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Enhanced enzyme production with the pelleted form of *D. squalens* in laboratory bioreactors using added natural lignin inducer

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Abstract White-rot fungi are extensively used in various submerged biotechnology processes to produce ligninolytic enzymes. Transfer of the process from the laboratory to the industrial level requires optimization of the cultivation conditions on the laboratory scale. An interesting area of optimization is pellet growth since this morphological form solves problems such as the decreased oxygen concentration, limited heat, and nutrient transport, which usually occur in dispersed mycelium cultures. Many submerged fermentations with basidiomycetes in pellet form were done with *Phanerochaete*, *Trametes*, and *Bjerkandera* species, among others. In our study, another promising basidiomycete, *D. squalens*, was used for ligninolytic enzyme production. With the addition of wood particles (sawdust) as a natural inducer and optimization of mixing and aeration conditions in laboratory stirred tank (STR) and bubble column (BCR) reactors on pellet growth and morphology, the secretion of laccase and the manganese-dependent peroxidase into the medium was substantially enhanced. The maximum mean pellet radius was achieved after 10 days in the BCR (5.1 mm) where pellets were fluffy and 5 days in the STR (3.5 mm) where they were round and smooth. The maximum Lac activity (1,882 U l^{-1}) was obtained after 12 days in the STR, while maximum MnP activity (449.8 U l^{-1}) occurred after 18 days in the BCR. The pellet size and morphology depended on the agitation and aeration conditions and consequently influenced a particular enzyme synthesis. The enzyme activities were high and comparable with the activities found for other investigations in reactors with basidiomycetes in the form of pellets.

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Introduction

The white-rot fungi, such as *Phanerochaete chrysosporium, Trametes versicolor*, and *Bjerkandera adusta*, are an important group of microorganisms that produce non-specific ligninolytic enzymes, e.g. lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and laccase (Lac) [20, 27]. These are products with a high potential for degrading a variety of persistent environmental pollutants [37] and lignocellulosic materials, such as wood [31]. The industrial production of enzymes is mainly performed by submerged fermentation (SmF). In this case, these fungi exhibit different morphological forms, ranging from dispersed mycelial filaments to densely interwoven mycelial masses, referred to as pellets [19]. Dispersed mycelia cause many problems in reactors due to the restricted mass transport of oxygen, heat, and nutrients, and biomass growth on the bioreactor walls, agitators, etc. [25, 36]. Such operational problems do not occur in pellet cultures where the fungi form aggregates of hyphae during growth. The fermentation broth exhibits low viscosity and approaches Newtonian flow behavior, while the mycelium does not adhere to any part of the bioreactor [23]. Pellet morphology strongly depends upon the cultivation conditions in bioreactors, e.g., agitation rate, temperature, medium composition and pH, dissolved oxygen, inoculum concentration, etc. [15, 16, 39, 40]. All of these parameters need to be considered in order to obtain a good product yield, therefore good control of mycelial morphology in SmF is important for many industrial applications. The influence of various parameters on fungal morphology and productivity was

recently reviewed [7]. It is well known that enzyme production can be enhanced by the addition of various inducers. For example, different reactor systems were used for enzyme production with the basidiomycete *Trametes versicolor* in pellet form in SmF but without addition of synthetic or natural inducers [1–3, 6, 33]. Olivieri et al. [18] cultivated *Pleurotus ostreatus* in the form of pellets in an air-lift loop reactor and used olive mill wastewater as a natural inducer, but enzyme production was no higher than in the SmF mentioned above without an inducer.

Filamentous fungi have another typical characteristic: a natural inclination to adhere to surfaces. For enzyme production, the chemical composition of the supporting material offering a surface for growth is especially important because it affects fungal activities. It was found that various types of wood with different physico-chemical compositions have diverse effects on enzyme production during cultivation of immobilized *Ganoderma lucidum* [4, 29]. It was indicated that beech wood, as an immobilization support, induced Lac production with *Dichomitus squalens* better than polyurethane foam [30]. This is due to natural lignin, which besides cellulose and hemicellulose is one of the three main polymeric constituents of wood [4].

