Bioprocess Engineering 11 (1994) 161–165 © Springer-Verlag 1994

Oxygen controlled batch cultivations of Schizophyllum commune for enhanced production of branched β-1,3-glucans

U. Rau, C. Brandt

Abstract Oxygen and shear stress are the key factors for enhanced glucan production with Schizophyllum commune. During batch cultivation control of p_{O_2} or q_{O_2} (specific oxygen uptake rate) was achieved by variation of the impeller speed. Biomass was modelled by using the carbon and oxygen balance derived from exhaust data. At mycel growth a q_{O_2} of 0.042 h^{-1} presents just the border before oxygen limitation arises and is simultaneously the optimum operation condition for maximum glucan formation. Related to an overall cultivation time of 72 h a maximum of both productivity (4.3 $kg\,m^{-3}\,d^{-1}$) and yield (13 $kg\,m^{-3}$) were obtained.

List of symbols

C	$kg\,m^{-3}$	concentration
$k_L a$	h^{-1}	volume related oxygen transfer coefficient
K_S	$mol\,m^{-3}$	substrate saturation constant
N	rpm	impeller speed
p_{O_2}	%	oxygen partial pressure of the liquid phase
Q_{O_2}	$kg\,m^{-3}\,h^{-1}$	oxygen uptake rate
q_{O_2}	h^{-1}	specific oxygen uptake rate, kg O_2 (kg biomass h)$^{-1}$
t	h	time
Y_{X/O_2}	–	yield coefficient (biomass formed/oxygen consumed)

Greek symbols

μ	h^{-1}	specific growth rate

Indices

O_2	oxygen
X	biomass
L	liquid phase
$*$	gas/liquid interface
S	substrate (glucose)

Received 24 January 1994

U. Rau, C. Brandt
Institut für Biochemie und Biotechnologie der Technischen Universität Braunschweig, Spielmannstr 7, D-38106 Braunschweig, Germany

Correspondence to: U. Rau

Dedicated to the 65th birthday of Professor Fritz Wagner.

This work was kindly supported in parts by B. Braun Biotech International. The authors are grateful to Prof. Dr. Fritz Wagner for scientific support and appreciate the technical assistance of Detlev Rasch

1 Introduction

The filamentously growing fungus Schizophyllum commune secretes a neutral homoglucan (trivial name Schizophyllan [1]) that 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. Many other fungi like Sclerotium rolfsii [2], Sclerotium glucanicum [3], Monilinia fructigena [4], Botrytis cinerea [5] etc. are able to produce the same glucan with a uniform, primary molecular structure. However, these polysaccharides differ substantially in molecular weights and in their tendency to form microgels. These characteristics directly influence their filtration and adsorption behaviour when being used as additives for polymer flooding in the scope of enhanced oil recovery. Recent studies have shown that Schizophyllan is very useful for this kind of application [6].

As pullulan [7] the glucan can also be used to form films hardly not permeable for oxygen [8] e.g. for the protection of foods [9] being sensitive to oxygen. A further application is the stimulation of the immune system by degraded glucans [10], and especially in Japan these antitumor glucans are currently used as cancer immunotherapeutic drugs in combination with other chemotherapeutic compounds [11].

The glucan dissolves in water as a triple helix [12] with protruding pendent β-1-6-linked D-glucose units originating from the outside of the triplex [13]. In DMSO and at a pH > 12 the triple helix melts to single, randomly coiled strains, equivalent to the reduction of the average molecular weight by one third [14]. Aqueous solutions show thixotropic, pseudoplastic [15] and viscoelastic [16] behaviour. Native suspensions, also containing the producing microorganism, reveal enhanced non-Newtonian characteristics [17] due to the filamentous network of the internal woven hyphae.

The glucan formation is strongly coupled with growth [9]. Therefore, under nitrogen starvation both growth and glucan formation cease [18]. The polysaccharide is as a mucilage either loosely associated with the outer cell wall or released into the medium. Mixing of these high viscous suspensions requires a proper agitator which allows short mixing times, high mass transfer and glucan release from the cell wall on the one hand, and low shear stress on the fungus and glucan on the other. All these requirements are fulfilled by a 4-bladed fan impeller [12] that has been tested in batch and in continuous cultivation [19]. Similarly good results were yielded by using a reciprocating jet bioreactor in mutual conducted cultivations at the Institute of Process Engineering (TU Berlin, FRG) [20, 21]. All these experiments have doubtlessly proven that oxygen and shear stress are the key factors for enhanced glucan formation. After the control of oxygen supply was established for the continuous

process [22] the following investigations present the results from batch cultivations using the control of p_{O_2} or specific oxygen uptake rate by variation of impeller speed.

