Antonie van Leeuwenhoek **62**: 189–199, 1992.

Formation of antigenic extracellular polysaccharides by selected strains of *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp., *Absidia corymbifera* and *Syncephalastrum racemosum*

Gerhard A. De Ruiter[1], Andrea W. Van Bruggen-van der Lugt[1], M.J. Robert Nout[1], Wouter J. Middelhoven[2], Pop S.S. Soentoro[3], Servé H.W. Notermans[3] & Frank M. Rombouts[1]*

[1] Wageningen Agricultural University, Department of Food Science, Bomenweg 2, 6703 HD Wageningen, The Netherlands; [2] Wageningen Agricultural University, Department of Microbiology, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands; [3] Laboratory for Water and Food Microbiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands (Requests for offprints)*

Received 6 September 1991; accepted in revised form 15 April 1992

Key words: antigen, ELISA, extracellular polysaccharides, *Mucor, Pichia, Rhizopus*

Abstract

In this study, polyclonal IgG antibodies raised against extracellular polysaccharides (EPS) of *Mucor racemosus* were characterised as almost specific for moulds belonging to the order of Mucorales. Cross-reactivity in the ELISA could be observed only towards the yeast *Pichia membranaefaciens*. EPS were isolated from various cultures of *M. hiemalis* growing on six different carbon sources and two nitrogen sources, with ratios varying from 0.13 to 0.44 relative to the amount of biomass. Other strains including *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp., *Absidia corymbifera* and *Syncephalastrum racemosum* also excreted EPS, with ratios varying from 0.05 to 0.23. In all cases, the excreted EPS had similar antigenic properties as determined by ELISA. No enzymatic degradation of the antigenic parts of the polysaccharides could be observed upon prolonged incubation. Considering that all tested strains formed similar amounts of antigenic EPS there might be scope for the specific detection of biomass of Mucoralean moulds using ELISA techniques for example in food.

Introduction

Filamentous fungi are able to excrete a large variety of extracellular substances (Martin & Adams 1956; Goatley 1968; Graham et al. 1976). Among these, water-soluble extracellular polymers are thought to play various roles in fungal biology which probably can be compared with their function in bacteria (Longbottom & Austwick 1986). Some of the excreted polymers may act as reserve nutrients, as adhesin or as moisture regulatory compound (Fett et al. 1989). Other polymers seemingly have no function in the organism, and may be by-products of the metabolism of the mould (Longbottom & Austwick 1986).

The extracellular polymers of Mucorales (Zygomycetes) mainly consist of carbohydrates (50 to 90%) and protein (10 to 50%) and are therefore often referred to as extracellular polymers or polysaccharides (EPS). The presence of fucose, galactose, mannose and glucose residues has been established (Martin & Adams 1956; Bartnicki-Garcia &

Reyes 1968; De Ruiter et al. 1991a, 1991b). Also, β-D-(1–4)-linked glucuronic acid polymers have been described as an important structural entity of these EPS (De Ruiter et al. 1992a). A part of these EPS from species of the order of Mucorales possess antigenic properties (Miyazaki & Irino 1972; Miyazaki et al. 1980; Notermans & Heuvelman 1985; Notermans et al. 1986; Notermans & Soentoro 1986). As demonstrated recently, the EPS preparations of various species belonging to Mucorales did give a clear immunological reaction with the IgG antibodies raised against EPS of *M. racemosus* and contain one common antigenic fraction (De Ruiter et al. 1991a). The sugar constituents of these EPS were found to be immunodominant. However, until now no detailed information is available about the exact composition of these carbohydrate epitopes.

It was proposed to use these antigenic properties for immunological ELISA detection of moulds in food (Notermans & Heuvelman 1985; Notermans et al. 1986; De Ruiter et al. 1992b) or for diagnosis of mucormycosis in man (Jones & Kaufman 1978; Kaufman et al. 1989). The IgG antibodies used in this study were raised against EPS of *M. racemosus* and were slightly different compared to those used by Notermans and Soentoro (1986).

Before antibodies could be used for the detection or quantification of biomass of a fungal order, e.g. Mucorales in foods, several prerequisites must be met. First the specificity of the antibodies must be significant. In addition, it must be established that the target fungi produce the antigenic compound when growing on various saccharides occurring in foods. For quantitative purposes the antigenic activity per unit biomass must be predictable (De Ruiter et al. 1991b).

In this study, the formation of antigenic activity of the EPS is assessed in the presence of several saccharides and lactate, and related to biomass production. In addition, the specificity of polyclonal IgG antibodies raised against EPS of *M. racemosus* is established by testing 39 strains of yeasts and 18 strains of moulds, many of them often occurring in food, on their ability to react with these antibodies.

