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Kinetics of Soluble Glucan Production by *Claviceps viridis*

M. FLIEGER[a], M. KANTOROVÁ[b], T. BENEŠOVÁ[b], S. PAŽOUTOVÁ[a], J. VOTRUBA[a]

[a]*Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia*
[b]*Faculty of Science, Charles University, 128 40 Prague, Czechia*

fax +420 241 062 347
e-mail flieger@biomed.cas.cz

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ABSTRACT. Among 18 tested strains of *Claviceps* spp., 7 produced significant amounts of exocellular polysaccharide (EPS). The maximum production of EPS was found in fermentation broth of *Claviceps viridis.* The kinetics of growth, substrate consumption, and EPS production in the batch, aerobic, submerged culture of this fungus were investigated in detail. The experimental data were processed by a simple mathematical model describing mass balance of growth, substrate consumption, formation of intermediates, and production of EPS. The parameters of the model were estimated from data obtained in cultivation performed in flasks and two laboratory fermentors of different size. Physiological similarity was obtained during process scale-up in volumetric ratio 1 : 100. The sugar consumption efficiency (52 %) and observed EPS productivity (1.9 kg/m^3 per d) were comparable with literature data.

Abbreviations

EPS	exocellular polysaccharide	S_1	total concentration of monosaccharides, g/L
k_1, k_2	rate constants in Eqs *1–2*, L g^{-1} d^{-1}	S_2	sucrose concentration, g/L
k_3, k_4	rate constants in Eqs *1–4*, 1/d	t	cultivation time, d
K_1, K_2	reciprocal saturation constants in Eqs *1–2*, L/g	T	characteristic time for achievement of maximum EPS or dry biomass concentration, d
K_S	saturation constant for sucrose in Eqs *2–3*, g/L		
μ	specific growth rate, 1/d	VVM	aeration rate, vessel volume per min
P	polysaccharide concentration, g/L	X	dry biomass concentration in fermentor, g/L
$r_{P\max}$	maximum specific rate of EPS formation, 1/d	Y	macroscopic yield coefficient, defined by Eq. *2*

Neutral polysaccharides are of increasing interest in food (Charalampopoulos *et al.* 2002), cosmetic (Steinbüchel *et al.* 2002), pharmaceutical, and biodegradable plastic (Flieger *et al.* 2003*a*) industries. Various forms of microbial -D-glucan have been recognized as having important positive therapeutic effects on coronary heart disease, on the reduction of cholesterol and glycemic response (Wasser 2002) and on enhancement of the humoral immune response (Mucksová *et al.* 2001; Borošková *et al.* 1998). Recently, also the preparation of nanoparticles of metal–polysaccharide complexes was described (Flieger *et al.* 2003*b*).

Among different microorganisms the filamentous fungi are very promising producers of -D-glucan because the hyphal cell wall and extracellular matrix contain more than 75 % of the polysaccharide (Gutierrez *et al.* 1996). A portion of EPS constitutes an extracellular sheath, surrounding the mycelium (Buck *et al.* 1968). Usually, this exocellular matrix is partially dissolved in the medium; therefore, the content of soluble EPS in the medium is proportional to biomass (Schilling *et al.* 1999; Selbmann *et al.* 2002; Osaku *et al.* 2002).

The production of soluble neutral polysaccharides is accompanied by considerable increase in viscosity of broth during cultivation (Koní ek *et al.* 1993). In addition, glucan solutions show thixotropic, pseudoplastic flow behavior and viscoelastic characteristics (Schilling *et al.* 1999). Due to it, the fast submerged cultivations of microorganisms, producing large amounts of EPS are connected with insufficient mixing and oxygen supply.

In the last two decades, attention was paid to submerged fermentation of various *Claviceps* species. These studies were mainly focused on the production of secondary metabolites, ergot alkaloids (Flieger *et al.* 1988, 1989, 1993, 1997; Kantorová *et al.* 2002). The aim of this study was to test the submerged production of EPS by different *Claviceps* species from the *Czech Collection of Clavicipitaceae* and to find the strain for possible industrial use with the highest yield of EPS, which allows avoiding the negative influence of dissolved oxygen limitation on the EPS production kinetics.