2 Materials and methods

2.1 Microorganism and conditions of cultivation

The wood rotting basidiomycete Schizophyllum commune ATCC 38548 was employed to produce extracellular glucan. The fungus was grown on agar slants supplemented with 39 $g\,l^{-1}$ potato dextrose agar and 5 $g\,l^{-1}$ yeast extract. After one week the covered slants were stored at 4°C. New slants were inoculated at intervals of 4 weeks.

The medium used for cultivation both in shake flasks and bioreactor consisted of (per 1 deonized water): glucose 30 g, yeast extract 3 g, KH_2PO_4 1 g and $MgSO_4.7H_2O$ 0.5 g. Cultivations were performed at 27°C with an initial pH of 5.3 not being adjusted. For the first subculture a piece of covered agar was used to inoculate 100 ml medium in 500 ml shake flasks. After 4 days on a rotary shaker (100 rpm) the second seed with 250 ml in 1 l shake flasks was inoculated with 10 ml of homogenized (Ultra Turrax, Ika, Staufen i.B., FRG) culture suspension and was cultivated as described above. Bioreactor cultivations were carried out in a vessel of 30 l working volume (B. Braun Biotech International, Melsungen, FRG), a height/width ratio of 2 with four baffles mounted inside using three 4-bladed fan impellers (blade pitch 45°, impeller/tank ratio of 0.64) installed on the shaft at a distance of 2/3 to the stirrer diameter (0.19 m). The inoculated suspension (5 vol%) was gassed with a volume related aeration rate of 0.08 V/VM. All data presented refers to at least two experiments.

2.2 Analysis

The cell dry weight was determined gravimetrically after homogenization, centrifugation and drying. Glucan was precipitated from the supernatand by i-propanol and subsequently dried to constant weight. The procedure is described in detail elsewhere [16].

The exhaust analysis of carbon dioxide and oxygen was performed by Unor 6N and Oxygor 6N (Maihak, Hamburg, FRG), respectively, additionally equipped with a 7-channel multiplier switching from one exhaust tube to the other. The crude data ($\%O_2$, $\%CO_2$) were transferred to a connected PC. After correction of the drift the data were used for calculation of oxygen uptake rate, carbon dioxide formation rate and respiration quotient using an exhaust manager (tables and graphics of these parameters with menu driven work sheets) developed in our lab [21]. This PC is connected to another with a multi-tasking operating system (Concurrent DOS, Digital Research, USA) which runs simultaneously a process manager (data input of exhaust manager, biomass simulation, data transfer to MFCS) and a MFCS (Multi-Fermenter-Control-System, B. Braun Biotech International, Melsungen, FRG) both controlling the bioreactor by using a FIS (Fermenter Interface System, B. Braun Biotech International, Melsungen, FRG) as a transfer unit.

2.3 Modelling of biomass

Provided that the rate of increase in cell mass is only a function of the cell mass:

$$\frac{dC_X}{dt} = \mu \cdot C_X, \tag{1}$$

and regarding oxygen as the first limiting substrate then

$$\mu = q_{O_2} \cdot Y_{X/O_2} = \frac{Q_{O_2}}{C_x} \cdot Y_{X/O_2} \tag{2}$$

is valid. The mass balance of oxygen solved in the liquid phase during instationary growth is

$$\frac{dC_{O_2,L}}{dt} = k_L a (C^*_{O_2,L} - C_{O_2,L}) - Q_{O_2}. \tag{3}$$

In short time intervals Eq. (3) is equal to zero and becomes:

$$Q_{O_2} = k_L a (C^*_{O_2,L} - C_{O_2,L}). \tag{4}$$

The combination of Eqs. (1), (2) and (4) gives:

$$\frac{dC_X}{dt} = \left[\frac{Q_{O_2}}{C_X} \cdot Y_{X/O_2}\right] \cdot C_X = \left[\frac{k_L a (C^*_{O_2,L} - C_{O_2,L}) \cdot Y_{X/O_2}}{C_X}\right] \cdot C_X. \tag{5}$$