Materials and methods

Organisms

The strain of *Mucor hiemalis* CBS 201.28 used in this study was grown on Malt Extract Agar (Oxoid CM 59) plates (14 cm diameter) at 22° C. After incubation for about one week, the spores were harvested by adding 30 ml sterile NaCl solution (9 g l^{-1} containing 0.05% Tween 20) and the surface growth was rubbed with a sterile Drigalski spatula. The spores were collected by centrifugation (1000 *g*, 5 min), washed with sterile distilled water and resuspended in a physiological NaCl solution and stored at − 20° C. A density of 10^7 spores ml^{-1} was established by counting in a Bürker-Türk haemocytometer and appropriate dilution. Other moulds used in this study were *M. circinelloides* M 40, *M. racemosus* H473-R5, *Rhizopus oryzae* LU 581, *R. stolonifer* CBS 609.82, *Rhizomucor miehei* CBS 371.71, *Rhizomucor pusillus* CBS 432.78, *Absidia corymbifera* LU 017 and *Syncephalastrum racemosum* CBS 443.59 grown under the same culture conditions.

Various yeasts and moulds commonly found in food (listed in Table 1) were tested on their activity towards IgG antibodies raised against *M. racemosus*. The yeast strains were obtained from the culture collection of the Laboratory of Microbiology, Wageningen Agricultural University. Yeast species known to occur in foods (Barnett et al. 1991) were represented by strains isolated from foods or silage if available.

Inoculation

In order to eliminate variability due to asynchronous spore germination, inoculation was carried out either with spore-suspensions or with germinated spores. In the time experiments cultures were inoculated with spore suspensions: 0.2 ml (10^7 spores per ml) per 400 ml medium in an Erlenmeyer flask of 1 l. In the other experiments microcolonies of previously germinated spores (Bartnicki-Garcia & Nickerson 1962; Solomons 1975)

Table 1. Perfect and imperfect Ascomycetous and Basidiomycetous yeast species and moulds species of nine genera often found in food, screened for the reactivity towards IgG antibodies raised against EPS of *Mucor racemosus*.

Species
Ascomycetous species
Arxula adeninivorans (Middelhoven, Hoogkamer-te Niet et Kruger-van Rij) Van der Walt, M. Th. Smith et Yamada M13–11
Candida famata (Harrison) Meyer et Yarrow D 74e[a]
Candida glabrata (Anderson) Meyer et Yarrow CBS 138
Candida holmii (Jörgensen) Meyer et Yarrow D 17f[a]
Candida krusei (Castellani) Berkhout D 4a[a]
Candida lambica (Lindner et Genoud) Van Uden et Buckley PB 1 B[b]
Candida magnoliae (Lodder et Kruger-van Rij) Meyer et Yarrow CBS 166
Candida milleri Yarrow PB 32[b]
Candida parapsilosis (Ashford) Langeron et Talice CBS 604t
Candida sake (Saito et Ota) Van Uden et Buckley CBS 159
Candida tropicalis (Castellani) Berkhout CBS 94t
Candida utilis (Henneberg) Lodder et Kreger-van Rij CBS 621
Candida vini (Vallot ex Desmazières) Van Uden et Buckley CBS 639t
Citeromyces matritensis (Santa Maria) Santa Maria CBS 2764t
Debaryomyces hansenii (Zopf) Lodder et Kreger-van Rij G 815
Geotrichum candidum Link D 74b[a]
Hanseniaspora vineae Van der Walt et Tscheuschner CBS 2171t
Hansenula anomala (Hansen) H. et P. Sydow D 25a[a]
Hansenula capsulata Wickerham CBS 1993t
Hansenula holstii Wickerham CBS 2028
Kluyveromyces marxianus (Hansen) Van der Walt var. *marxianus* Van der Walt CBS 1555
Metschnikowia pulcherrima Pitt et Miller CBS 5833t
Pichia guillermondii Wickerham G 26[c]
Pichia membranaefaciens (Hansen) Hansen CBS 107t
Saccharomyces cerevisiae Meyen ex Hansen K 1502
Saccharomyces dairensis Naganishi WM 1K[b]
Saccharomyces exiguus Reess ex Hansen WM 5[b]
Saccharomyces unisporus Jörgensen CBS 398t
Schizosaccharomyces pombe Lindner CBS 356t
Stephanoascus ciferrii Smith, Van der Walt et Johannsen P2–25[c]
Torulaspora delbrueckii (Lindner) Lindner CBS 1146t
Yarrowia lipolytica (Wickerham et al.) Van der Walt et Von Arx CBS 6124t
Zygosaccharomyces bailii (Lindner) Guillermond Na
Zygosaccharomyces rouxii (Boutroux) Yarrow CBS 732t
Basidiomycetous species
Cryptococcus laurentii (Kufferath) Skinner CBS 7140
Leucosporidium scottii Fell, Statzell, Hunter et Pfaff M 1
Rhodosporidium toruloides Banno CBS 14t
Rhodotorula minuta (Saito) Harrison K2–7[c]
Trichosporon cutaneum (De Beurmann, Gougerot et Vaucher) Ota B1-301[c]
Mould species
Aspergillus fumigatus RIVM M3
Cladosporium cladosporioides CBS 143.65
Cladosporium herbarum CBS 673.69
Fusarium oxysporum RIVM M28
Fusarium sambucinum CBS 291.91
Fusarium poae CBS 446.67
Mortierella polycephala Coemans CBS 327.72
Mortierella reticulata CBS 452.74
Mortierella hyalina (Harz) W. Gams CBS 654.68
Paecilomyces variotti RIVM M113
Penicillium funiculosum RIVM M50
Penicillium digitatum RIVM M58
Penicillium tardum RIVM M59
Penicillium aurantiogriseum CBS 342.51
Penicillium charlesii NRRL 1887
Talaromyces flavus RIVM M86
Trichoderma roseum RIVM M34
Wallemia sebi v. Arx RIVM M114

