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Yeast β(1-3),(1-6)-D-glucan films: Preparation and characterization of some structural and physical properties

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ABSTRACT

Cell wall polysaccharide suspensions (mainly β-glucan) was isolated from baker's yeasts (*Saccharomyces cerevisiae*) and used for the preparation of films. Glycerol was added as a plasticizer. Purity and composition of the films were tested by elemental analysis, enzymatic assay of α- and β-glucans, monosaccharide composition analysis (total hydrolysis, HPAEC) and vibration spectroscopy (FTIR, FT Raman). Surface properties and the degree and type of crystallinity, together with ageing effects, were estimated by scanning electron microscopy (SEM), atomic force microscopy (AFM) and X-ray diffraction (XRD). Mechanical and thermal properties were characterized by tensile tests and difference scanning calorimetry (DSC), respectively. The prepared films were water insoluble, compact, non-porous, exhibit no pronounced crystallinity and consist of granular-like and fibre microstructures, which could be assigned as cell wall residues and released polysaccharide macromolecules. Certain structural changes in the film surface during one-year shelf storage can be related to reorientation and decomposition of surface macromolecules due to reaction with the ambient atmosphere, rather than to crystallization phenomena.

1. Introduction

Films formed from biologically active substances are used as an alternative to textile based materials. These modern wound dressing are usually composed by polysaccharides, *e.g.*, chitin (Su et al., 1997, 1999), chitosan (Howling et al., 2001) and/or β(1-3),(1-6)-D-glucan (Kofuji et al., 2010), in certain cases combined with other suitable substances, *e.g.*, gelatine (Lee, Jeon, Lee, Cho, et al., 2003), collagen (Delatte et al., 2001), and gelatine and hyaluronic acid (Lee, Jeon, Lee, Lee, et al., 2003).

Cell wall β-glucan, a potent immunomodulator isolated from yeasts or higher fungi (Novák & Větvička, 2009), has been reported to increase collagen deposition and tissue tensile strength in experimental models of wound repair (Duo, Leiying, Williams, & Browder, 2002). According to an earlier proposed mechanism, β-glucan supports wound healing by macrophage activation (Ovington, 1998). Macrophages are vital in the inflammatory phase of healing, providing phagocytosis and secretion of cytokines that promote the formation of the new tissue (Portera et al., 1997). Other products of cytokines, fibroblasts, play a particularly important role in the generation of the new skin tissue. Recent data have shown the presence of β-glucan receptors on normal human dermal fibroblasts, thus possibility of direct stimulation of fibroblast collagen biosynthesis (Duo et al., 2002; Son et al., 2005).

The biological benefits of β-glucan on wound healing is thus well known. In practical use the immunological benefits represent only one side of the problem for dressings or skin substitutions based on β-glucan films. The package of β-glucan macromolecules in a film depends on their conformation, which determines the presence of ordered or disordered structures. It is generally believed that biologically active preparations should contain β-glucan in the triple-helix conformation (*e.g.*, Bohn & BeMiller, 1995), however it is known that the β-glucan helices dissociate into random coils when the strength of the bonds keeping the helix together are decreased below a critical limit (Sletmoen & Stokke, 2008): the triple helix of β-glucan is unravelled, *e.g.*, by increased temperature and/or high pH (Miura et al., 1995; Saitô et al., 1991; Young & Jacobs, 1998); after some time the non-crystalline stage slowly renaturated to the crystalline one (McIntire & Brant, 1998; Sletmoen & Stokke,

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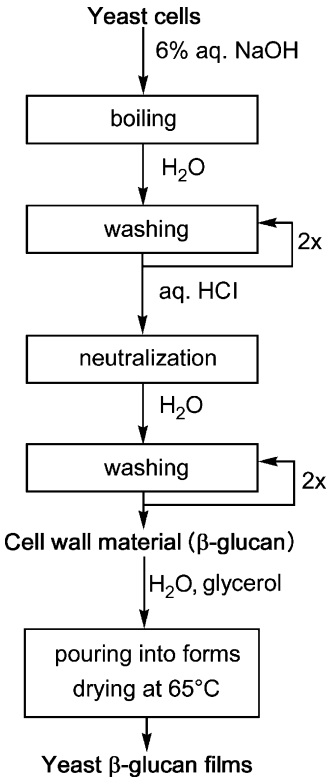

Fig. 1. Scheme of preparation of yeast β-glucan films.

2008). High pH and temperature are common conditions during β-glucan isolation processes, so freshly prepared β-glucan is probably denatured.

Knowledge of physicochemical, morphological and mechanical properties of β-glucan films, *e.g.*, a shape of surface, porosity and, last not least, a tensile strength, enables appreciation of suitability of the material for intended application, as well as tracing of mechanisms of healing. Modern physical methods, vibration spectroscopy (FTIR and FT Raman), scanning electron microscopy (SEM) (Simi & Abraham, 2010) and atomic force microscopy (AFM) (Abu-Lail & Camesano, 2003; Ding & Himmel, 2006; Morgan, Roberts, Tendler, Davies, & Williams, 1999) represent necessary tools for film characterization.