MATERIALS AND METHODS

Microorganism and cultivation conditions. Flask cultivation. Eighteen isolates of *Claviceps* spp. from the *Czech Collection of Clavicipitaceae* (*Institute of Microbiology, Academy of Sciences of the Czech Republic*, Prague; CCC) were tested. These strains were specified and described in detail by Pažoutová (2001). Sucrose–asparagine based media T1 and T2 (Pažoutová *et al.* 1981) were used for submerged cultivation. Medium T1 (in g/L distilled water: sucrose 100, L-asparagine 10, L-cysteine-HCl 0.1, yeast extract 0.1, $Ca(NO_3)_2{\cdot}4H_2O$ 1, KH_2PO_4 0.25, $MgSO_4{\cdot}7H_2O$ 0.03, $ZnSO_4{\cdot}7H_2O$ 0.02; pH adjusted to 5.2 with NaOH) was inoculated with a suspension of mycelial fragments from T2 slant agar. The culture was cultivated for 7–10 d. Five mL of the inoculum were transferred to fermentation medium T2 (in g/L distilled water: sucrose 100, L-asparagine 10, yeast extract 0.1, $Ca(NO_3)_2{\cdot}4H_2O$ 1, KH_2PO_4 0.25, $MgSO_4{\cdot}7H_2O$ 0.25 g, KCl 0.12, $FeSO_4{\cdot}7H_2O$ 0.02, $ZnSO_4{\cdot}7H_2O$ 0.015; pH 5.2) at 24 °C. Cultivation (18 d) was done in the dark on a rotary shaker (4 Hz) in Erlenmeyer flasks (300 mL) containing 60 mL of the medium.

Fermentor cultivation. The isolate of *Claviceps viridis* CBS 125.63 was found in preliminary experiments to be the most suitable for further scale-up of the fermentation. The inoculum for fermentor cultivation was prepared in flasks (*see above*). The cultivation was done in two stirred tank-bioreactors of different volume – 2 L (1 L working volume, BioFlo; *New Brunswick Scientific Instruments*, USA) and 7 L (5 L working volume, MBR Bioreactor; *Sulzer*, Switzerland). Both bioreactors had a height-to-width ratio of 2 and were equipped with four baffles and three 6-bladed Rushton impellers (with impeller-to-tank ratio of 0.45) installed on the shaft at ¼, ½ and ¾ of the liquid level. Rotation frequency of both impellers was 5 Hz with bioreactor aeration rate 0.5 VVM. The cultivation was performed in both bioreactors for 5 d at 24 °C without pH control. The slightly modified CS2 medium (in g/L distilled water: sucrose 20 or 30, $(NH_4)_2SO_4$ 10, KH_2PO_4 6, $CaCl_2$ 1, $Ca(NO_3)_2{\cdot}4H_2O$ 1, $MgSO_4{\cdot}7H_2O$ 0.25, KCl 0.12, $FeSO_4{\cdot}7H_2O$ 0.02, $ZnSO_4{\cdot}7H_2O$ 0.015; disodium citrate 80 mmol/L; pH 5.2, adjusted with NaOH; Pažoutová *et al.* 1981) was inoculated with a mycelial suspension (1 : 10, *V*/*V*) of 7-d-old culture of *C. viridis* growing on T1 medium. These experimental conditions allow a shorter time of cultivation (5 d) with minimized catabolic repression by sucrose on process kinetics. The data from both fermentors represent the means of triplicates.

Assays. The content of biomass was determined gravimetrically (Pažoutová *et al.* 1981). Production of EPS was determined as acetone precipitate from culture filtrate. Fermentation broth after removal of polysaccharides was directly used for the HPLC analysis of mono- and disaccharides. All HPLC analyses were carried out on Waters HPLC pump 600 controller and 717 plus autosampler (*Waters*, USA) conducted to differential refractometer RIDK 101 (*Laboratory Instruments*, Czechia). The data were processed using a ChromJet integrator (*Spectra-Physics*). HPLC analyses were carried out on a Separon SGX NH_2 column, (150 × 3.3 mm; *Tessek*, Czechia). Chromatographic conditions: mobile phase acetonitrile–water 4 : 1 (*V*/*V*), flow rate 1 mL/min, RI detection. The content of each compound in the mixture was calculated from appropriate calibration curve.

Statistical procedures. SPSS Base v.10 package was applied for the statistical analysis (*SPSS Inc.*, USA). The nonlinear programming technique was used for the fitting of the model to experimental data; for details *see* Voleský and Votruba (1992).