[a] Described by Middelhoven & Van Baalen (1988).
[b] Described by Middelhoven & Franzen (1986).
[c] Described by Middelhoven, De Jong & De Winter (1990).

were used as an inoculum. These were prepared by making pour plates (14.5 cm diameter) of several dilutions of spore suspensions in 20 ml of a solution of 1.5% (w/v) malt extract (Oxoid L 39) and 0.25% (w/v) agar (Oxoid L 13), which were incubated at 25° C till the germinated spores had grown out to micro-colonies with a diameter of 2–5 mm and could be harvested with a small spatula. From plates with 20–50 well isolated micro-colonies, five micro-colonies were transferred to 1 l Erlenmeyer flasks containing 200 ml of culture medium.

Culture media

Yeast nitrogen base (YNB) and yeast carbon base (YCB) (Difco Labs, Detroit, U.S.A.) served as basal medium with concentrations of 6.7 g l^{-1} and 11.7 g l^{-1} respectively. Both media are composed of defined quantities of minerals, some amino acids, vitamins and some trace elements. In addition, YNB contains ammonium sulphate (5 g l^{-1}) as nitrogen source but no carbon source while YCB contains glucose (10 g l^{-1}) as carbon source but no nitrogen source. Six different carbon sources (30 g l^{-1}), *viz.* glucose monohydrate (Merck), saccharose (Merck), lactose (Difco), maltose monohydrate (Merck), mannose (Merck) and lactate (CCA Biochem, Gorinchem, The Netherlands) were used. Two nitrogen sources, *viz.* ammonium sulphate (Merck) (5 g l^{-1}) and urea (Merck) (2.25 g l^{-1}) containing the same amount of nitrogen, were used in YCB. Solutions of ten-fold concentrated basal media were filter-sterilised (0.45 μm) and added to autoclaved solutions of nitrogen and carbon sources. Incubation was performed at 22° C, on a rotary shaker at 100 rpm (Gallenkamp Ltd, Loughborough, UK), except with *A. corymbifera* and *S. racemosum* which were incubated at 37° C.

Isolation of mycelium and water-soluble extracellular polymers

The culture fluid (200 ml) was separated from the mycelium by filtration through pre-dried and pre-weighed filter paper (589 Schwarzband, Schleicher and Schüll, Dassel, Germany) on a Büchner funnel. The mycelial dry weight was measured after oven-drying the mycelium residue at 80° C for 24 h.

The filtrates of two flasks were combined (400 ml) and concentrated five-fold with a vacuum rotary evaporator in a water bath at 40° C, and heated for 5 min at 100° C to inactivate cellulase enzymes damaging dialysis bags. The filtrate was poured into a dialysis bag (pre-boiled with distilled water), dialysed overnight against running tap water and subsequently against distilled water for 24 h. The solution was concentrated five times with a rotary evaporator and lyophilized. The solid was dissolved in 20 ml of distilled water and any water-insoluble material was removed by centrifugation (15 min, 19600 *g*). Finally, the water-soluble material supposed to consist of the extracellular polymers (EPS) was freeze-dried and weighed.

Chemical analyses of the extracellular polymers

The glucuronic acid content of the mixture of extracellular polymers was measured using the automated *m*-hydroxy diphenyl assay (Thibault 1979) slightly modified by the addition of 0.0125 M sodium tetraborate to the sulphuric acid as described by Blumenkrantz and Asboe-Hansen (1973), with glucuronic acid as the standard. Neutral sugars were determined using the automated orcinol method (Tollier & Robin 1979), using glucose as the standard. Corrections in the latter analysis were made for interference from glucuronic acid.

HPLC detection of the glucose level in the culture medium

The amount of residual glucose was measured using the HPLC method according to Voragen et al. (1986). Samples of the culture filtrates (1 ml) were treated with 50 μl of a 1 M solution of lead nitrate. The mixture was stored for at least 1 hour in the freezer (– 20° C) and centrifuged (1000 *g*; 10 min). An aliquot of 20 μl of the supernatant was injected into an SP 8800 HPLC-system (Spectra Physics, San Jose, CA U.S.A.). The system was equipped

with a Merck Polyspher CH PB HPLC-column (85° C) and a guard column (20° C) packed with a mixture of equivalent amounts of dried AG50W-X4 (H^+, 400 mesh) and AG3-X4A (OH^-, 200–400 mesh) (Bio-Rad Labs., Richmond, CA U.S.A.). Distilled water filtered in a Millipore system (Millipore Corp., Bedford, MA U.S.A) was used as eluent at a flow rate of 0.5 ml min^{-1}. Sugars were detected with a Shodex RI SE-61 (Showa Denko, Tokyo, Japan) Refractive Index detector. Standard solutions of glucose, saccharose, mannose, lactose and maltose were used for calibration in the respective experiments.