In this study β-glucan films were prepared from cell wall polysaccharide suspensions isolated from baker's yeasts (*Saccharomyces cerevisiae*). Structure and physical properties of these films were examined by elemental analysis, enzymatic assay of α- and β-glucans, vibration spectroscopy (FTIR, FT Raman), microscopic methods (SEM, AFM), X-ray diffraction (XRD), tensile test and thermal analysis (DSC). Structural changes of the films during ageing are to some extent also studied in this paper.

2. Materials and methods

2.1. Preparation of yeast β-glucan films

The scheme of preparation of yeast β-glucan films is shown in Fig. 1. Intact cells of commercial baker's yeasts (*S. cerevisiae*) were used for isolation of the cell wall material by alkali digestion (6% aq. NaOH) followed by centrifugal separation of the cell wall residues according to the method of Manners, Masson, and Paterson (1973). The isolated material was assigned as β-glucan because it contained this polysaccharide as the main component, mixed with small amount of other polysaccharides. Yeast β-glucan films were prepared from the isolated material by re-suspending in water at the concentration corresponding to an area density of 10 mg cm^{-2}. Glycerol (25% related to weight of the solid phase) was added as a plasticizer directly into the suspension before drying. The suspension was poured onto a metal dish with known area and then dried out at 65 °C in a hot-air oven for 2 h. After drying, films were peeled off and examined. For ageing studies, films were stored at steady air-conditioned laboratory conditions at known temperature and air humidity, protected from direct light and dust deposition. All chemicals were used as received. Water used in the experiments was purified by reverse osmosis and deionization.

2.2. Analyses of film composition

Content of carbon, hydrogen and nitrogen in the parent yeast β-glucan as well as yeast β-glucan films was performed on the Elementar Vario EL III analyzer (Elementar Analysensysteme GmbH, Germany).

Monosaccharide composition was detected after 1-h hydrolysis by 3 M trifluoroacetic acid (TFA) at 100 °C. After removal of TFA the monosaccharides were analysed by High Performance Anion-Exchange Chromatography system with pulsed amperometric detection (HPAEC-PAD), Dionex BIO LC (Dionex, USA), equipped with an analytical column 2 mm × 250 mm CarboPac PA1, guard column 2 mm × 50 mm CarboPac PA1 (Dionex Corporation, USA) and a gradient pump (Dionex Corporation Sunnyvale, USA), with mobile phase 16 mmol NaOH.

Content of total, β- and α-glucans was determined by an enzymatic kit KYBGL (Megazyme International Ireland Ltd.). Content of β-glucans was calculated as a difference between the determined contents of total glucans (obtained by acidic hydrolysis) and α-glucans (obtained by enzymatic hydrolysis).

2.3. Solubility in water

Dry films were weighed and immersed into 50 ml of distilled water containing traces of sodium azide (0.02%, w/v) and agitated very slowly at 25 °C for 24 h. Then the films were removed, dried at 60 °C and weighed again. The solubility was calculated as per cents of weight loss.

2.4. Tensile tests

The specimens for mechanical testing were rectangular strips 90 mm × 10 mm; their actual thickness and width were measured with a micrometer (average from five different points) or by stereo microscopy (average from three different points), respectively. Before testing the strips were conditioned for at least 15 days at room temperature in closed chambers with fixed relative humidity (RH) of 43% over saturated solution of potassium carbonate. The equilibrium RH was checked by a hygrometer (Thermo-Hygro-Barometer/Logger COMET D4141). Moisture content in the films stored at the same conditions was determined by drying of four representative samples at 105 °C for 1 h.

The tensile tests of the specimen strips up to their rupture were performed in the Instron™ universal testing machine (type 33R 4464) according to the corresponding software Bluehill (Instron Bluehill™ Material Testing Software, 2004) based on ASTM standards. The testing machine was equipped with a temperature controlled environmental chamber (type 3119-506). The temperature and humidity in the chamber during the test were the same as the conditions at which the tested films were stored. The initial grip separation was set at 50 mm, so that the exposed area of the specimens was 50 mm × 10 mm, and the stretching rate was set at 1 mm min^{-1} or 2 mm min^{-1}, respectively. Actual values of the load

and the grip extension were recorded with a frequency of 10 s^{-1} or 5 s^{-1}, respectively.

2.5. Differential scanning calorimetry

DSC measurements were performed in the DSC 131 (SETARAM, France). The DSC was calibrated using an indium standard. Samples (10 mg) were placed into closed platinum cells and heated under nitrogen atmosphere from −40 to +400 °C at a heating rate of 5 °C min^{-1}.

2.6. Vibration spectroscopy

FT Raman spectra were recorded on the Bruker FT Raman (FRA 106/S, Equinox 55/S) spectrometer equipped with a quartz beamsplitter, and a liquid-nitrogen cooled germanium detector; excitation at 1064 nm from the Nd:YAG laser (Bruker, USA). The laser power was set to 250 mW, and 1026 scans were accumulated with spectral resolution of 2.0 cm^{-1}. FTIR ATR spectra (spectral region 4000–400 cm^{-1}, 64 scans, spectral resolution 2.0 cm^{-1}) of prepared β-glucan films were recorded on a Nicolet 6700 spectrometer using HATR smart accessory and Omnic 8.0 software (Nicolet Analytical Instruments, USA). All obtained FT Raman and FTIR spectra were smoothed and baselines corrected by the Origin 6.0 (Microcal Origin, USA) and Omnic 8.0 (Nicolet Analytical Instruments, USA) software, respectively. The second derivative algorithm was applied for determination of overlapped bands positions.