MODELING OF FERMENTATION PROCESS

Identification of the model and its parameters was based on experimental data obtained from batch laboratory scale cultivations. Its mathematical formulation is a set of differential mass balances of products (glucose, fructose, EPS), biomass and substrate (sucrose) in a perfectly mixed vessel. The laws of process kinetics are based on the five following assumptions:

(*i*) Polysaccharides are part of the cell wall. Soluble polysaccharide originates from the extracellular sheath surrounding the mycelium.
(*ii*) The kinetics of soluble polysaccharide formation is 1st-order with respect to biomass concentration (Dean 1978).
(*iii*) The increase in concentration of biomass and polysaccharide is proportional to the total consumption of mono- and disaccharides. The proportionality constant reflects the macroscopic yield coefficient (Y) (Schilling *et al.* 1999; Osaku *et al*. 2002; Selbmann *et al*. 2002).
(*iv*) The rate-controlling step of growth kinetics can be described by competitive Langmuir isotherm for sucrose and total concentration of other mono- and disaccharides.
(*v*) Monod's formula is sufficient to describe sucrose consumption kinetics.

Based on these assumptions the following set of differential equations may be written:

Biomass concentration (X):

$$dX/dt = (k_1S_2 + k_2S_1)X/(1 + K_1S_2 + K_2S_1) - k_3X \quad (1)$$

The first term on the right side of biomass balance represents the rate of growth of cell mass with extracellular sheath formed by EPS. The second term is the rate of extracellular sheath dissolving in the medium.
The balance of total monosaccharide concentration (S_1):

$$dS_1/dt = k_4S_2X/(K_S + S_2) - YX(k_1S_2 + k_2S_1)/(1 + K_1S_2 + K_2S_1) \quad (2)$$

The first term on the right side of Eq. *2* is the rate of monosaccharide formation from sucrose; the second one represents the rate of monosaccharide conversion into cell mass covered with extracellular sheath of EPS. Y is the macroscopic yield coefficient of total monosaccharide conversion to biomass and EPS.
The balance of sucrose (S_2):

$$dS_2/dt = -k_4S_2X/(K_S + S_2) \quad (3)$$

The balance of polysaccharide concentration (P):

$$dP/dt = k_3X \quad (4)$$

RESULTS AND DISCUSSION

Only 7 out of the 18 tested strains were found to produce exocellular glucan (Table I). In 2 cases (*C. fusiformis*, *C. zizaniae*) the production of EPS was accompanied by the production of ergot alkaloids. Maximum production of EPS was achieved during cultivation of *C. viridis* and *C. sulcata*. The maximum specific growth rate of all strains was similar with the exception of *C. zizaniae*. Based on these findings, the strain of *C. viridis* was selected as the most suitable for further studies.

Table I. Basic physiological parameters of *Claviceps* spp. strains producing EPS

Strain	r_{Pmax}, 1/d	P_{max}, g/L	T_P, d	μ_{max}, 1/d	X_{max}, g/L	T_X, d	Alkaloids, mg/L
C. fusiformis 129	0.15	3.9	5	0.40	11.4	19	>1000
C. maximensis	0.11	4.0	12	0.54	14.0	14	0
C. sulcata EMBR1	0.27	5.1	12	0.42	19.4	21	0
C. sulcata PF2	0.50	8.6	9	0.45	17.5	19	0
C. viridis CBS 125.63	0.35	13.0	18	0.59	11.3	18	0
C. zizaniae CCM 8240	0.27	4.0	7	1.67	10.6	9	<5
Claviceps subsp. HYP	0.01	1.4	21	0.61	23.8	16	0

The typical features of *C. viridis* submerged cultivation in Erlenmeyer flasks, *i.e*. EPS and biomass production, formation of monosaccharides on sucrose-rich medium T2 are given in Fig. 1A. The fermentation is characterized by two distinct phases in biomass growth and EPS formation. In the first phase (day 1–7) sucrose is consumed for the production of biomass and monosaccharides. Unlike in *C. fusiformis*, in *C. viridis* is the production of EPS suppressed during this phase. When the concentration of sucrose fell below 30–40 g/L, the growth of biomass ceased and the culture switched to the second phase (day 10–19), which can be characterized by exhaustion of sucrose, consumption of monosaccharides, growth of biomass and EPS production.

