



Glucans secreted by fungi

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Summary

The production of Schizophyllan by *Schizophyllum commune* and Scleroglucan by *Sclerotium rolf-sii* is growth associated. Shear stress as a result of the impeller type and rotation speed as well as an optimum but not maximum oxygen supply are the key factors for enhanced production. Batch culti-vations resulted maximum yields of 13 g L^{-1} for both β -glucans. In oxygen limited chemostats with biomass feedback maximum productivities of $40 \text{ g L}^{-1} \text{ d}^{-1}$ (Schizophyllan) and $95 \text{ g L}^{-1} \text{ d}^{-1}$ (Sclero-glucan) were attained. The subsequent downstream process was carried out by cross flow filtration techniques.

Introduction

Many fungi are able to form extracellular polysaccharides. Unlike bacteria, heteropolysaccharides occupy only a subordinate position among fungi. Homopolysaccharides occur most frequent with D-glucose differently linked as building block. *Schizophyllum commune* (Rau, 1999), *Sclerotium rolf-sii* (Schilling et al., 2000), *Sclerotium gluanicum* (Rau et al., 1992a), *Monilinia fructigena* (Cordes, 1990), *Botrytis cinerea* (Gawronski et al., 1996) are some of the more investigated fila-mentously growing fungi which secrete the same β -glucan with a uniform, primary molecu-lar structure (Fig. 1). However, these polysaccharides differ substantially in molecular weights and in their tendency to form microgels.

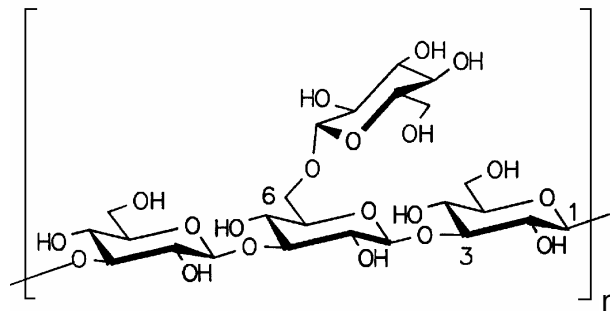


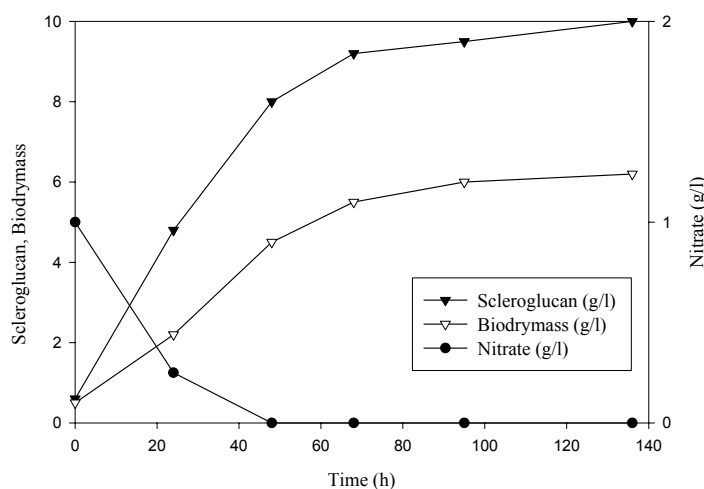
Fig. 1 Primary molecular structure of β -glucan. $n=9,000 - 18,000$

The neutral homoglucon consists of a backbone chain of 1,3- β -D-glucopyranose units linked with single 1,6-bounded β -D-glucopyranoses at about every third glucose molecule in the basic chain. The molecular weight varies between 6 and $12 \cdot 10^6$ g mol⁻¹. The following investigations are related to *Sclerotium rolfsii* ATCC 15205 and *Schizophyllum commune* ATCC 38548 whose secreted polysaccharides are known as Scleroglucan and Schizophyllan, respectively.

Production

The production of Schizophyllan as well as Scleroglucan is strongly coupled with growth and, as known for primary metabolites, the secretion under nitrogen starvation reduces to zero when the stationary phase is attained (Fig. 2). Both fungi need yeast extract as complex nitrogen source in different amounts. Only *S. rolfsii* is able to utilize nitrate at reduced concentration of yeast extract.

Fig. 2



30-L batch cultivation of *S. rolfsii* equipped with three 4-bladed fan impellers at 200 rpm, 27°C, at an initial pH of 2.5 and an aeration rate of 120 L h⁻¹. Glucose (30 g L⁻¹) was used as carbon source and nitrate (1 g L⁻¹) as nitrogen source (Schilling, 1997).

The gum is as a mucilage either loosely associated with the outer cell wall or released into the medium. Shear stress, created by the agitator used during bioreactor cultivation, reduces pellet growth as well as enhances release of β -glucan from the cell wall. However, too high shear stress causes damage to the hyphae and even the β -glucan itself. In addition to this, the resulting cell fragments impede cell separation during subsequent downstream processing. The agitator and speed applied must, therefore, present a compromise between mixing and mass transfer of the highly viscous, pseudoplastic suspension as well as Schizophyllan release from the cell wall on the one hand and low shear stress on the fungus and β -glucan on the other (Rau et al, 1992). In direct comparison, *S.*

commune shows a lower shear stability than *S. rolfsii*. Therefore, the low shear fan impeller is suitable for the cultivation of *S. commune* (Fig. 3).