At the beginning of oxygen limitation ($C_{O_2,L} \to 0$) dC_X/dt will be constant and at its maximum and is expressed as $[dC_X/dt]_{max}$. For simulation of biomass in the decelaration phase a Monod-term is included:

$$\frac{dC_X}{dt} = C_X \cdot \mu = C_X \cdot \mu_{max} \cdot \frac{C_S}{K_S + C_S}, \tag{6}$$

with

$$\left[\frac{dC_X}{dt}\right]_{max} = C_X \cdot \mu_{max} \Rightarrow \tag{7}$$

$$\frac{dC_X}{dt} = k_L a (C^*_{O_2,L} - C_{O_2,L}) \cdot Y_{X/O_2} \cdot \frac{C_S}{K_S + C_S}. \tag{8}$$

It is assumed that $k_L a$ does not depend on the concentration of glucan, $> 3\ kg\,m^{-3}$, because in this region $k_L a$ is only slightly decreasing [23, 24]. Thus, $k_L a$ depends only on the impeller speed (Fig. 1) and is approximated by a polynom. The actual concentration of oxygen in the liquid phase, $C_{O_2,L}$, is proportional to p_{O_2}. The estimation of glucose C_S was achieved by using the carbon balance presented in Table 1. The molar ratios of products depend on the cultivation conditions e.g. differences in glucan and ethanol. However, the summarized amount of carbon in ethanol, glucan and biomass is almost constant and varies between 74% to 77%. Therefore, starting from the on-line measurement of CO_2, the estimation of the other products is possible and finally the amount of glucose consumed is calculable. $Y_{X/O_2} = 0.114$ and $K_S = 5.5\ mol\,m^{-3}$ are set to constant data over the whole period of cultivation.

3 Results and discussion

Preliminary investigations have revealed that during batch cultivation enhanced glucan formation is achieved by increased, but limited, oxygen supply. Linear increase of the impeller speed, which started with 100 rpm at the beginning of limitation, to

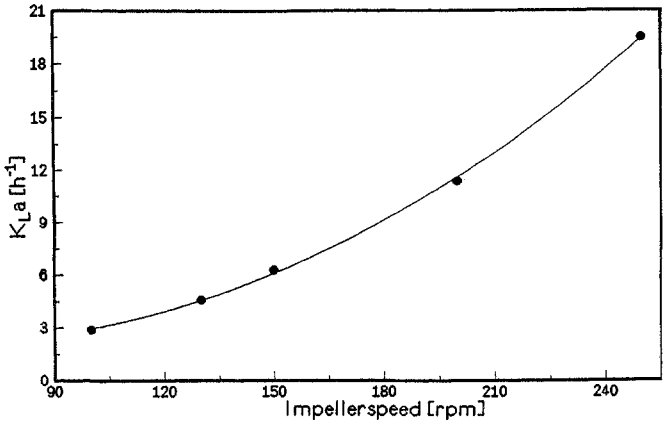

Fig. 1. Volume related oxygen transfer coefficient (measured by exhaust analysis) vs. impeller speed (4-bladed fan impellers) at glucan concentration $>3\ kg\,m^{-3}$

Table 1. Carbon distribution of products related to the cultivations presented in Figs. 2–4

(%C)	Fig. 2	Fig. 3	Fig. 4
Glucan	31.9	37.9	39.4
Ethanol	31	27.9	20.2
CO_2	20.1	20	19.8
Biomass	11.1	11.3	16.3
Remainder	4.6	2.3	2.8

200 rpm in a time interval between 8 to 32 h resulted in a yield of glucan of 9.5 $kg\,m^{-3}$ related to an overall cultivation time of 100 h [25]. In this uncontrolled cultivation the level of p_{O_2} varied between 5% and 35%. First of all, in order to establish a controlled batch process p_{O_2} was selected as master variable. Thus, we tried to maintain a constant p_{O_2} of 5% by applying gradual changes of the impeller speed (Fig. 2). The following simple control algorithm was used: If $p_{O_2}<5\%$ then $N=N+10$.