Sandwich ELISA for the detection of antigenic EPS

Polyclonal IgG antibodies 1000/1201 were obtained by immunization of rabbits with EPS isolated from *Mucor racemosus* as described by Notermans and Heuvelman (1985). The IgG fraction of the serum was purified using selective precipitation with octanoic acid as described (Steinbuch & Audran 1969). The sandwich ELISA was carried out as described earlier (Notermans & Heuvelman 1985; De Ruiter et al. 1991a) in wells of polyvinyl chloride microtitre plates (Dynatech, Chantilly, VA U.S.A.). The substrate solution was 3,3′,5,5′-tetramethylbenzidine in dimethyl sulphoxide containing H_2O_2 prepared according to Bos et al. (1981). The horseradish peroxidase reaction was stopped by adding 50 μl of 2 M H_2SO_4 to each well. The absorbance of the yellow colour was measured spectrophotometrically at 450 nm. The ELISA reactivity was expressed as the titre of the isolated EPS, defined as the reciprocal dilution of a solution of 10 μg ml^{-1} EPS in distilled water just giving a positive reaction, i.e. an absorption ≥ 0.1 above that of a blank containing no antigenic EPS.

Results

Specificity of the polyclonal IgG antibodies used in this study

The polyclonal IgG antibodies (1000/1201) raised in rabbits against the EPS of *M. racemosus* were further tested on their specificity. A sandwich ELISA based on these antibodies was used to test EPS of different strains of yeasts and moulds, of which many often occur in foods. The yeast strains tested belonged to ascomycetous and basidiomycetous species (Table 1). The EPS of (imperfect) ascomycetous yeasts are almost species-specific (Middelhoven & Notermans 1988), therefore a large variety of ascomycetous species were tested. Moreover, 18 strains of moulds, belonging to nine different genera were tested. No cross-reactivity with the strains of yeasts and moulds tested could be observed, except with the yeast *Pichia membranaefaciens* (Hansen) Hansen. This strain gave a clear positive reaction with the polyclonal IgG antibodies raised against EPS of *M. racemosus*. Therefore, EPS from *P. membranaefaciens* was isolated and purified. Its ELISA titre determined on a 10 μg ml^{-1} preparation of EPS in distilled water was 200. A similar ELISA reactivity was obtained in the same assay for EPS of *M. racemosus* and *Rhizopus stolonifer*.

Time experiments

The production of extracellular polymers by *Mucor hiemalis* and its content of antigenic EPS was tested as a function of growth. The yield of extracellular polymers and the mycelial dry weight as function of the age of the culture using ammonium sulphate as the nitrogen source are shown in Figure 1. After removing the mycelial pellets by filtration, fractions of the culture medium were examined with the sandwich ELISA also shown in Fig. 1. Mycelial growth and production of antigenic EPS followed similar trends stabilizing after 6 days. The production of total EPS was more gradual and stabilized after 12 days. The initial pH (5.1) dropped to 2.3, while the amount of glucose was only consumed partially after 20 days of incubation as indicated. Apparently, the low pH limited further growth and substrate assimilation. In similar experiments with urea as sole source of nitrogen, the initial pH of 5.9 increased to 8.8 after 21 days of incubation. The use of equal amounts of urea and ammonium calculat-

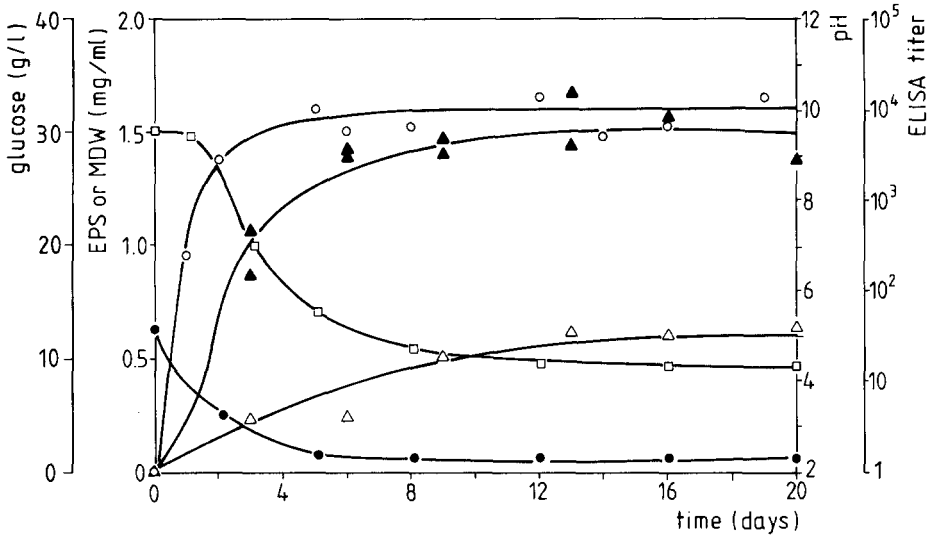

Fig. 1. Growth and production of antigenic extracellular polysaccharides of *Mucor hiemalis* with glucose as the carbon source and ammonium sulphate as the nitrogen source. Symbols: -▲-▲-, Mycelial dry weight; -△-△-, yield of EPS; -○-○-, sandwich ELISA titre; -●-●-, pH; , -□-□-, glucose consumption.

ed on N-weight, resulted in a decrease of the pH from 6.5 to 4.7 during the first nine days followed by an increase to 7.4 after 21 days of incubation. Under these circumstances the amount of glucose was consumed completely after 8 days. Also, the mycelial dry weight and antigenic EPS increased during six days, while no significant decrease of the titre of the culture medium could be observed during the stationary phase.

