m^{-3}$): sucrose, 30; $(NH_4)_2SO_4$, 0.6: KH_2PO_4, 5.0: NaCl, 1.0: $MgSO_4 \cdot 7H_2O$, 2.0; Yeast extract (Oxoid Ltd), 0.4. The pH of the medium was adjusted to the appropriate setting using 1 M NaOH or 10% H_2SO_4.

Fermentations

Fermentations were carried out in a 10 dm^3 working volume (15 dm^3 total volume) stirred tank reactor (MBR Bioreactor AG, Switzerland). Airflow rate was 1 vvm; temperature was maintained at 28°C±0.1; agitation speed 300 rpm; pH was controlled by automatic addition of either 1 M NaOH or 10% H_2SO_4.

Analytical methods

Biomass estimation

Biomass was measured by means of dry weight estimation. 5 ml samples of culture were filtered through predried and weighed nylon mesh (Henry Simon Ltd) with a pore size of 45 μm, in order to separate yeast like cells from the filamentous cells. The mycelial matt was washed twice with distilled water, dried and weighed. The filtrate and washings thus obtained were filtered through predried and weighed Whatman GFC filters (Whatman UK Ltd), washed, dried and weighed, in order to obtain the yeast like cell dry weight.

Polysaccharide estimation

The exopolysaccharide fractions were precipitated from cell free filtrate using two volumes of absolute ethanol. Two distinct fractions resulted, the upper fraction was removed by rotating spatula, washed and filtered through GFC filters, dried and weighed. The lower fraction was recovered by filtration through GFC filter,

dried and weighed. For IR, GC-MS compositional and methylation analysis, 0.4 g of polysaccharide obtained from each of the fractions was redissolved in 200 ml distilled water, allowed to stand at room temperature for 4 h to ensure complete solubility and then re-precipitated with two volumes of absolute alcohol, dried, weighed and ground to a fine powder. The samples taken for these analyses were from a 48 h broth of the standard culture.

Molecular weight determination

Molecular weight was estimated by gel permeation chromatography, using a two column system. The first column (800×1 mm i.d.) was packed with fractogel TSK HW-75 (Merck Ltd) and the second (300×1 mm i.d.) packed with TSK HW-55. Elution was carried out at room temperature with 0.1 M NaCl buffer which had been filtered through Whatman membrane filters (0.45 μm), deaerated and protected from microbial contamination with 0.05% sodium azide. The flow rate through the columns was adjusted to 20 ml h^{-1}. Columns were calibrated using dextran standards of known molecular weights, ranging from 9.3×10^3 to 2×10^6 (Sigma Ltd). A refractive index detector was used (model 1755 Bio-Rad Labs Ltd, UK) to detect the separated polysaccharides eluting from the columns.

Sucrose analysis

Sucrose was determined using the colorimetric assay of Dubois *et al.* (1956).

Ammonium analysis

Ammonium was measured using a colorimetric method (Sigma diagnostics).

Elemental compositional analysis

The elemental composition of both materials recovered by ethanol precipitation from cell free filtrates was determined. C, H, N percentage by weight was determined using a Carlo ERBA 1106 analyser. Products of combustion in pure oxygen at 1800°C were carried through a tube packed with CuO, Cr_2O_4 and Ag gauze by a helium carrier gas. Excess O_2 and oxides of nitrogen were removed by passage through a reduction tube containing copper at 650°C. CO_2, H_2O_2 and N_2 are then passed through a chromatographic column and peak areas are used to compute relative percentages. Determination of the percentage of sulphur in the samples was done with a sulphanazo 111 analyser. The sample was combusted in O_2, after 30 min the flask was washed with distilled water and the solution boiled to drive off the excess H_2O_2. The flask was cooled to 25°C, acetone and indicator were added and the solution was titrated with barium perchlorate. Phosphorus determination involved sample digestion in concentrated H_2SO_4 and $HClO_4$ for 30 min. After cooling, the flask contents were transferred to a conical flask, treated with conc HCl, sodium molybdate and quinoline hydrochloride. The resultant phosphomolybdate complex was assayed gravimetrically.

Infrared analysis

The IR spectra were determined using the potassium bromide technique of Barker *et al.* (1956) on a Mattson 1000 FTIR spectrometer (Unicam Ltd).