The increase of the specific oxygen uptake rate at the beginning of cultivation is due to a slight rise of oxygen consumption for cell internal adaption to changed environment during the lag phase. During the control interval the specific oxygen uptake rate q_{O_2} runs through a maximum which results in a value of 0.042 h^{-1} at the maximum rate of glucan formation (indicated by arrows in Fig. 2). This is corresponding to the data derived from continuous cultivation. Therefore, we started a cultivation maintaining a constant q_{O_2} of 0.042 h^{-1} at the beginning of oxygen limitation set at $p_{O_2}<5\%$ (Fig. 3). For on-line calculation of biomass the model mentioned in "Materials and Methods" was used. The control of q_{O_2} was achieved by the following algorithm:

If $q_{O_2}<0.04$ then $N=N+5$ and if $q_{O_2}>0.04$ then $N=N-5$. The gentle variation of impeller speed is specifically adapted to the low growth rate ($\mu_{max}=0.08\ h^{-1}$) of the fungus. First the actual value of control variable q_{O_2} is compared to its setpoint. In case of a positive answer a temporary variation of the correction variable N follows. Then a trend is calculated equal to the slope of some data points of the control variable. When the trend is negative the actual value approaches its setpoint. The combination of both control functions may result in the

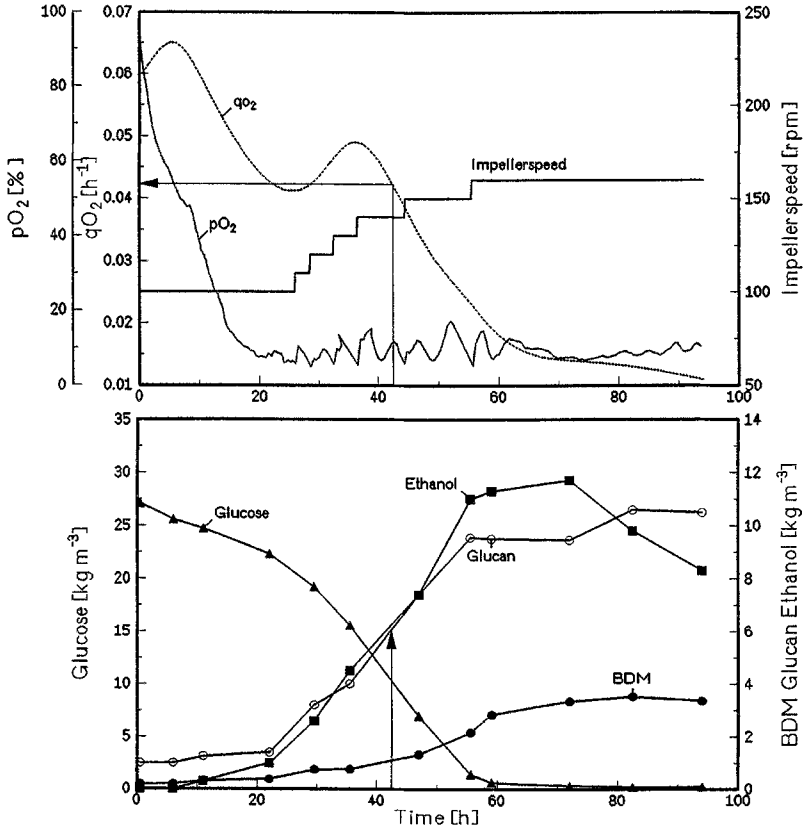

Fig. 2. 30l-batch cultivation of S. commune equipped with three fan impellers at 27°C, 0.08 V/VM and an initial pH of 5.3. At the time when the culture reached a p_{O_2} of 5% impeller speed was stepwise increased