The basidiomycete *Dichomitus squalens* produces various extracellular enzymes such as MnP and Lac, but not LiP and consequently efficiently degrades both natural [38] and synthetic lignins [22, 35]. Research on this interesting lignin-degrading fungus, which offers a good potential for industrial applications, intensified in the last few years since high enzyme activities have been obtained. The idea for the present work was suggested by research results on enzyme production with this fungus, immobilized on wooden cubes in stationary cultures [21, 30]. The advantage of the natural fungal tendency to grow on a solid surface and the effect of beech wood as a natural inducer was considered. Using beech wood sawdust, the specific surface area available for fungal attack on inducing wood constituents like lignin, was enlarged. The substantially increased enzyme activities demonstrated the important role of lignin in the medium and revealed the potential for optimization of enzyme production processes with other ligninolytic fungi. There are no reports of SmF in reactors in the form of pellets with this basidiomycete. Therefore, the aim of this study was to find optimal agitation and aeration conditions for enzyme production with pellets of this fungus in a laboratory stirred tank reactor (STR) and a bubble column reactor (BCR) in cultivation media containing beech wood sawdust. For quantitative evaluation of the growth and product formation parameters, such as pellet growth rate and enzyme productivity, the mean pellet radius and ligninolytic enzyme activities were measured during cultivation.

Table 1 Maximum activities of Lac and MnP in shaken submerged cultures at an agitation rate of 150 rpm at 28°C in growth medium with two different sizes of NLI, i.e., 0.125–0.3 and 1.0–1.4 mm

NLI concentration ($g\ l^{-1}$)	Size of NLI (mm)	Time of max activity (day)	Max Lac activity ($U\ l^{-1}$)	Max MnP activity ($U\ l^{-1)}$
1.0	0.125–0.3	7	315.0	5.4
2.0	0.125–0.3	7	565.7	8.5
5.0	0.125–0.3	7	927.2	13.4
10.0	0.125–0.3	7	1,094.2	14.3
2.0	1.0–1.4	5	73.4	0.6
5.0	1.0–1.4	5	44.6	9.7

The activity of Lac and MnP increased with the available specific surface area of NLI, which is inversely proportional to the particle size. The activities also increased with the amount of NLI available

Materials and methods

Microorganism

The fungus *D. squalens* MZKI B1233 was obtained from the MZKI culture collection (National Institute of Chemistry, Ljubljana, Slovenia). The strain was maintained on 2% malt agar plates at 4°C as described previously [32].

Natural lignin inducer

Beech wood particles were used as a natural lignin inducer (NLI). They were prepared by drilling 10-mm holes in a beech wood slab. The chips were then separated with standard round sieves with a 200-mm frame diameter (Saulas, France) to obtain particles with sizes 0.125–0.3 and 1.0–1.4 mm. Prior to media preparation, the particles were dried for 2 h at 130°C. NLI particles were added to culture media in concentrations from 1.0 to 5.0 $g\ l^{-1}$ (see Table 1).

Mycelial suspension

The mycelial suspension of *D. squalens* was prepared by inoculation of four 1-cm-diameter plugs from the fungus-growing zone on malt agar, in 50 ml of N-limited mineral medium [34] in a 250-ml Erlenmeyer flask. This was incubated at 28°C in an incubator (Heraeus, Function Line IP 20, Germany). After 7 days, a dense mycelial mass was formed. To prepare the mycelium suspension for inoculation, the suspension was disrupted with an Ultra-Turrax T25 (Janke & Kunkel, IKA-Labortechnik, Germany) at 9,000 rpm under sterile conditions prior to inoculation.

Shaken cultures

The shaken cultures were prepared by inoculating 100 ml of the growth mineral media containing NLI in a 250-ml Erlenmeyer flask with 5% (v/v) of the mycelial suspension [21, 34]. The fungus was incubated on an RVI-403 rotary shaker (Tehtnica, Železniki, Slovenia) at 28°C at a constant agitation of 150 rpm. Aliquots of the culture liquid were collected for determination of extracellular Lac and MnP activities and mean pellet radius. All experiments were performed in duplicate. The inoculum for laboratory bioreactors was prepared on the shaker in the same way.

Bubble column reactor

A 4.0-l bubble column reactor (BCR) was constructed from a 50-cm-long and 10-cm-ID glass cylinder. A stainless-steel plate with a gas inlet and a stainless-steel plate with several standard ports for air filters and media sampling were used at the bottom and at the top of the reactor to obtain a 2.0-l working volume. A cylinder of 0.8 cm radius and 1 cm height, and with six 0.5-mm holes on the circumference, was used as a gas distributor. The reactor was filled with 1.8 l of growth mineral medium with 5 g l^{-1} of 0.125–0.3 mm NLI, sterilized and inoculated with 200 ml of 3-day-old pellets previously cultivated in an Erlenmeyer flask on a shaker. The cultivation continued in separate experiments at airflow rates of 50, 75, and 100 l h^{-1} at 28°C.