Inoculation using micro-colonies

During the above-mentioned growth experiments (Fig. 1), inoculation with spore suspensions caused rather large standard deviation, variation coefficient 19.1%, of mycelial dry weight. It was attempted to reduce this variability by inoculation with pre-germinated spores. The reproducibility was tested in 12 replications. In nearly all cases every single micro-colony grew out into one mycelial pel-

Table 2. Mycelial dry weight, yield and ELISA reactivity of excreted EPS of *M. hiemalis* using different glucose concentrations in the liquid culture medium of YNB with ammonium sulphate (5 g l^{-1}) as nitrogen source after 8 days of incubation[a].

Glucose		Mycelial biomass	Excreted EPS			Ratio[c]	ELISA titre[d]
initial (g l^{-1})	final (g l^{-1})	(mg l^{-1})	weight (mg l^{-1})	% n.s.[b] (wt/wt)	% glcA[b] (wt/wt)		
5	0	1395 ± 50	330 ± 5	34 ± 1	23 ± 0	0.13	++
10	0	1515 ± 30	445 ± 25	35 ± 0	25 ± 1	0.18	++
20	4	1655 ± 65	600 ± 20	34 ± 1	24 ± 0	0.21	++
30	12	1560 ± 45	610 ± 40	35 ± 2	23 ± 1	0.23	++
50	25	1510 ± 25	650 ± 5	34 ± 1	21 ± 1	0.24	++

[a] Average of four replications ± SD.
[b] Neutral sugar and glucuronic acid content respectively.
[c] Calculated by taking the saccharide content (neutral sugars + glucuronic acid) of the EPS and the mycelial biomass.
[d] ELISA titre of the isolated EPS fractions as determined on 10 μg ml^{-1} solutions in distilled water: -, titre < 10; +, 10 ≤ titre ≤ 100; ++, 100 ≤ titre ≤ 1000; +++, titre > 1000.

Table 3. Mycelial dry weight, yield and ELISA reactivity of excreted EPS of *M. hiemalis* using different carbon and nitrogen sources after 8 days of incubation[a].

Carbon source[e]	Nitrogen source[f]	Mycelium Biomass	Excreted EPS			Ratio[c]	ELISA titre[d]
		(mg l^{-1})	weight (mg l^{-1})	% n.s.[b] (wt/wt)	% glcA[b] (wt/wt)		
glucose	$(NH_4)_2SO_4$	1605 ± 60	710 ± 15	36 ± 2	22 ± 1	0.26	++
mannose	$(NH_4)_2SO_4$	1865 ± 15	765 ± 15	39 ± 2	21 ± 1	0.25	++
saccharose	$(NH_4)_2SO_4$	1945 ± 55	600 ± 30	32 ± 1	27 ± 2	0.18	++
maltose	$(NH_4)_2SO_4$	765 ± 25	665 ± 5	44 ± 1	7 ± 0	0.44	+
glucose	urea	1285 ± 90	240 ± 25	24 ± 2	7 ± 2	0.06	++
glucose	$(NH_4)_2SO_4$/urea	1755 ± 15	130 ± 5	30 ± 1	17 ± 1	0.03	++

[a], [b], [c] and [d] as in Table 2.
[e] Concentration of the sugars was 30 g l^{-1}.
[f] Concentration of the nitrogen source 1.06 g N l^{-1}.

let under shaking conditions. The average of the biomass produced per Erlenmeyer flask was 281.2 ± 13.6 mg (variation coefficient 4.8% with confidence limit 95%; Student's *t* test). Consequently, in the experiments reported in Tables 2, 3 and 4 micro-colonies were used as inoculum.

Growth and production of EPS as a function of the glucose concentration

The effect of glucose concentration on mycelial dry weight and production of EPS of *M. hiemalis* was assessed using ammonium as the nitrogen source. As the sugar constituents of the EPS were found to be immunodominant (Miyazaki et al. 1980; De Ruiter et al. 1991b), the ratio between the amounts of EPS and of biomass of the moulds was calculated based on the total sugar content of the EPS. As shown in Table 2, production of EPS of *M. hiemalis* occurred at all glucose concentrations tested. The chemical composition of the respective EPS was similar, as can be judged from the percentages of neutral sugars and glucuronic acid (Table 2). After

Table 4. Mycelial dry weight, yield and ELISA reactivity of excreted EPS of various strains belonging to Mucorales after 8 days of incubation at their optimum temperature[a].