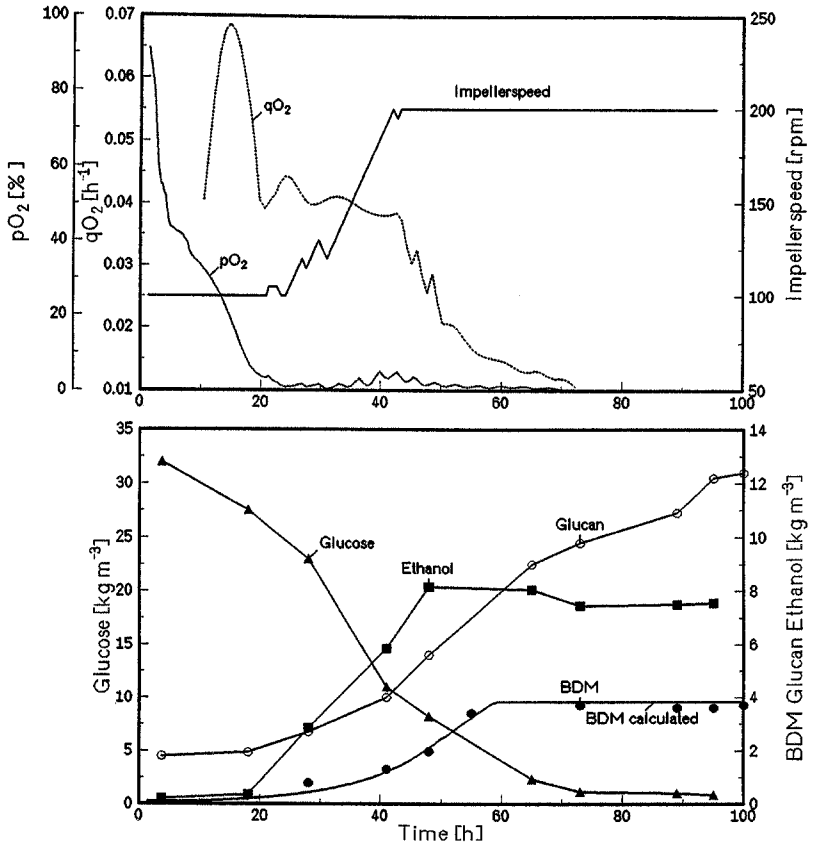

Fig. 3. 301-batch cultivation of S. commune with controlled specific oxygen uptake rate by variation of impeller speed. For further cultivation conditions see Fig. 2

following answer of the system: At an actual value for example lower as the setpoint but positive trend the variation of N can be compensated as q_{O_2} still improves.

Using the control sqn/m mentioned above a nearly constant q_{O_2} of 0.04 h^{-1} was maintained for about 30 h resulting in an increased yield of glucan (12.5 kg m^{-3}) at equal biomass produced. A further important result is the decreased amount of ethanol primary channelled into glucan which was caused by improved oxygen supply (Table 1). Partly existing anaerobic domains inside the mycelia can never be fully avoided, stated by the fact that ethanol is always formed in spite of $p_{O_2} > 5\%$ [21]. However, the p_{O_2} is not a representative quantity in this connection as it reflects only one local point of the liquid phase and not the situation inside the mycels. Otherwise local excess of oxygen is primarily used for biomass production. With these thoughts in mind a q_{O_2} of 0.042 h^{-1} at a given morphology of the fungus presents just the border before oxygen limitation arises and growth is, therefore, unlimited. In other words, this state is characterized by a minimum of ethanol and biomass as well as a maximum of glucan produced.

The upper and lower limit of the impeller speed was set at 100 and 200 rpm, respectively. The upper value derived from batch cultivations with constant speed of 200 rpm [19] which resulted in decreased growth rates and related low yields due to high shear stress. In order to investigate how the fungus responds to higher shear stress and whether q_{O_2} could be prolonged at its optimum the upper limit of the impeller speed was set at 300 rpm (Fig. 4). As a result, compared to the preceding cultivation ethanol was further decreased from 8 to 5.5 kg m^{-3}; adequate amount of glucan (13 kg m^{-3}) but also biomass (5 kg m^{-3}) was produced. A constant q_{O_2} could be maintained up to 225 rpm. The subsequent sharp decrease in q_{O_2} could possibly be due to higher stress caused by the impeller and might not be a consequence of insufficient oxygen transfer. The argument for this assumption may be derived from Fig. 1. For example, 250 rpm is equivalent to a $k_L a$ of 20 h^{-1} and assuming a $C^*_{O_2,L} = 0.008$ kg m^{-3} an OTR of 0.160 kg m^{-3} h^{-1} will be obtained ($p_{O_2} \rightarrow 0\%$). At a given biomass of 2 kg m^{-3} at this time (45 h) the theoretical maximum value of q_{O_2} is twice as high as its optimum. Using a logarithmic depiction of biomass versus time a straight line results up to 55 h with a slope of $\mu = 0.075$ h^{-1} (data not shown). This is a further evidence for the just oxygen unlimited conditions as mentioned before.