Stirred tank reactor

A 3.1-l stirred tank reactor (*Benchtop Laboratory fermentor KLF2000*, Bioengineering, Switzerland) with a 2.1-l working volume was used in the experiments. The reactor was constructed from a 30.5-cm-long and 10.5-cm-ID glass cylinder, a stainless-steel bottom plate and top lid with air filters, pH electrode, O_2 electrode, cooling and warming unit, and air distributor. Two Rushton turbines of 4.0 cm in diameter were used for mixing. Growth mineral medium (1.8 l) with NLI was prepared, and then sterilized and inoculated. The initial NLI concentration and inoculum size were the same as in the BCR. The fungi cultivation was performed in separate experiments at an airflow rate of 75 l h^{-1} and stirrer speeds of 175, 250, and 350 rpm, initial pH 4.5, initial pO_2 100% and T 28°C.

Pellet size

A media sample was dispensed on Petri dishes and photographed for morphology determination and image analysis. Assuming a spherical shape, the pellet radius and pellet morphology were estimated from the photograph by applying commercial image analysis software (Motic Images Plus 2.0).

Substrate analysis

The quantitative detection of reducing sugar in the culture filtrate was made according to the standard Miller method [14]. The presence of free carbonyl groups was determined spectrophotometrically with a Perkin-Elmer spectrophotometer, type Lambda 25 (USA), at a wavelength 540 nm (A540).

Enzyme assays

The Lac activity was measured by monitoring the oxidation of 5 mM 2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonate) (ABTS) at A420 [8] and MnP activity by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at A469 [5]. One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 µmol of the corresponding substrate per minute. All spectrophotometric measurements were conducted using a Perkin-Elmer spectrophotometer, type Lambda 25 (USA). The substrates for the Lac and MnP activity assays were both purchased from Sigma (USA).

Results and discussion

Optimization of amount and size of beech wood particles

White-rot fungi are known to be capable of using lignin as their sole carbon and energy source and it is generally believed that lignin breakdown is necessary to gain access to cellulose and hemicellulose [10, 24]. In previous research with *D. squalens* cultivation on various types of immobilization support in the form of 1-cm cubes, it was established that beech wood cubes acted as a good inducer of lignino-lytic enzyme production due to their lignocellulosic structural cell-wall components [21]. To check the effect of the available surface area available for fungal attack and consequently Lac and MnP production, two particle sizes of beech wood with different specific areas and various concentrations were used in the preliminary experiments. The highest Lac and MnP enzyme activities were obtained on the seventh day of cultivation. The results (cf. Table 1) show that the activity of Lac and MnP increased with the surface area of NLI available. Smaller particles (0.125–0.3 mm) gave approximately twice as high activities, since the specific area is inversely proportional to the particle size. The activities also increased with the amount of NLI available. Both activities for concentrations of 5.0 g l^{-1} (927.2 U l^{-1}) and 10.0 g l^{-1} (1,094.2 U l^{-1}) were high. However, the broth was thicker at 10.0 g l^{-1} of beech wood particles and could hinder mixing and oxygen transfer in the bioreactors. Therefore, we decided to use 5.0 g l^{-1} of 0.125–0.3 mm particles during further investigations.

Table 2 Effect of aeration and agitation on ligninolytic enzyme activity, productivity, and pellet radius of fungi *D. squalens* in the BCR and STR

	BCR			STR		
	1	2	3	1	2	3
N (rpm)	/	/	/	175	250	350
Aeration rate (l h^{-1})	50	75	100	75	75	75
P/V (W l^{-1})	0.018	0.028	0.036	0.02	0.06	0.18
γ (s^{-1})	9	13	18	32	46	64
Max Lac activity (U l^{-1})	1,460.0	1,112.1	958.3	1,571.6	1,882.3	444.6
t_{max} Lac (day)	10.0	14.0	12.0	10.0	12.0	15.0
Lac productivity (U $l^{-1}day^{-1}$)	146.0	79.4	79.9	157.2	156.9	29.6
Max MnP activity (U l^{-1})	449.8	56.5	154.8	114.4	188.8	139.2
t_{max} MnP (day)	18.0	12.0	9.0	13.0	13.0	12.0
MnP productivity (U $l^{-1}day^{-1}$)	25.0	4.7	17.2	8.8	14.5	11.6
r_{start} (mm)	0.7	0.7	0.6	0.8	0.7	0.6
r_{max} (mm)	3.9	4.6	5.1	3.5	3.1	2.7
t_{max} (day)	8.0	10.0	10.0	5.0	5.0	4.0
k_r (mm day^{-1})	0.39	0.41	0.42	0.55	0.50	0.47