2.7. Microscopic analysis

SEM images were obtained by the FEG-SEM microscope (Hitachi S-4700). Observed films were cut into small pieces and fixed to a sample holder by carbon suspension #05006-AB (SPI). In addition to film surface observation, cross sections of the films were examined as well. To prepare the brittle fractures of the films and to avoid changes of morphology of the cross sections by plastic deformation, all fractures were done in liquid nitrogen, and the cross sections prepared were again fixed to sample holders. Finally, to increase contrast of the pictures, all prepared samples were coated by a 10-nm layer of Au/Pd alloy by the sputter coater (Bal-Tec SCD-500). The SEM images were recorded by secondary electrons, so that topographic and phase contrasts might be superimposed in all figures. The lateral resolution of the original figures taken at high magnification (10 k) is below 10 nm; lower magnification proportionally decreases the lateral resolution. Depth resolution can be in all cases estimated to about 10 nm. Hence, each point in the presented figures (10 k-magnification) represents information yielded by the 10 nm^3 volume. Values of accelerating voltages, working distances, and original magnifications are given at individual snapshots.

Surface roughness and morphology of films were determined by the AFM in a contact mode on the Digital Instruments CP II engine with Veeco silicon P-doped probes CONT20A-CP having the spring constant 0.9 N/m.

2.8. X-ray diffraction

Powder XRD data were collected at room temperature on the X'Pert PRO θ–θ powder diffractometer with parafocusing Bragg–Brentano geometry using CuKα radiation (λ = 1.5418 Å, U = 40 kV, I = 30 mA). Data were scanned with the ultrafast detector X'Celerator or with a scintillator detector equipped with a secondary curved monochromator over the angular range 5–60° (2θ) with a step size of 0.02° (2θ) and a counting time of 0.3 s $step^{-1}$. Data were evaluated by the software package HighScore Plus.

3. Results and discussion

3.1. Composition of the films

Composition of yeast β-glucan films was tested by elemental analysis (C, H and N contents), enzymatic assay (α- and β-glucans) and neutral sugar analysis (total hydrolysis, HPAEC). Acording to elemental analysis the films contained on average 45.57%, m/m of carbon, 7.38%, m/m of hydrogen and 0.76%, m/m of nitrogen. Small amounts of nitrogen can originate from chitin and/or protein fragments retained after the alkali hydrolysis. If we suppose that all nitrogen comes from chitin, an average content of nitrogen corresponds to about 5% of this polysaccharide. A monosaccharide composition analysis confirmed homopolymer character of the yeast β-glucan preparation, which contained only glucose.

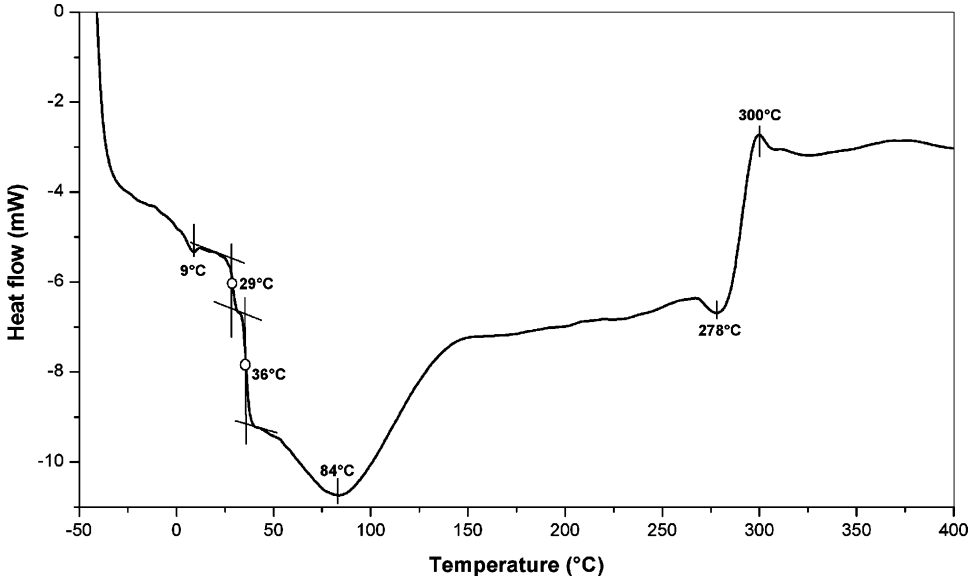

Fig. 2. DSC curve of the yeast β-glucan film (5 °C min^{-1}, under N_2).