In conclusion, deriving from continuous cultivation [22] a controlled batch process was established using the specific oxygen uptake rate as master variable as well as the impeller speed as correction variable. At a given morphology (mycel growth) a $q_{O_2} = 0.042$ h^{-1} enables Schizophyllum commune just an oxygen unlimited growth with maximum glucan and minimum ethanol yield. The use of the 4-bladed fan impeller at speeds > 225 rpm, also in a slowly increasing manner, influences the fungus by a sharply decreasing q_{O_2}. Through the use of the presented control system both productivity (4.3 kg m^{-3} d^{-1}) and yield (13 kg m^{-3}) of glucan could be markedly increased compared to a conventional, uncontrolled batch (productivity 2.4 kg m^{-3} d^{-1}, yield < 10 kg m^{-3}) [3, 19].

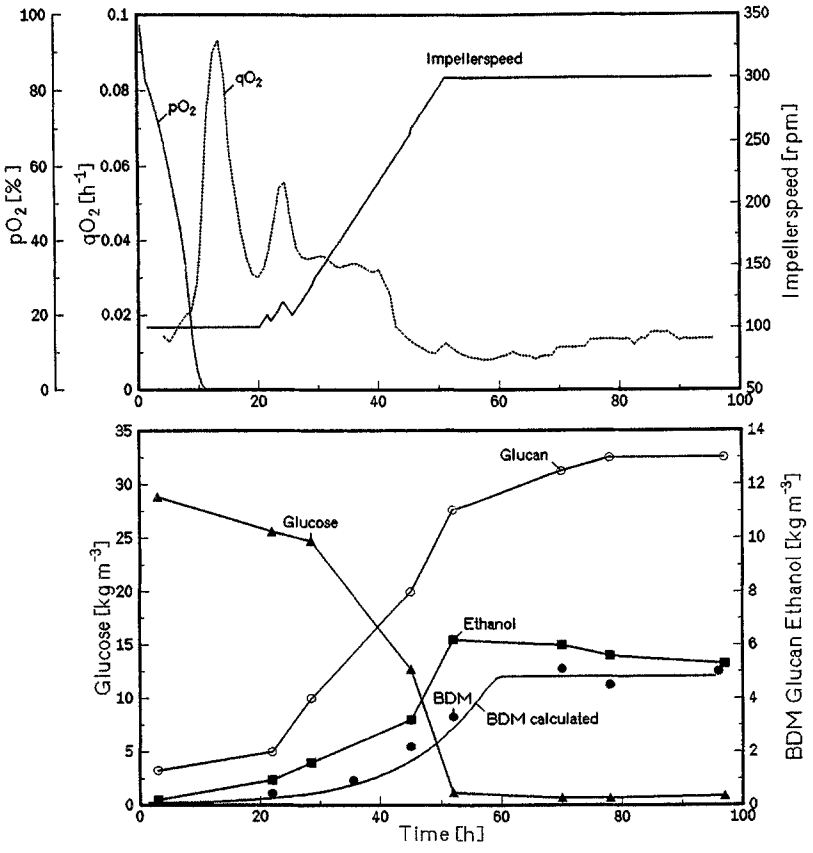

Fig. 4. 301-batch cultivation of S. commune with controlled specific oxygen uptake rate. Upper limit of impeller speed was set at 300 rpm