Methylation analysis

Five milligrams of each sample typical and jelly-like was first subjected to partial acid hydrolysis (250 μl, 0.1 M HCl, 100°C for 2 h). The acid was removed by passage through 0.2 ml Dowex AG3 (OH) eluted with water to give a stock solution of 2–5 mg ml^{-1}. Fifty microgram samples were taken for methylation analysis following the method of Ferguson (1992). The programme described below was run on an HP 5890-5970 GC-MS system. Mass spectra were recorded using electron impact (70 eV) ionisation at a source temperature of 150°C and a pressure of $2\text{–}4\times10^{-5}$ Torr.

The GC was equipped with an on column injector. Column head pressure was 5 psi with a carrier gas flow (He) of around 0.5 ml min^{-1} through a 30 m×0.25 mm Econocap SE-54 bonded phase column (Alltech). The GC to MS transfer line was made from a 1 m×0.25 mm piece of deactivated fused silica (Supelco) and was kept at 10°C above maximum oven temperature. See Table 1 for GC-MS programme.

RESULTS AND DISCUSSION

Figure 1 shows the time course of a typical batch pullulan production process in a stirred bioreactor at a controlled pH 4.5. Under these conditions the morphology is mixed with both the unicellular and the filamentous forms being present. The concentration of pullulan achieved is higher than reported elsewhere (McNeil and Kristiansen, 1987, 1990).

Table 1. GC-MS programme used in this study

Analysis	Column	Initial temp.	Gradient 1	Intermediate temp.	Gradient 2	Final temp.	Mass spectral aquisition
Methylation	SE54	80°C (1')	30°C min^{-1}	140°C (0')	5°C min^{-1}	250°C (20')	Linear scanning MHz 40–350

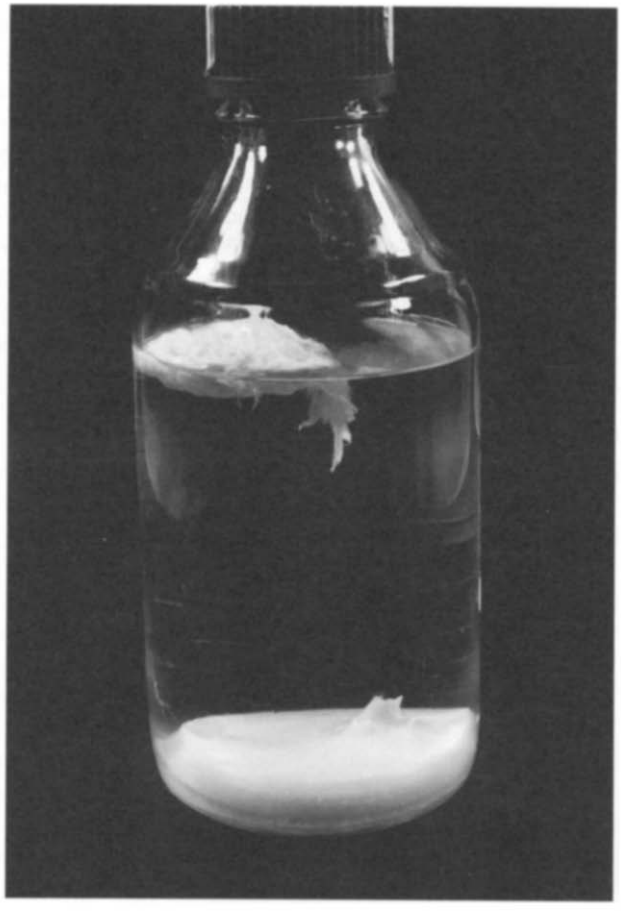

Fig. 2. Two distinct polymer fractions recovered by ethanol precipitation.

Figure 2 shows the two distinct polymer fractions recovered by ethanol precipitation. The recovery was performed very gently without the normal vigorous agitation. Consistently, two fractions of different gross appearance formed: the upper fraction appeared 'strand like', fibrous in nature, similar to the usual appearance of pullulan in ethanol/water mixtures, the lower fraction was much more gel-like in character. This trait was constant within this strain, and processes operated under these conditions separated by some 9 months, consistently produced these two fractions.

Given the uncertainty surrounding the biosynthetic capabilities of this organism, and the impact 'impurities' might have upon the functionality of pullulan, it was felt essential to analyse these two fractions fully.

Elemental analysis

The starting point for the characterisation was to compare the elemental composition of the two fractions with that of pullulan supplied by Sigma. The results of these analyses are presented in Table 2.