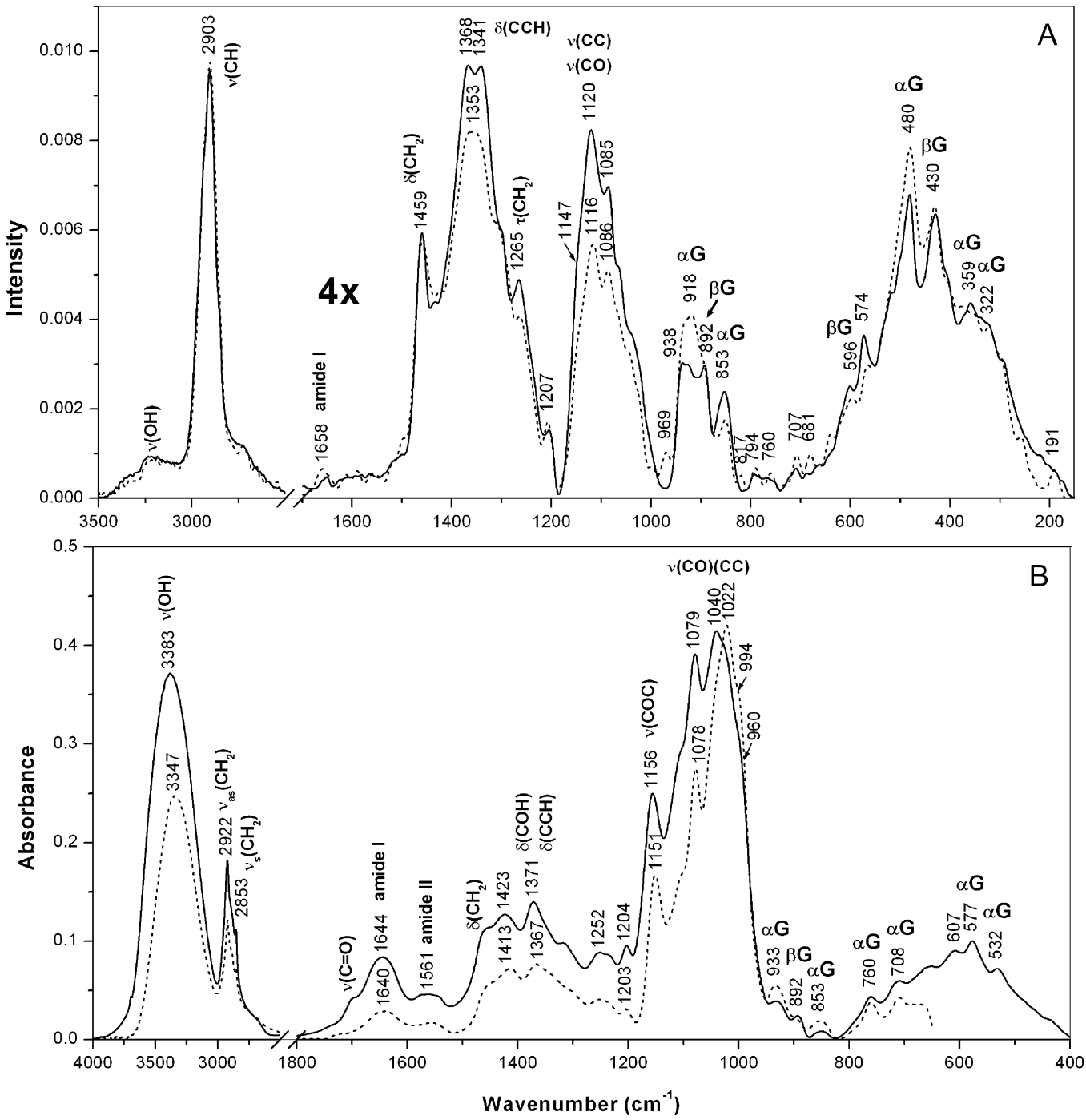

Fig. 3. FT Raman (A) and FTIR (B) spectra of the isolated yeast cell wall material (solid line) the representative sample of yeast β-glucan film (dashed line) G = glucan.

Enzymatic assay showed that β-glucans (~58%, m/m) predominate in the films, while α-glucans are present as well but in much smaller amount (~13%, m/m). Thus chitin together with α-glucans do not exceed 20%, m/m of the films. Presence of the contaminants including these polysaccharides is given by the used isolation method and generally did not influence the intended use of the films.

3.2. Solubility of the films

When immersed in distilled water, films demonstrated partial solubility; the weight loss was about 26%, m/m, which corresponded to amount of glycerol in the films and supported the idea that processing of β-glucan did not affect its solubility.

3.3. Tensile experiments

Used yeast glucan films containing 25% of glycerol were equilibrated at relative humidity of 43%. Modulus of elasticity *E*, yield stress, strength and strain at rupture of these films (21 measurements) were found to be 712 ± 31 MPa, 9.45 ± 0.37 MPa, 17.48 ± 0.61 MPa and 14.16 ± 1.11 MPa, respectively (Blahovec, Hejlová, Čopiková, & Novák, 2011). Dependence of these tensile characteristics on the amount of plasticizers (glycerol and water) and on ageing are analysed in the cited article.