Mould species	Mycelium biomass	Excreted EPS			Ratio[c]	ELISA titre[d]
	(mg l^{-1})	weight (mg l^{-1})	% n.s.[b] (wt/wt)	% glcA[b] (wt/wt)		
Mucor hiemalis	1560 ± 100	610 ± 40	35 ± 2	23 ± 1	0.23	++
Mucor circinelloides	1455 ± 100	410 ± 25	30 ± 1	14 ± 0	0.12	++
Mucor racemosus	1170	190	34	16	0.08	++
Rhizopus oryzae	1405 ± 145	290 ± 5	30 ± 2	22 ± 1	0.11	++
Rhizopus stolonifer	1885	300	33	21	0.09	++
Rhizomucor miehei	2270	325	28	22	0.07	++
Rhizomucor pusillus	1555	220	19	15	0.05	++
Absidia corymbifera	1250 ± 80	175 ± 20	37 ± 2	9 ± 1	0.06	+
Syncephalastrum racemosum	1125 ± 90	140 ± 10	35 ± 2	13 ± 1	0.06	++

[a] Glucose (30 g l^{-1}) was used as the carbon source and ammonium sulphate as the nitrogen source (5 g l^{-1}), average of four replications ± SD. If no SD is given, the values are the average of two experiments.
[b], [c] and [d] as in Table 2.

8 days of growth, glucose was exhausted in the media initially containing 5 and 10 g l^{-1} of glucose. An increase of the initial glucose concentration to 20–50 g l^{-1} resulted in higher amounts of carbon source consumed, approximately the same amount of mycelial biomass produced and significantly higher ratios between excreted amounts of EPS and biomass of the mould. The antigenicity of the EPS isolated from culture media with increasing amounts of glucose as carbon source, was similar as could be revealed from their ELISA reactivity in Table 2. In all cases between 10 and 100 ng per ml of the isolated EPS could be detected. Under all conditions, the initial pH of 5.1 dropped to 2.6 within three days, probably as a result of ammonia utilization and production of organic acids (Slaughter 1988).

Effect of the nutrients

To investigate the effect of different carbon sources, glucose was replaced by 30 g l^{-1} of a monosaccharide (mannose), disaccharides (saccharose, maltose and lactose) and lactate in YNB, using ammonium sulphate as the nitrogen source (Table 3). Replacement by mannose did not have significant influence on production of biomass and EPS. Also, the antigenicity of the isolated EPS, tested in the ELISA, was similar to that of glucose-grown *M. hiemalis*. With disaccharides (saccharose and maltose) as carbon source, these features changed somewhat more as shown in Table 3. Growth of *M. hiemalis* on maltose as sole carbon source resulted in an EPS preparation with a relatively high neutral sugar content, low glucuronic acid content and a low antigenicity. The initial pH (5.1) of the culture media with various carbon sources dropped to a final value of 2.4 (mannose and saccharose) and 2.9 (maltose). On lactose and lactate, no growth occurred and consequently no EPS could be detected.

If ammonia was replaced entirely or partly by urea, the amount of EPS was significantly lower (Table 3). If urea was used as the only nitrogen source the initial pH of 7.1 increased to a value of 8.0, and the ratio between the sugar part of the EPS and biomass was as low as 0.06, which is approximately four times less than with ammonium as nitrogen source. The combined use of urea and ammonium had a stabilizing effect on the pH (final value 4.7), enabling rapid growth. However, the amount of EPS was even lower as shown in Table 3. The change in nitrogen source did not have a significant influence on the antigenicity of the respective EPS (Table 3).

Growth and production of extracellular polymers and antigenic EPS of various species belonging to Mucorales

In order to assess growth, formation of EPS and ELISA titre of species of the Mucorales, other than *M. hiemalis*, a number of strains of major genera were grown under the same conditions at their optimum temperature for growth. Strains tested belonged to species of the genera of *Mucor*, *Rhizopus* and *Rhizomucor* as well as *Absidia corymbifera* and *Syncephalastrum racemosum*. As shown in Table 4, all species belonging to five different genera excreted EPS, containing neutral sugars and glucuronic acid. However, the proportion of EPS to biomass was significantly lower than for *M. hiemalis*. The EPS produced by *Absidia corymbifera* contained much more glucose compared to the EPS of the other Mucorales strains tested (De Ruiter et al. 1991a). The antigenicity of the EPS of *A. corymbifera* as tested in the sandwich ELISA was lower compared to the other EPS which showed similar antigenicity as EPS from *M. hiemalis* as shown in Table 4.

Discussion

The polyclonal IgG antibodies raised against EPS of *M. racemosus* used in this study were found to be specific for moulds belonging to Mucorales. Only the yeast *Pichia membranaefaciens* (Hansen) Hansen did cross-react with these antibodies. This yeast often has been isolated from soil and is frequently found in foods such as various beverages, yoghurt and yeast cake (Kreger-van Rij 1984). No cross-

reaction of the IgG antibodies was observed with different strains of moulds other than those belonging to Mucorales. Hence, these polyclonal antibodies are more specific than those described by Notermans & Soentoro (1986) which cross-reacted with some other mould genera. Therefore, our IgG antibodies can be considered as almost mono-specific for Mucoralean moulds, enabling specific detection of these moulds.