Infrared spectroscopy

Typical IR spectra for the two fractions and for Sigma pullulan are presented in Fig. 3.

GC-MS

Initial compositional analysis by GC-MS confirmed that both fractions contained only glucose. Samples were then taken for methylation analysis by the method indicated above. Both fractions gave identical results, as shown in Fig. 4. Representative mass spectra for the typical fraction are shown in Fig. 5. The spectra from the gel fraction were identical in all respects.

Gel permeation chromatography

The molecular masses of the two fractions and Sigma pullulan were estimated by gel permeation chromatography. Table 3 summarises the results of these analyses. Taken as a whole, the range of analyses performed permit identification of the two fractions. From Table 2 it can be seen that in terms of elemental composition all three materials are similar, the only significant difference being the presence of phosphorus, probably as phosphate groups attached to the polymer in the typical fraction.

On the basis of these analyses, one can reasonably state that both materials are essentially carbohydrates, with no protein contamination present.

The IR spectra of the two fractions and the commercial Sigma pullulan confirm the similarity of all three materials. All three spectra exhibited the absorption characteristics of α-(1–4) and α-(1–6) glucosidic linkages in the region 750–950 cm^{-1} as reported by Bouveng *et al.* (1963b) and Sowa *et al.* (1963) for pullulan. This points to the materials being largely α-glucans very similar to commercial pullulan, but in order to fully determine the structure, GC-MS analysis was used.

Figure 4 again confirms the close similarity of the two fractions. The peak at around 20.7 min is glucitol-hex-

Table 2. Elemental composition of two fractions and Sigma pullulan as determined by microanalysis

	Element (%)				
	C	H	N	P	S
Sigma	42.0	6.4	trace or nil	trace or nil	trace or nil
Upper/typical	40.3	6.5	trace or nil	0.5	trace or nil
Lower/gel like	41.5	6.5	trace or nil	trace or nil	trace or nil