References

1. **Kikumoto, S.; Miyajima, T.; Yoshizumi, S.; Fujimoto, S.; Kimura, K.:** Polysaccharide produced by Schizophyllum commune. I. Formation and some properties of an extracellular polysaccharide. Nippon Nogei Kagaku Kaishi 44 (1970) 337–342
2. **Pilz, F.; Auling, G.; Rau, U.; Stephan, D.; Wagner, F.:** A high affinity Zn^{2+} uptake system and oxygen supply control growth and biosyntheses of an extracellular, branched β-1,3-glucan in Sclerotium rolfsii ATCC 15205. Exp. Macol. 15 (1991) 181–192
3. **Rau, U.; Gura, E.; Olszewski, E.; Wagner, F.:** Enhanced glucan formation of filamentous fungi by effective mixing, oxygen limitation and fed-batch processing. J. Ind. Microbiol. 9 (1992) 19–26
4. **Cordes, K.:** Ph.D. Thesis, Technical University of Braunschweig 1990
5. **Dubourdieu, D.; Ribereau-Gayon, P.; Fournet, B.:** Structure of the extracellular β-D-glucan from Botrytis cinerea. Carb. Res. 93 (1981) 294–300
6. **Rau, U.; Haarstrick, A.; Wagner, F.:** Eignung von Schizophyllanlösungen zum Polymerfluten von Lagerstätten mit hoher Temperatur und Salinität. Chem.-Ing.-Tech. 64 (1992) 576–577
7. **Hijiya, U.; Shiosuka, M.:** Pullullan containing films. JP 2235991 (1973)
8. **Schulz, D.; Rau, U.; Wagner, F.:** Characteristics of films prepared by native and modified branched β-1,3-D-glucans. Carb. Polym. 18 (1992) 295–299
9. **Yuen, S.:** Pullulan and its applications. Proc. Biochem. (1974) 7–22
10. **Münzberg, J.; Rau, U.:** Investigations to the regioselective hydrolysis of a branched β-1,3-glucan. Carb. Polym. (1994) (accepted)
11. **Kishida, E.; Yoshiaki, S.; Misaki, A.:** Effects of branch distribution and chemical modifications of antitumor (1,3)-β-D-glucans. Carb. Polym. 17 (1992) 89–95
12. **Kashiwagi, Y.; Norisuye, T.; Fuyita, H.:** Triple helix of Schizophyllum commune polysaccharide in dilute solution. Macromol. 14 (1981) 1220–1225
13. **Bluhm, T.L.; Deslandes, Y.; Marchessault, R.H.:** Solid state and solution conformation of scleroglucan. Carb. Res. 100 (1982) 117–130
14. **Norisuye, T.; Yanaki, T.; Fujita, H.:** Triple helix of a Schizophyllum commune polysaccharide in aqueous solution. J. Polyu. Sci. 18 (1980) 547–558
15. **Rau, U.; Wagner, F.:** Non-Newtonian flow behaviour of colloid-dispers glucan solutions. Biotechnol. Letters 9 (1987) 95–100
16. **Oertel, R.; Kulicke, W.-M.:** Viscoelastic properties of liquid crystals of aqueous biopolymer solutions. Rheol. Acta 30 (1991) 140–150
17. **Rau, U.; Müller, R.J.; Cordes, K.; Klein, J.:** Process and molecular data of branched 1,3-β-D-glucan in comparison with xanthan. Bioproc. Eng. 5 (1990) 89–93
18. **Münzer, S.:** Ph.D. Thesis, Technical University of Braunschweig 1989
19. **Rau, U.; Olszewski, E.; Wagner, F.:** Gesteigerte Produktion von verweigten β-1,3-Glucanen mit Schizophyllum commune durch Sauerstofflimitierung. GIT Fachz. Lab. 4 (1992) 331–337
20. **Brauer, H.; Korn, A.:** Biotechnologische Produktion von Pilzen im Hubstrahl–Bioreaktor. Chem.-Ing.-Tech. 65 (1993) 1128–1230
21. **Gura, E.:** Ph.D. Thesis, Technical University of Braunschweig 1992
22. **Rau, U.; Gura, E.; Haarstrick, A.:** Prozeßintegrierte Aufarbeitung verzweigter β-1,3-Glucane (Schizophyllan). GIT Fachz. Lab. 12 (1992) 1233–1238
23. **Olszewski, E.:** Ph.D. Thesis, Technical University of Braunschweig 1992
24. **Schügerl, K.:** Oxygen transfer into highly viscous media. Adv. Biochem. Eng. 19 (1981) 71–174
25. **Gura, E.; Rau, U.:** Comparison of agitators for the production of branched β-1,3-glucans by Schizphyllum commune. J. Biotechnol. 27 (1993) 193–201