We concluded that excess of sucrose in the original medium inhibited EPS formation; it was verified in fermentor cultivation (Fig. 1B, C) on CS2 medium. As shown earlier ((Pažoutová *et al*. 1981) there is no difference between the course of biomass growth and EPS formation on CS2 or T2 medium during the early phase of submerged cultivation. However, there are smaller discrepancies during the second phase of cultivation coupled with alkaloid production. Due to the content of calcium carbonate the CS2 medium is less suitable for experiments in a flask than medium T2. In contrast, the CS2 medium is much cheaper and therefore more prospective for scale-up. As shown in Table II the estimated values of the rate constants are of the same order which supports our assumption of physiological similarity of the growth phase in both T2 and CS2 cultivation media.

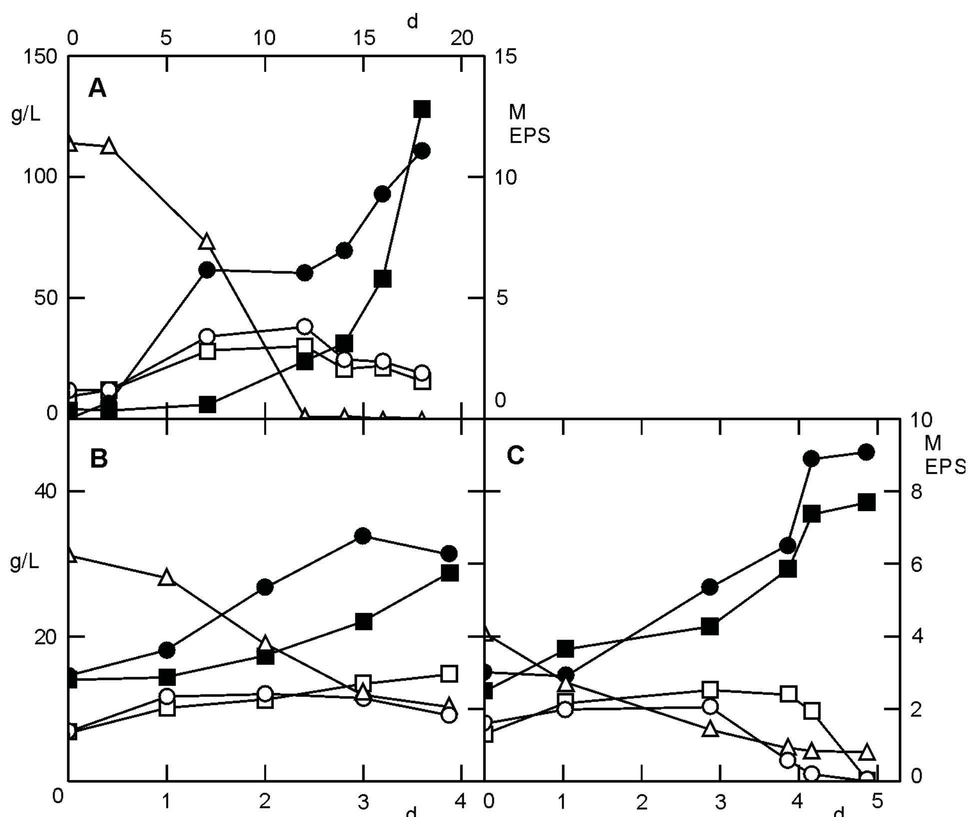

Fig. 1. Sucrose metabolism and production of biomass and EPS in submerged culture of *C. viridis* cultivated on (**A**) sucrose rich medium T2 in Erlenmeyer flasks, and (B, C) CS2 medium in laboratory fermentor with volume of (**B**) 1 L (BioFlo, *New Brunswick*) and (**C**) 5 L (MBR Bioreactor, *Sulzer*); *triangles* – sucrose (g/L), *open circles* – glucose (g/L), *open squares* – fructose (g/L), *closed circles* – biomass (M, g/L), *closed squares* – EPS (g/L).