3.4. DSC analysis

DSC curve of the representative sample of a yeast β-glucan film is shown in Fig. 2. A weak endothermic peak at 9 °C could be assigned to a reversible inter-helix transition of the crystalline form of yeast β-glucan (Wang, Zhang, Zhang, & Ding, 2009). Two glass transitions of the glycerol–water–glucan system were found at 29 and 36 °C. A broad endothermic peak centered at 84 °C was attributed mainly to evaporation of residual water, while disordering of the β-glucan package accompanying by the breakage of intra- and intermolecular hydrogen bonds might contribute at this region (Wang et al., 2009; Zhang, Huang, Nishinari, Watase, & Konno, 2000; Zhang, Nishinari, Williams, Foster, & Norton, 2002). The next thermal events registered represented the combination of endothermic and exothermic peaks centered near 278 and 300 °C, respectively. This is a region of polysaccharide thermal decomposition. The onset temperature of the endothermic peak (267 °C) was similar to that of pure yeast β-glucan, and the position of the exothermic peak (300 °C) was typical for chitosan, a deacetylated derivative of chitin. Partial deacetylation of the chitin component of the yeast β-glucan film could take place during alkali digestion of yeast cell walls. Based on the literature (Pawlak & Mucha, 2003; Wanjun, Cunxin, & Donghua, 2005), it was suggested that the endothermic effect corresponded to the fragmentation of both major β-glucan and minor chitin components, whereas the exothermic effect might result from cross-linking reactions between polysaccharide macromolecules involving destruction of free amino groups in chitosan. Endo- and exothermic peaks were not so pronounced as in the case of pure polysaccharides due to overlapping of these opposite effects. It is possible that carbonyl products of polysaccharide fragmentation are involved into cross-linkage reactions with chitosan amino groups, so the endothermic process is substituted by the exothermic one at higher temperatures.

3.5. Vibrational spectroscopy

FT Raman and FTIR spectra of parent yeast β-glucan and the representative sample of a yeast β-glucan film are shown in Fig. 3. The broad IR band centered around 3350–3380 cm^{-1} indicates OH stretching vibration of hydroxyls and water. The very strong Raman band near 2903 cm^{-1} was assigned mainly to CH stretching vibrations in polysaccharides pyranoid rings. IR bands at 2922 cm^{-1} and 2853 cm^{-1} and corresponding shoulders in the Raman spectra have contribution of CH_2 stretching vibrations of CH_2OH groups in sugars. The region of carbonyl vibrations (1500–1800 cm^{-1}) has weak Raman band at 1658 cm^{-1} (amide I) and two IR bands at 1640–1644 cm^{-1} (amide I and in-plane bending of water) and near 1560 cm^{-1} (the amide II). These peaks indicate the presence of chitin, a minor component of the yeast cell wall, and probably some products of protein degradation. The Raman band at 1459 cm^{-1} as well as the corresponding IR shoulder at 1465–1468 cm^{-1} were assigned to CH_2 in-plane bending in CH_2OH of sugars. The IR/Raman features in the region of 1200–1440 cm^{-1} were assigned mainly to in-plane ring deformation including CH and OH bending modes. Intense highly overlapped IR/Raman bands in the region of 990–1200 cm^{-1} (COC and CC stretching vibrations) are characteristic for polysaccharides and can be used for their identification (Kačuraková, Belton, Wilson, Hirsch, & Ebringerová, 1998; Kačuraková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The Raman bands and shoulders at 794, 1086 and 1145 cm^{-1} are typical for β-glucans (Šandula, Kogan, Kačuráková, & Machová, 1999), as well as several IR features near 1080, 1040, 994, 960 and 893 cm^{-1}. Several IR bands at 1022, 933, 853, 760, 708, 577 and 532 cm^{-1} are characteristic for α-glucans