The time experiment in this study showed that growth and carbohydrate assimilation of *M. hiemalis* is limited by the decreased pH. However, more carbohydrate may have been incorporated into the EPS under these conditions. It is shown that using selected carbon and nitrogen sources, often found in foods, significant amounts of EPS are excreted if growth of Mucorales moulds occurs. The chemical composition of these EPS, previously established consisting of neutral sugars (19–37%), glucuronic acid (14–42%) and protein (11–40%) is similar under all growth conditions tested, except using maltose as carbon source and if *Absidia corymbifera* is inoculated. In these cases EPS is produced with a much higher neutral sugar content, due to the presence of glucans, not reactive in this sandwich ELISA (De Ruiter et al. 1991a).

The antigenic polysaccharides constitute only a minor part of the total amount of EPS excreted by different moulds under various growth conditions and evidently, their production only occurs if biomass is formed. The increase of the ELISA titre in the growth experiment follows the growth curve of the mould closely, indicating that at all stages of growth the antigenicity of the EPS is of the same order of magnitude. This agrees with results of Middelhoven et al. (1988) who demonstrated that the amount of EPS excreted by the yeasts *Hansenula wickerhamii* and *Stephanoascus ciferri* was proportional to the amount of biomass under a great variety of culture conditions. However, in cultures of *Saccharomyces cerevisiae* antigenic EPS excretion occurred in the stationary growth phase (Middelhoven et al. 1988). In Mucorales and in yeasts under various conditions no enzymes are produced that degrade the antigenic part of the EPS. This phenomenon is important in view of practical application of the ELISA. However, as shown for growth experiments with selected culture liquids, a quantification of the amount of biomass based on ELISA titre is inaccurate. This is due to the variations of the excreted amounts of EPS related to biomass, and to the relative amount of antigenic polysaccharides in the EPS.

Hence, our results allow the conclusion that the use of the sandwich ELISA developed by Notermans and coworkers (Notermans & Heuvelman 1985; Notermans et al. 1986; De Ruiter et al. 1991a, 1992b) for the detection of *Mucor* and *Rhizopus* species is almost specific for moulds belonging to Mucorales including species belonging to the genera *Rhizomucor*, *Absidia* and *Syncephalastrum*. However, as shown in this study, quantification of these moulds in liquid cultures with the ELISA may only be indicative as a result of variations in the excretion and composition of the EPS due to differences in growth conditions as well as the reproducibility of the ELISA system.

The use of the sandwich ELISA method in complex samples such as food, characterized by complex matrices of various components, requires the detection of false-positive immunological reactions in order to enhance the reliability of the assay. This can be accomplished by the use of synthetic antigens in control assays as clearly shown by Notermans and coworkers (Notermans et al. 1988; Kamphuis et al. 1989). Detailed knowledge about the structure of the epitopes is necessary to make their synthesis possible. A general application of this sandwich ELISA requires also more detailed information about the excretion of antigenic polysaccharides during growth of these moulds on solid substrates.

Acknowledgements

We gratefully acknowledge Dr. R.A. Samson and Dr. W. Gams (Centraalbureau voor Schimmelcultures, Baarn, the Netherlands) for providing some strains of moulds used in this study. These investigations were supported by the Netherlands' Foundation for Chemical Research (SON) with financial aid from the Netherlands' Technology Foundation (STW).

References

Barnett JA, Payne RW & Yarrow D (1991) Yeasts; Characteristics and Identification (2nd ed.). Cambridge University Press, Cambridge

Bartnicki-Garcia S & Nickerson WJ (1962) Nutrition, growth, and morphogenesis of *Mucor rouxii*. J. Bacteriol. 84: 841–858

Bartnicki-Garcia S & Reyes E (1968) Polyuronides in the cell walls of *Mucor rouxii*. Biochim. Biophys. Acta 170: 54–62

Blumenkrantz N & Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484–489

Bos ES, Van der Doelen AA, Van Rooy N & Schuurs AHWM (1981) 3,3',5,5'-tetramethylbenzidine as an Ames test negative chromogen for horse-radish peroxidase in enzyme-immunoassay. J. Immunoassay 2: 187–203

De Ruiter GA, Van der Lugt AW, Voragen AGJ, Rombouts FM & Notermans SHW (1991a) High-performance size-exclusion chromatography and ELISA detection of extracellular polysaccharides from Mucorales. Carbohydr. Res. 215: 47–57

De Ruiter GA, Smid P, Van der Lugt AW, Van Boom JH, Notermans SHW & Rombouts FM (1991b) Immunogenic extracellular polysaccharides of Mucorales. In: Latgé JP & Boucias D (Eds) Fungal Cell Wall and Immune Response, Vol H 53 (pp 169–180). NATO ASI Series, Springer Verlag, Berlin/Heidelberg

De Ruiter GA, Josso SL, Colquhoun IJ, Voragen AGJ & Rombouts FM (1992a) Isolation and characterization of β(1–4)-D-glucuronans from extracellular polysaccharides of moulds belonging to Mucorales. Carbohydr. Polym. 18: 1–7

De Ruiter GA, Hoopman T, Van der Lugt AW, Notermans SHW & Nout MJR (1992b) Immunochemical detection of Mucorales species in food. In: Samson RA, Hocking AD, Pitt JI & King AD (Eds) Modern Methods in Food Mycology (pp 221–227). Elsevier Publishers, Amsterdam