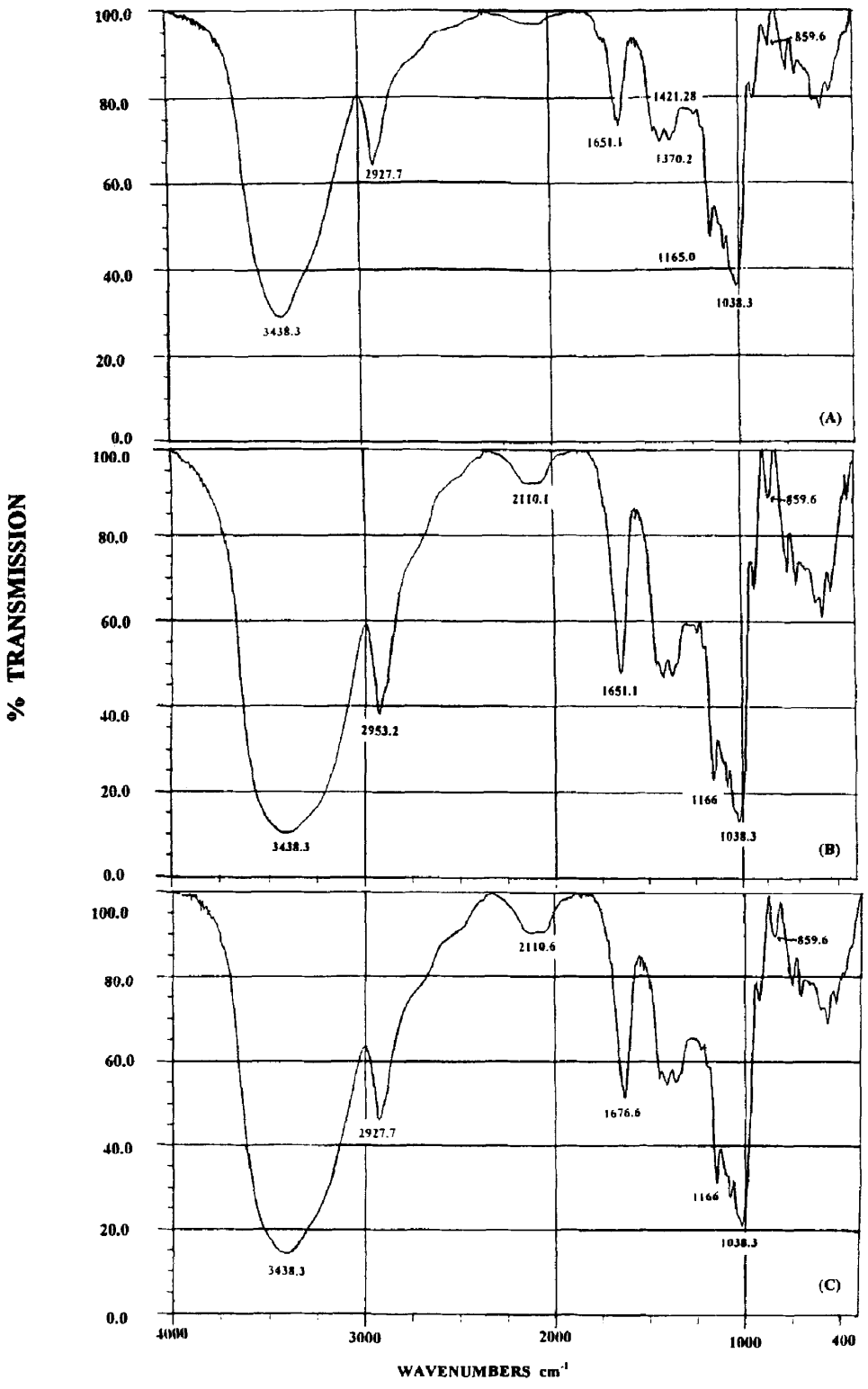

Fig. 3. Infrared spectra for the exopolysaccharides: (A) upper fraction; (B) lower fraction; (C) commercial Sigma pullulan.

acetate (an artefact of the analysis method chosen) while that at 21.2 min is the internal standard (scyllo-inositol hexacetate). The peak at 14.1 min was identified as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-[1-^{2}H]-glucitol, corresponding to non-reducing terminal glucose residues; the peak at 16.1 min was identified from its spectrogram as 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-[1-^{2}H]-glucitol, corresponding to 4-O-substituted glucose residues, and the peak at 16.5 was 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-[1–^{2}H]-glucitol, corresponding to 6-O-substituted glucose residues. The respective ratios were 1.0:1.3:1.0 for the typical fraction and 1.0:1.4:1.0 for the gel fraction. From these results it can be concluded that these two fractions are essentially of identical structure