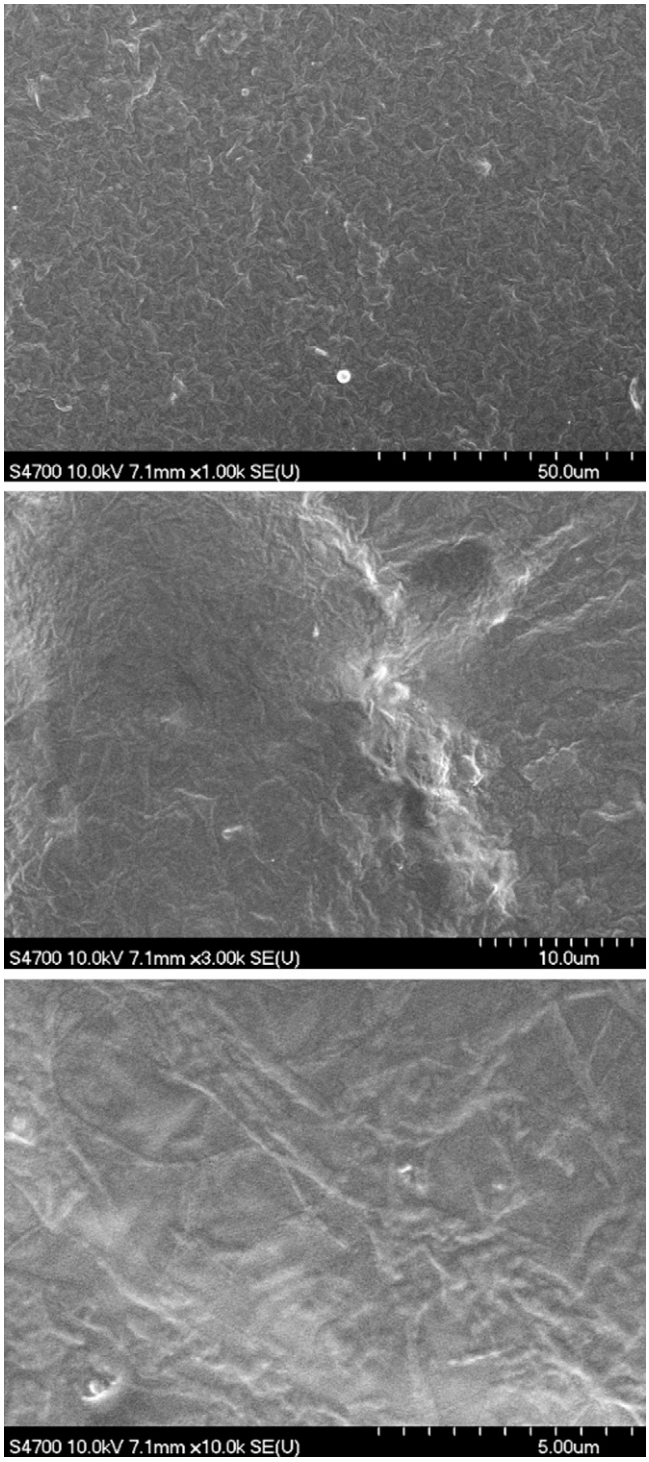

Fig. 4. SEM microphotographs of morphology of the upper side of the yeast β-glucan film.

(Kačuraková et al., 1998; Zhbankov, Andrianov, & Marchewka, 1997). The vibration bands near 892 cm^{-1} and 853 cm^{-1} are sensitive to anomeric structure around glycosidic bonds (Cael, Koenig, & Blackwell, 1974) and confirmed, respectively, β- and α-configuration of the polysaccharides (Mohaček-Grošev, Božac, & Puppels, 2001). The skeletal Raman bands at 430 and 596 cm^{-1} are indicative for β-glucans, whereas those at 322, 359 and 480 cm^{-1} could be assigned to α-glucans (Corbett, Zichy, Goral, & Passingham, 1991; Šandula et al., 1999; Zhbankov et al., 1997).

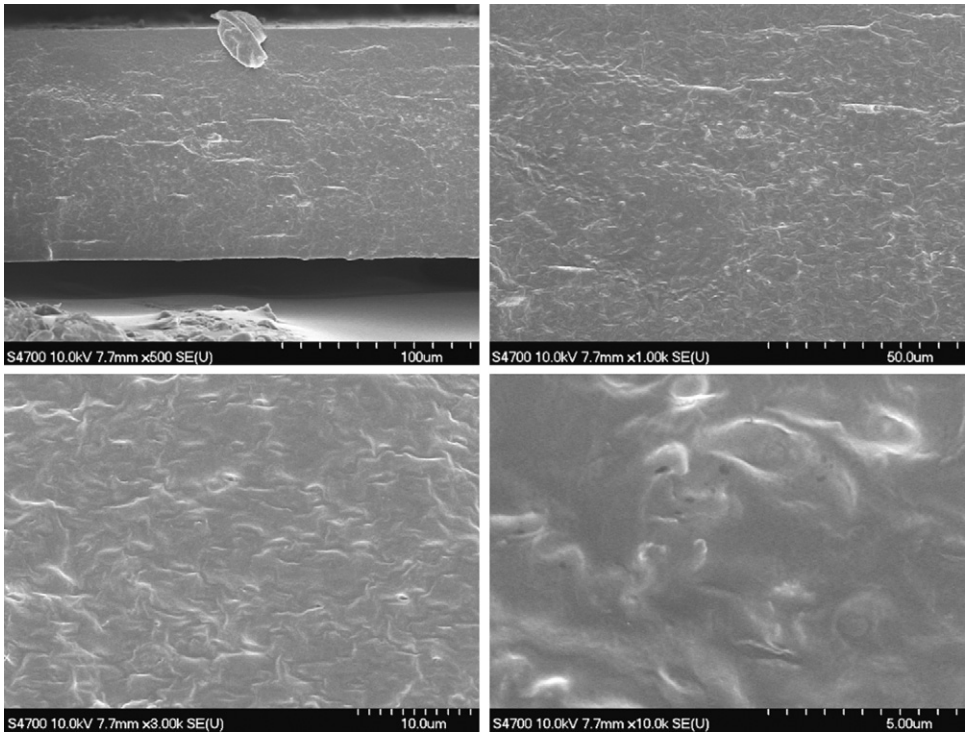

Fig. 5. SEM microphotographs of the cross sections of the yeast β-glucan film.

From the measured spectra can be estimated that the yeast cell wall preparation contains β-glucan as the main component, while chitin and α-glucans are also present but in smaller amounts.