Fett WF, Osman SF & Dunn MF (1989) Characterization of exopolysaccharides produced by plant-associated fluorescent Pseudomonads. Appl. Environ. Microbiol. 55: 579–583

Goatley JL (1968) Production of exocellular polysaccharides by *Alternaria solani*. Can. J. Microbiol. 14: 1063–1068

Graham DCW, Steinkraus KH & Hackler LR (1976) Factors affecting production of mold mycelium and protein in synthetic media. Appl. Environ. Microbiol. 32: 381–387

Jones KW & Kaufman L (1978) Development and evaluation of an immunodiffusion test for diagnosis of systemic Zygomycosis (Mucormycosis) Preliminary report. J. Clin. Microbiol. 7: 97–103

Kamphuis HJ, Notermans S, Veeneman GH, Van Boom JH & Rombouts FM (1989) A rapid and reliable method for the detection of molds in foods: using the Latex Agglutination assay. J. Food Prot. 52: 244–247

Kaufman L, Turner LF & McLaughlin DW (1989) Indirect enzyme-linked immunosorbent assay for Zygomycosis. J. Clin. Microbiol. 27: 1979–1982

Kreger-Van Rij NJW (Ed) (1984) The yeasts, a taxonomic study. (3rd ed). Elsevier, Amsterdam

Longbottom JL & Austwick PKC (1986) Fungal antigens. In: Weir DM (Ed) Immunochemistry: Handbook of Experimental Immunology, Vol 1 (pp 7.1–7.11). Blackwell Scientific Publications, Oxford

Martin SM & Adams GA (1956) A survey of fungal polysaccharides. Can. J. Microbiol. 34: 715–721

Middelhoven WJ & Franzen MM (1986) The yeast flora of ensiled whole-crop maize. J. Sci. Food Agric. 37:855–861

Middelhoven WJ & Notermans S (1988) Species-specific extracellular antigen production by ascomycetous yeasts detected by ELISA. J. Gen. Appl. Microbiol. 34: 15–26

Middelhoven WJ & Van Baalen AHM (1988) Development of the yeast flora of whole-crop maize during ensiling and during subsequent aerobiosis. J. Sci. Food Agric. 42: 199-207

Middelhoven WJ, Slingerland RJ & Notermans S (1988) The effect of growth conditions on production and excretion of extracellular antigens by three ascomycetous yeasts. Antonie van Leeuwenhoek 54: 235–244

Middelhoven WJ, De Jong IM & De Winter M (1990) Yeasts and fungi occurring in ensiled whole-crop maize and other ensiled vegetable crops. Antonie van Leeuwenhoek 57: 153–158

Miyazaki T & Irino T (1972) Studies on fungal polysaccharides. X. Extracellular heteroglycans of *Absidia cylindrospora* and *Mucor mucedo*. Chem. Pharm. Bull. 20: 330–335

Miyazaki T, Yadomae T, Yamada H, Hayashi O, Suzuki I & Ohshima Y (1980) Immunochemical Examination of the polysaccharides of Mucorales. In: Sandford PA & Matsuda K (Eds) Fungal Polysaccharides, ACS Symposium Series 126 (pp 81–94). American Chemical Society, Washington DC

Notermans S & Heuvelman CJ (1985) Immunological detection of moulds in food by using the enzyme-linked immunosorbent assay (ELISA); preparation of antigens. Int. J. Food Microbiol. 2: 247–258

Notermans S, Heuvelman CJ, Beumer RR & Maas R (1986) Immunological detection of moulds in food: relation between antigen production and growth. Int. J. Food Microbiol. 3: 253–261

Notermans S & Soentoro PSS (1986) Immunological relationship of extra-cellular polysaccharide antigens produced by different mould species. Antonie van Leeuwenhoek 52: 393–401

Notermans S, Veeneman GH, Van Zuylen CWEM, Hoogerhout P, Van Boom JH (1988) (1–5)-linked β-D-galactofuranosides are immunodominant in extracellular polysaccharides of *Penicillium* and *Aspergillus* species. Mol. Immunol. 25: 975–979

Slaughter JC (1988) Nitrogen metabolism. In: Berry DR (Ed) Physiology of Industrial Fungi (pp 58–76). Blackwell Scientific Publications, Oxford

Solomons GL (1975) Submerged culture production of mycelial biomass. In: Smith JE & Berry DR (Eds) The Filamentous Fungi, Industrial Mycology, Vol 1 (pp 249–264). Edward Arnold Publishers, London

Steinbuch M & Audran R (1969) The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys. 134: 279–284

Thibault J-F (1979) Automatisation du Dosage des Substances Pectiques par la Méthode au Méta-hydroxydiphényl. Lebensm. Wiss. Technol. 12:247–251

Tollier MT & Robin J-P (1979) Adaptation de la méthode à l'orcinol-sulfurique au dosage automatique des glucides neutres totaux: conditions d'application aux extraits d'origine végétale. Ann. Technol. Agric. 28: 1–15

Voragen AGJ, Schols HA, Searle-van Leeuwen MF, Beldman G & Rombouts FM (1986) Analysis of oligomeric and monomeric saccharides from enzymatically degraded polysaccharides by high-performance liquid chromatography. J. Chromatogr. 370: 113–120