Fig. 3. 30-L Batch cultivation of *S. commune* equipped with three fan impellers at 100 rpm, 27°C, an initial pH of 5.3 and an aeration rate of 150 L h⁻¹.
pO₂: oxygen partial pressure of the liquid phase.

A major difference between both fungi exists in the formation of diverse by-products. While *S. commune* forms ethanol *S. rolfsii* does not possess a fermentation pathway. However, *S. rolfsii* secretes oxalic acid by the glyoxylate pathway (Schilling et al., 2000). Therefore, considerable attention has to be paid to the factors stimulating oxalic acid synthesis. High initial pH values were observed to favour oxalic acid and glucan production while a low initial pH resulted in oxalic acid reduction accompanied by poor growth. In addition, oxygen limitation was found to repress oxalic acid formation during cultivation due to the oxalic acid synthesizing enzyme glycolate oxidase which is stimulated in the presence of molecular oxygen. An early onset of oxygen limitation in the culture is favorable. Oxalic acid is also known to play a key role in the plant pathogenicity of *Sclerotium rolfsii* (Kritzman et al. 1977; Punja 1985).

Both fungi underlie a glucose induced repression of β -glucanases (Prokop et al, 1994; Rapp, 1992) which degrade the preformed glucan for the use as carbon source if glucose is consumed. Therefore, cultivations were terminated after glucose consumption. Prolonged cultivation under carbon limited conditions led to the release of β -glucan degrading enzymes which cause a slight increase in glucose concentration accompanied by a decrease in concentration as well as a sharp drop in the specific viscosity (mPa g⁻¹) of the β -glucan. For this reason not carbon limited but oxygen limited continuous cultivations were carried with both fungi. Depending of the process mode (batch or

continuous) a optimum specific oxygen uptake rate exists for maximum β -glucan yield and productivity, respectively. Compared to batch cultivation the continuous mode revealed 3-fold increase of productivity. In order to achieve a further increase of productivity combined with facilitated downstream processing biomass feedback was used. A cross flow filtration unit comprising a stainless steel membrane was employed for separation of biomass from the viscous culture suspension. For *Schizophyllum* (Rau et al., 2002) a maximum productivity of $40 \text{ g L}^{-1} \text{ d}^{-1}$ was achieved at a feedback rate (permeation flow / medium feed flow) of 0.92 and dilution rate of 0.2 h^{-1} (maximum specific growth rate $0,08 \text{ h}^{-1}$). An improved system was used for the production of Scleroglucan (Maier et al., 2003) and yielded $95 \text{ g L}^{-1} \text{ d}^{-1}$ at a feedback rate of 0.95 and dilution rate of 0.65 h^{-1} (maximum specific growth rate $0,12 \text{ h}^{-1}$). Optimized process and filtration conditions result a cellfree and undiluted β -glucan solution at the outlet of the bioreactor.

Downstream processing

The suspensions from batch cultivations contain cells which have to be separated by either centrifugation or microfiltration. Best results in centrifugation are yielded if the diluted and homogenized suspension is fed to a solid ejecting disc separator (5.700 g). The resulting supernatand contains only small amounts of hyphal fragments (concentration $<0.1 \text{ g L}^{-1}$) which can be easily separated by dead end filtration using glass-fibre filters. A more effective alternative method for cell separation is cross flow microfiltration. Undiluted suspension can be employed if a sintered stainless steel membrane is used at high tangential feed velocity. Cellfree β -glucan solutions without fragments but with the same concentration as at the end of batch cultivation are yielded as permeate.

The cellfree β -glucan solution originating either by high-speed microfiltration or by continuous cultivation with integrated biomass feedback has to be purified or eventually further concentrated in a next step. Owing to parallel investigations using different crossflow systems (Haarstrick et al., 1991), the application of low-shear PROSTAKTM (Millipore Corp., USA) flat membrane modules ($0.1 \mu\text{m}$) can be recommended. Best results related to a high permeation rate are achieved when the tangential feed velocity is at its individual maximum avoiding a transmembrane pressure >0.8 bar. Purification of the β -glucan solution is attained by the use of the diafiltration mode if all β -glucan molecules are fully rejected and low molecular compounds ($<0.1 \mu\text{m}$), such as proteins, glucose, and salts, permeate through the membrane. The permeate flow corresponds to the input solvent (water) flow, so that the volume of the retentate remains constant. The concentration mode starts by cutting the input solvent flow. During this process the negative influence of fouling at the membrane surface is increased with the consequence of a continual decrease of the permeation rate (Fig. 5). A highly viscous, colourless and transparent solution is the result of this procedure. Drying

or lyophilization of the product solution has to be avoided because only 50% (w/w) of the dried β -glucan is resoluble in water. Otherwise dimethylsulphoxide dissolves the dried β -glucan to 100%. However, this solvent degrades the triple helix to single coiled chains with drastic reduction of viscosity.