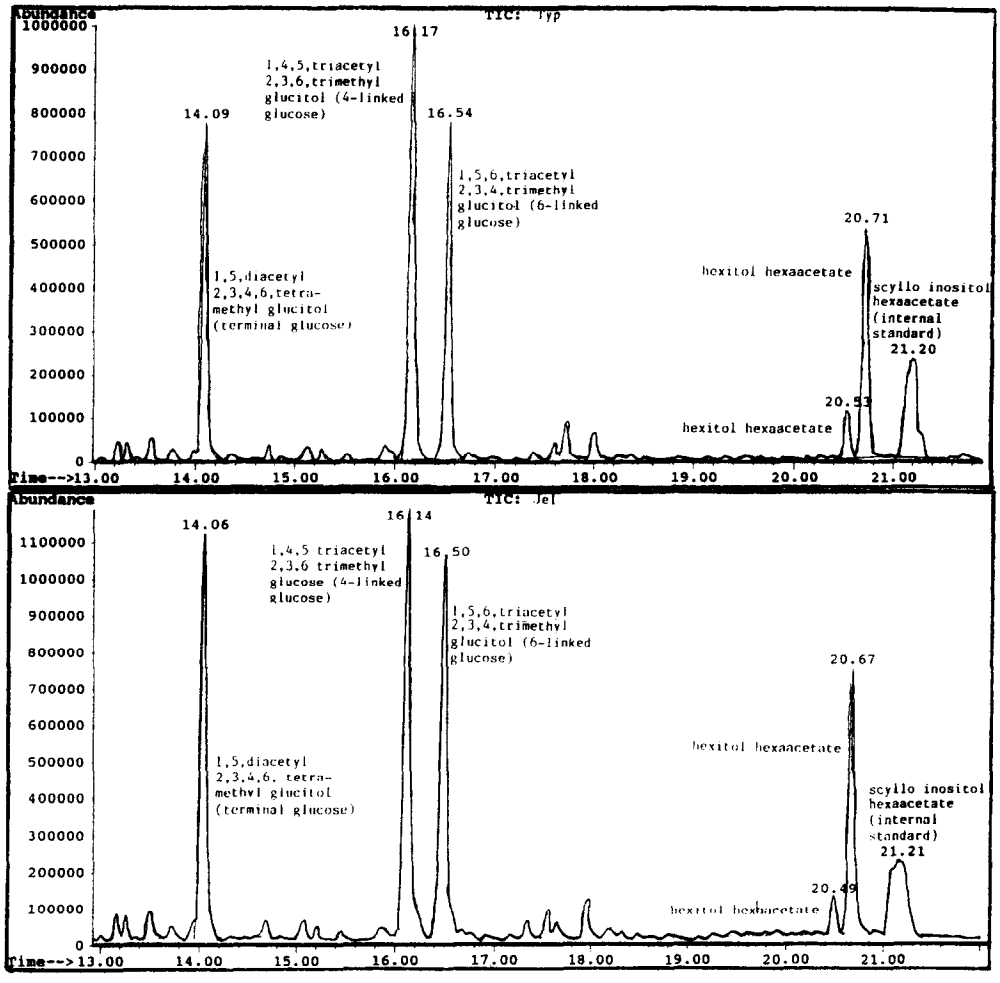

Fig. 4. GC-MS of the partially methylated alditol acetates of the two different exopolysaccharides after partial acid hydrolysis (method of Ferguson, 1992).

and composition, predominantly linear polysaccharides with a mixture of 1,4- and 1,6-linkages.

Considering the results of all the analyses performed on these two fractions the conclusion is that both materials are pullulan and that the difference in their physical behaviour is due to differences in their molecular mass.

These results may clarify the findings of McNeil and Kristiansen (1987) in assigning an unambiguous identity to the second exopolysaccharide they recovered from culture filtrates.

It is possible that as in *Zoogloea* (Kwon *et al.*, 1994) or *Sclerotium glucanicum* (McNeil and Harvey, 1993), there are two distinct polymers formed, one tightly adherent to the outside of the cells, and usually described as gel-like in nature, while the second is released freely into the extracellular medium. Another possible mechanism by which the two species of pullulan may have arisen is proposed by Simon *et al.* (1993) who indicated that a polysaccharide was present in some culture fluids of *A. pullulans* which matured into commercial pullulan. Although the exact nature of this material was not characterised, this indicates the possibility of modifications to the polysaccharides during formation. A third possibility is that specific cell types may be responsible for different fractions. Under the bioreactor conditions chosen, both yeast like and mycelial type morphology was noted, and from 30 h onwards increasing numbers of swollen cells/chlamydospores (said to be the main source of pullulan by Simon *et al.*, 1993) were present. However, most other reports on pullulan synthesis firmly point to the yeast like cells (blastospores) being the main synthetic form of the microorganism (McNeil *et al.*, 1989; Reeslev *et al.*, 1991). It is noteworthy that the method of separation of 'yeast' and mycelial cells used in these latter studies (filtration through a 45 μm mesh) would tend to include swollen cells in the former category, thus, the findings of Simon *et al.* (1993) may not be entirely at variance with other studies.

The phenomenon of formation of two physically distinct but structurally identical polymers may be more prevalent than might be thought, as, unless care is taken during recovery to separate the fractions, the presence of more than one type could easily be missed, since molecular mass analysis is not routine in most studies on microbial polysaccharide synthesis.