Table II. The estimated parameters of the kinetic model (Eqs *1–4*) describing growth and EPS production by *C. viridis*

Parameter	Dimension	Fermented in		
		flasks, 50 mL	BioFlo, 1 L	MBR Sulzer, 5 L
k_1	L g^{-1} d^{-1}	0.695	0.453	0.748
k_2	L g^{-1} d^{-1}	0.130	0.136	0.152
k_3	1/d	8.100	10.700	6.500
k_4	1/d	0.129	0.201	0.218
Y	–	3.100	1.980	1.940
K_1	L/g	1.340	0.910	3.510
K_2	L/g	0.520	0.350	0.040
K_S	g/L	29.300	18.500	19.400

In order to evaluate the macroscopic stoichiometry of the process, the hypothesis of Gutierrez *et al.* (1996) that the soluble polysaccharide originates from the extracellular sheath surrounding the mycelium was tested; it was found that the consumption of total sugar from the medium was proportional to the dry biomass and EPS content (Fig. 2). These findings on process kinetics and stoichiometry served as basic axioms for the formulation of a mathematical model describing the fermentation. The model was fitted to experimental data (Table II). Parameters k_1, k_2 and k_3 characterizing the growth rate and product formation were found to be of the same magnitude. This means that the biosynthetic rates were identical in both the flask and the fermentor. The value of K_S was similar to S_2, which can be attributed to the link between the first and the second phase of the cultivation. The different catabolism of sucrose and monosaccharides in both phases increased the maintenance energy necessary to overcome the physiological shunt typical of *Claviceps purpurea*. (Taber 1964).

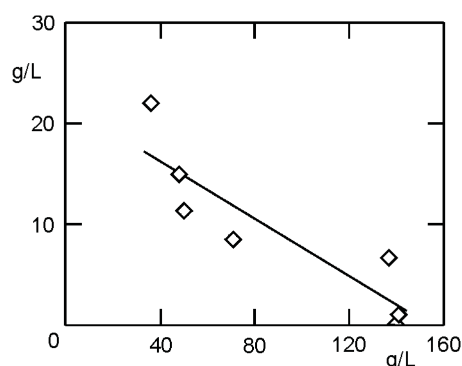

Fig. 2. Linear dependence of total sugar concentration (*abscissa*) on the sum of EPS and dry biomass concentration (*ordinate*).

In the production phase of the submerged fermentation of *C. viridis*, confined by the concentrations of sucrose below the K_S value, the concentration of EPS reached its maximum after 3 d of cultivation and the maximum biomass concentration ranged from 10 to 14 g/L. In order to compare our results with previously published data of other authors we used coefficient Y (which defines the amount of sugar consumed per total mass of soluble glucan and dry biomass). Thus, the efficiency of sugar conversion is related to soluble and solid parts of fungal biomass. The coefficients Y have very close numerical values for all aerobic cultivations (Table III). Strains of *C. viridis* and *Sclerotium rolfsii* exhibited the maximum efficiency of sugar conversion to biomass and EPS, whereas the physiological efficiency of *Lactobacilus* sp. was the lowest due to simultaneous production of lactic acid under anaerobic conditions (van Geel-Schutten *et al.* 1998). *C. fusiformis* and an Antarctic isolate of the fungus *Phoma herbarum* achieved the maximum specific productivity of soluble glucan but both strains exhibited high glucanase activity when maximum production of EPS was reached (Selbmann *et al.* 2002). For these reasons, the *C. viridis* (used here) and *S. rolfsii* (Schilling *et al.* 1999) strains can be considered to be the best candidates for future industrial application.

Table III. Comparison of productivity and specific sugar consumption for different microorganisms producing exocellular polysaccharide

Strain	Duration d	Biomass g/L	EPS g/L	Y^a g/g	Productivity g/L per d	Source
Claviceps viridis	4	9.0	7.7	1.98	1.9	*this paper*
fusiformis	6	14.8	4.5	2.40	0.8	Buck *et al.* (1968)
fusiformis	3	10.0	4.2	2.31	1.4	Banks *et al.* (1974)
fusiformis	7	14.0	34.0	2.57	4.9	Pažoutová *et al.* (1981)
Achromobacter sp.	3	–	17.0	2.40	5.7	Koní ek *et al.* (1993)
Lactobacillus sp.	3	13.0	1.2	4.38	0.4	van Geel-Schutten *et al.* (1998)
Phoma herbarum	3	13.6	13.0	3.61	4.5	Selbmann *et al.* (2002)
Sclerotium rolfsii	3	14.9	10.0	1.96	3.3	Schilling *et al.* (1999)
Thelephora terrestris	3	0.4	0.4	2.69	0.1	Osaku *et al.* (2002)

[a]Sugar consumed per g biomass and glucan formed.

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