3.6. SEM analysis

Obtained SEM images of the yeast β-glucan films at different scales are presented in Fig. 4 (the upper side surface) and Fig. 5 (the

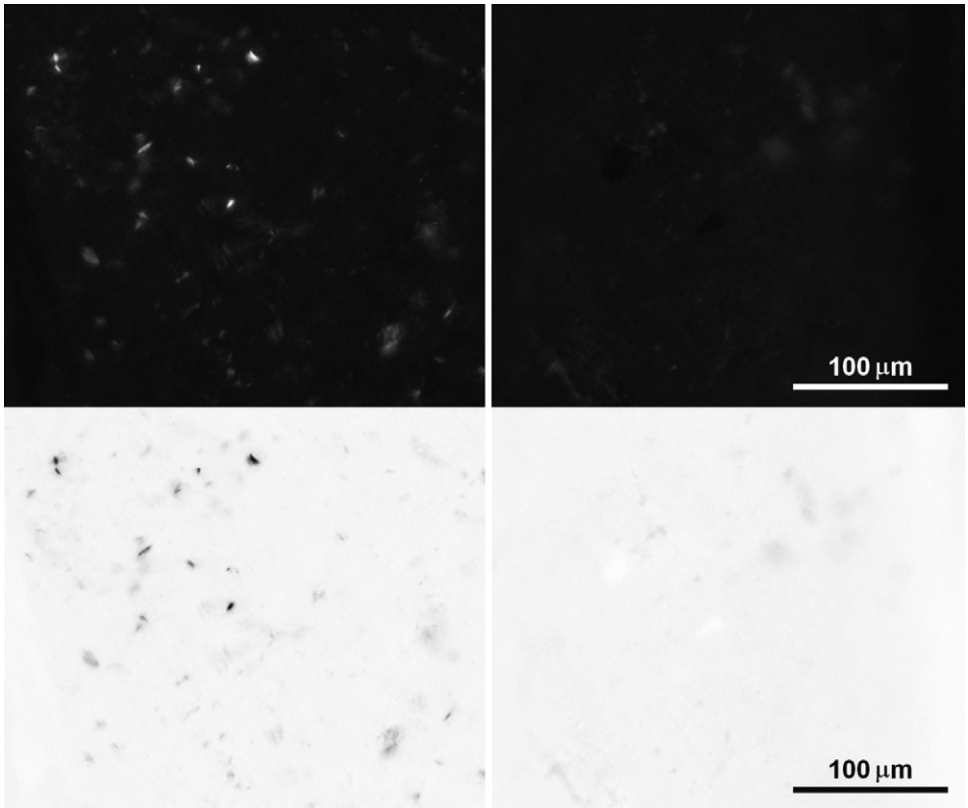

Fig. 6. Positive (top) and negative (bottom) microscopic images of yeast β-glucan films before (left) and after (right) washing with acidic ethanol in passing light with crossed polarizers.

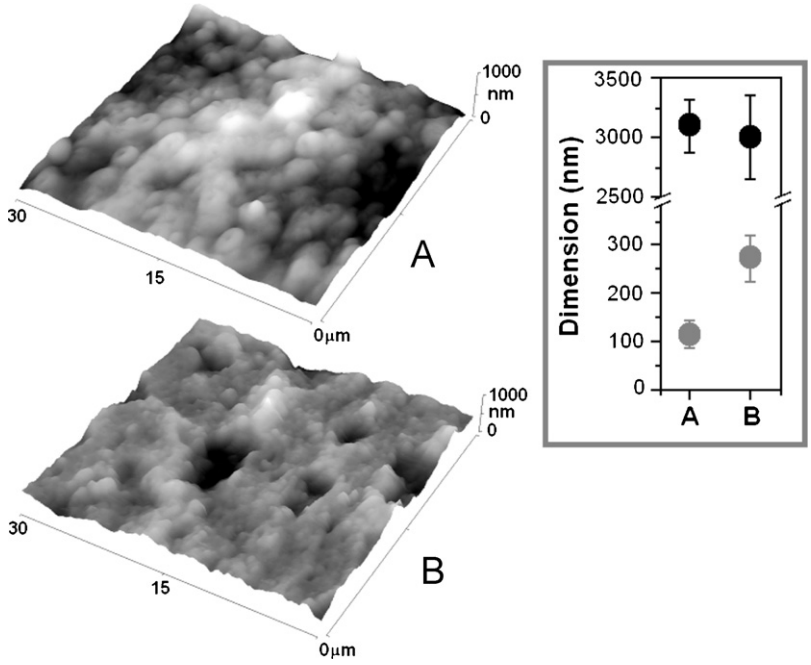

Fig. 7. AFM images (left) of the yeast β-glucan films immediately prepared (A) and after 12-month storage (B); influence of 12-month storage on the height (black circles) and width (grey circles) of the granular particles in yeast β-glucan films according to AFM (right).

cross section). Surface morphology reflects the complexity of interactions within the film as well as interactions between the film and the atmosphere during film solidification (Fig. 4). Unevenness of the surface on the microscopic level can be predominantly attributed to desiccation, non-homogeneity of a suspension containing cell wall fragments and, in a lesser extent, to high viscosity (long relaxation times) in the final stages of solidification. Nevertheless, high magnification pictures show the presence of string-like structures exceeding 10 μm that may be attributed to fibre biopolymers; these structures probably cannot be ascribed to non-destructed yeast envelopes, for their size hardly exceeds 10 μm. The inner morphology of the film was examined in cross sections (Fig. 5). Possible influence of a material flow caused by plastic deformation was eliminated by brittle fracture, giving more realistic morphology observation. The film thickness was estimated as about 120 μm, and the homogeneity of the film was proved on the level of tens of micrometers. However, high magnifications clearly show that the film contains pseudo-circular structures of 1–3 μm size, probably residues of yeast cell wall envelopes. Films are compact and non-porous. From general point of view, porosity of membranes serving as lesion coverings is a matter of discussion. Porous structure is considered as important for developing new products for replacement surgery, in scaffolds for tissue culture, and last not least, as wound coverings (*e.g.*, Kil'deeva et al., 2006; Lewin & Penhasi, 1998; Friess & Lee, 1996, *etc.*). Many of these products are polyurethane based and in this case presence of pores is indispensable. Yeast β-glucan films are of different nature and enable water and wound exudates leak through. We believe that in the case of polysaccharide based films porosity is not the crucial parameter for their applications.