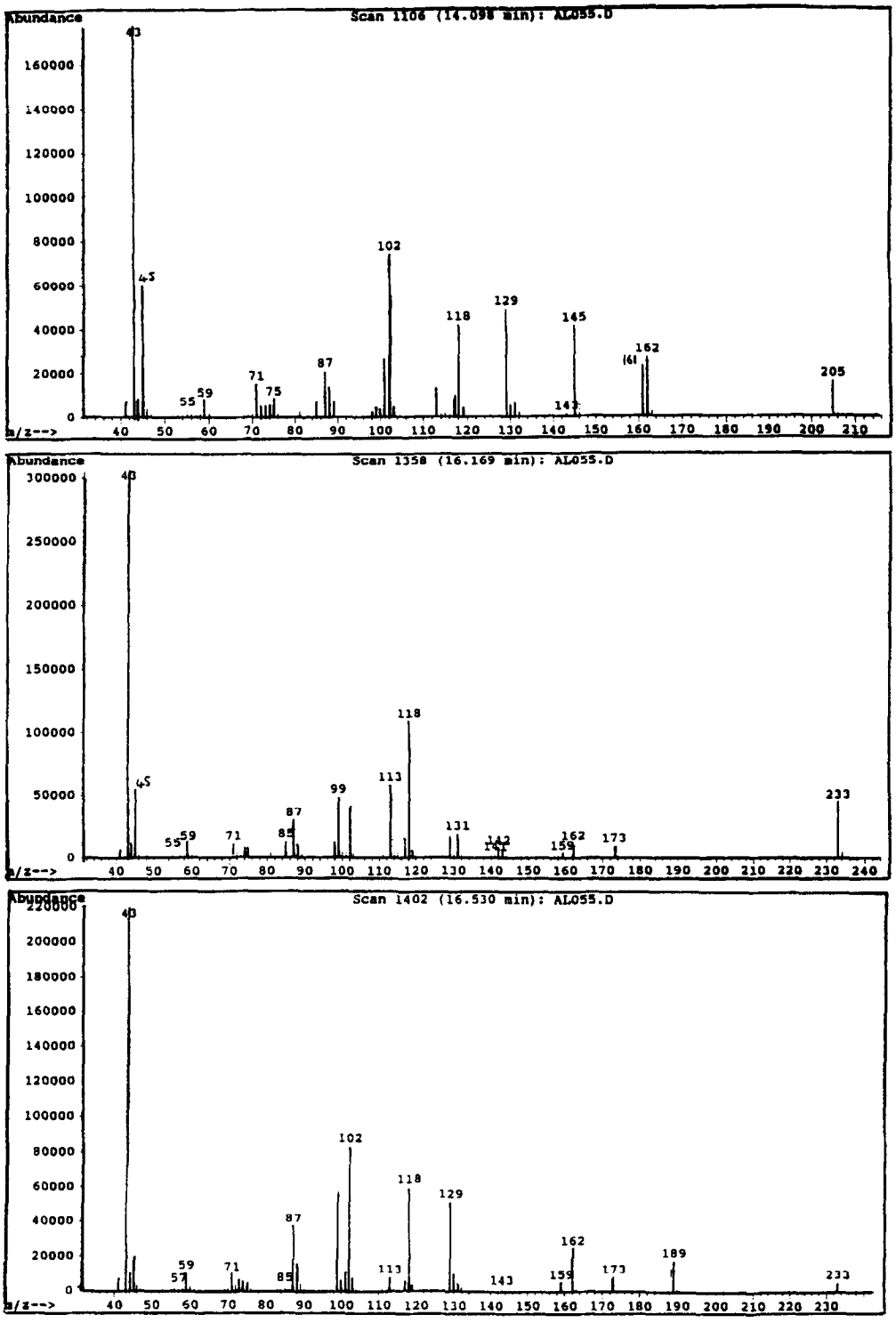

Fig. 5. Representative mass spectra for the typical polysaccharide fraction.

That one organism should be capable of releasing into the extracellular medium such a range of materials, including α-glucans, β-glucans, glucans with proteins attached, should not be entirely suprising. Strain differences may explain, in part, this diversity, but, more fundamentally, all of the materials isolated from *A. pullulans* cultures can be found in or attached to the cell wall. Thus, any change in the processes of wall formation, maturation and destruction may result in release of elements or components of the cell wall. In particular, in examining the nature of materials said to be released by *A. pullulans*, it should be borne in mind that in other fungi, a wide range of materials, including, β-1,3 and β-1,6 glucans, mannoproteins linked to glucans, and a range of α-glucans (during autolysis) (Gooday, 1995) have been shown to be released into the extracellular medium. Given

Table 3. Mean molecular weights of two fractions and Sigma pullulan

Polymer	Mean molecular weight
Sigma pullulan	9.8×10^4
Upper/typical pullulan[a]	$>2.0\times10^6$
Lower/gel like pullulan[a]	4.3×10^5

[a]Samples taken from a standard batch production process at 72 h.

the wide range of experimental conditions used in studying *A. pullulans*, it is, therefore, not surprising that materials which are essentially cell wall related have been reported in the extracellular medium, in addition to the predominant exopolysaccharide, pullulan.

The nature of cell wall associated materials is clearly not fixed in time, the polysaccharides within and passing through the cell wall may interact in a dynamic fashion with other components before achieving their 'final' condition (Gooday, 1995; Simon *et al.*, 1993). Further study relating to 'maturation' of polysaccharides and to regulation of molecular mass is clearly merited. In particular, much greater attention must be paid to establishing the 'authenticity' (structural and compositional) of polymers produced from microbial sources.

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