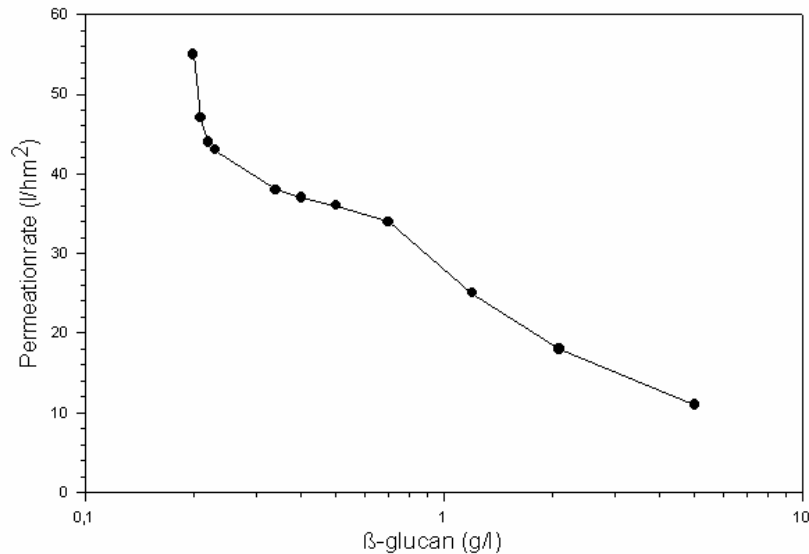


Fig. 5 .Decrease of the permeation rate during the concentration run of a cellfree β -glucan solution applying crossflow microfiltration.

Characteristics

In aqueous solution Schizophyllan and Scleroglucan are arranged as triple helix with protruding pendent β -1,6-linked D-glucose units originating from the outside of the triplex. In DMSO, at temperatures $>135^{\circ}\text{C}$ and at a $\text{pH}>12$ the triple helix melts to single, randomly coiled strains, equivalent to the reduction of the average molecular weight by one third (Norisuye et al., 1980). Aqueous solutions show thixotropic, pseudoplastic (Fig. 6) and viscoelastic behaviour. Native suspensions, additionally containing the producing fungus, reveal enhanced non-Newtonian characteristics due to the filamentous network of the internal woven hyphae.

β -glucans can also be used to form films hardly not permeable for oxygen (Schulz et al., 1992) e.g. for the protection of foods. A further application is the stimulation of the immune system by regioselectively degraded β -glucans (Münzberg et al., 1995) and especially in Japan these antitumor glucans are currently used as cancer immunotherapeutic drugs in combination with other chemotherapeutic compounds (Kishida et al., 1992). Scleroglucan has been found to exhibit anti-inflammatory properties, rendering them valuable as active ingredients in after-sun preparations for the treatment of sun burn (Maier et al., 1999).

In comparison to Schizophyllan the Scleroglucan shows an enhanced tendency to form microgels. This characteristic directly influence their filtration and adsorption behaviour when being used as additives for polymer flooding in the scope of enhanced oil recovery. Studies have shown that Schizophyllan is more useful for this kind of application than Scleroglucan (Rau et al., 1992b).

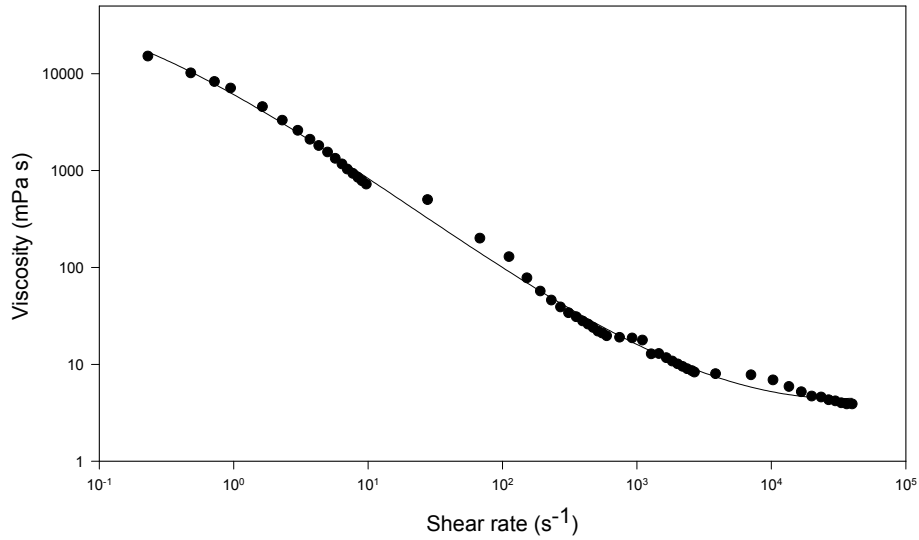


Fig. 6 .Pseudoplastic flow behaviour of an aqueous β -glucan solution ($5 g L^{-1}$). Shear viscosity was measured by a rotary viscometer (Haake, Karlsruhe) at $25^{\circ}C$ and at different constant shear rates until a constant shear stress resulted.

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