3.7. Polarized light microscopy

In viewing using crossed polarizers, automatically sharpened from vertical sequence and after inversion of colours, acicular crystals were found in the film (Fig. 6, left), with refractive index very close to that of the surrounding matter and the lowest along the crystal. Diffuse and isometric objects are prevalently crystals out of focus or a dust on the film's surface. The visible crystals represent less than 1% of the film volume. It seems that the crystals observed represent some impurities in the yeast β-glucan preparation used. Most of the crystals disappeared after washing with diluted HCl solution in 80% aqueous ethanol (Fig. 6, right) that supports an assumption of carbonates present in the film. However, carbonates were not detected by FTIR/FT Raman both in the film and in parent yeast β-glucan, so their possible inclusions are disputable.

3.8. AFM analysis

Morphology and roughness of the yeast β-glucan films, together with an effect of ageing, were studied by AFM (Fig. 7). The first series of measurements was performed immediately after the film preparation (Fig. 7A) and the second one after 12 months (Fig. 7B). The surface of freshly prepared films consisted of granular-like structures. The height of the granular particles is about 100 nm and their width about 2.70 μm. After ageing of samples, protected from direct light and dust deposition in an air-conditioned laboratory (20 °C), mild increase from 70 to 80 nm of surface roughness was observed, whereas the characteristic granular structure of the film disappeared. The resulting structure consisted of various formations, which were several hundred micrometers high and up to few micrometers wide.

3.9. XRD analysis

Crystallinity and its changes with time were observed by XRD (Fig. 8). All films were homogenous and transparent. Diffraction peak near 6° (2θ) corresponds to an interlayer basal d spacing of 1.498 nm. No evidence of a crystalline peak was observed within the range of 10–60° (2θ). Only very slight crystallinity around 38.1° (2θ) occurred after 12 month ageing (see above). Comparing AFM results with XRD ones enables it to be concluded that structural changes of the film surface detected by the AFM are probably not related to crystallization phenomena, however, with reservation that XRD measures crystallinity in the whole film, whereas the AFM only at the surface. Disorganization of the granular structure cannot probably be attributed to renaturation of the polysaccharide

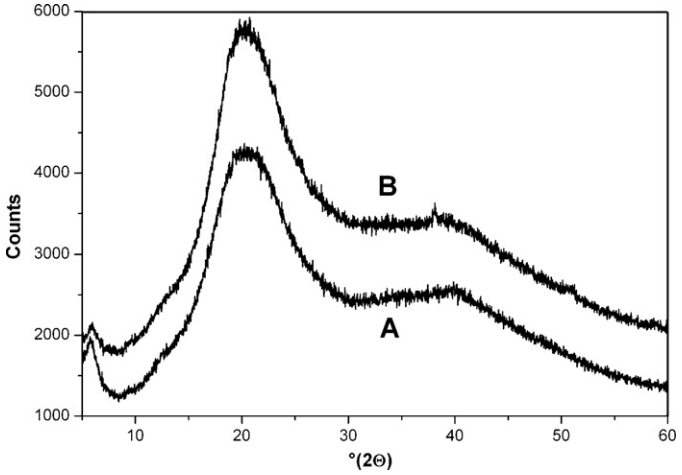

Fig. 8. XRD curves of the yeast β-glucan films immediately prepared (A) and after 12-month storage (B).

chains; it is likely to be caused by reorientation and/or decomposition of surface macromolecules due to reaction with the ambient atmosphere.

4. Conclusions

The aim of this work was to prepare and analyse yeast β-glucan films. The cell wall material prepared contains about 20% by weight of other yeast cell wall polysaccharides, predominantly α-glucans and chitin, in addition to β-glucan. Nevertheless, the presence of these polysaccharides as minor components cannot influence significantly the properties and applications of the films for both α-glucans and chitin derivatives (chitosan, oxidised chitin, *etc.*) are biocompatible materials. Films prepared from yeast β-glucan of given quality are water insoluble, compact, non-porous and exhibit no pronounced crystallinity. The film surface consists of granular-like structures, probably due to the presence of cell wall residues in a parent β-glucan suspension and uneven process of desiccation. Fibre structures exceeding 10 μm were observed as well; they are probably formed by β-glucan macromolecules released from the yeast cell walls. One year ageing of films induced few changes in the whole film, although the surface structure is sensitive to ageing. An improved way for preparing yeast β-glucan films as well as tensile properties of the films of various glycerol/glucan ratios will be the subjects of further publications.

Acknowledgements

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