The maximum mean pellet radius was achieved after 10 days in the BCR (5.1 mm) and after 5 days in the STR (3.5 mm). The highest linear growth rate constant (0.55 mm day^{-1}) was achieved in the STR. The maximum Lac activity (1,882 U l^{-1}) was obtained after 12 days in the STR, while maximum MnP activity (449.8 U l^{-1}) occurred after 18 days in the BCR.

N agitation speed, *P/V* power input, γ shear rate, t_{max} *Lac/MnP* time for achievement of maximum Lac/MnP activity, r_{start} mean pellet radius at the beginning of experiment, r_{max} maximum mean pellet radius achieved at time t_{max}, t_{max} time for r_{max} appearance, k_r linear radial growth rate

Pellet growth and morphology in BCR and STR with beech wood particles

The growth medium with 0.125–0.3 mm beech wood particles was inoculated with mycelial suspension of fungus *D. squalens*. Pellet growth in medium with particles was studied with the graphical analysis of mean pellet radius during cultivation. The linear radial growth rate constant (k_r) expressed in mm per day was determined from the slope of the curve in the phase of linear increase of pellet radius versus time [39]. To obtain a better insight into the growth conditions, the substrate concentration was also measured. The results are displayed in Table 2. In shaken cultures, a 2-day lag phase for pellet formation occurred (Fig. 1). A linear increase in pellet radius appeared after 3 days and lasted until the eighth day of cultivation. Then the substrate was consumed, growth stopped, and the pellet size slightly decreased. The stirred tank reactor and bubble column reactor were inoculated with small pellets from 3-day-old shaken cultures. Their mean pellet radius at the beginning of the experiments in reactors (r_{start}) was 0.7 ± 0.1 mm (Table 2). Linear growth in the STR lasted 4 days long (Fig. 2a), while in the BCR it lasted 7–10 days (Fig. 2b) and afterwards remained constant. In the STR at up-agitation speed (N) of 350 rpm, growth was reduced due to pellet breakdown to a pellet size of 1.0 mm, which remained constant over the next 3 days (Fig. 2a). The linear radial growth rate constants (k_r) were 0.36 mm day^{-1} for shaken cultures, 0.47–0.55 mm day^{-1} for the STR and 0.39–0.42 mm day^{-1} for the BCR (Table 2). The results show that maximum mean pellet radius (r_{max}) in the BCR increased faster at higher aeration rates with the highest linear radial growth rate constants $k_r = 0.42$ mm day^{-1}, giving the largest pellets ($r_{max} = 5.1$ mm) at 100 l h^{-1}. In the STR, the highest linear growth rate constant $k_r = 0.55$ mm day^{-1} and the largest pellets ($r_{max} = 3.5$ mm) were obtained at the lowest stirrer speed of 175 rpm. The maximum mean pellet radius in the cultivation vessels investigated was achieved at time t_{max}. This was after 8 days in shaken cultures (2.5 mm) (Fig. 1), 10 days in the BCR (5.1 mm) and 5 days in the STR (3.5 mm) (Table 2). The results from shaken cultures were more comparable with those from the BCR than those from the STR. The cultivation time up to maximum pellet size (t_{max}) and linear radial growth rate was practically the same in shaken cultures and the BCR, while growth in the STR was faster and pellets were smaller. The shear rate caused by shaking was lower in the Erlenmeyer flasks compared to mixing with stirrers in the STR. This on one hand provides more convenient agitation conditions for fungal growth in shaken cultures [13] and better oxygen supply in STR.

Figure 3 shows the morphological form of pellets from the BCR and the STR, which is different due to the different shear stress conditions in the two reactors. The average shear rates (γ) [11, 17] and power inputs (P/V) [9, 12, 26] in STR and BCR were estimated according to literature corre-

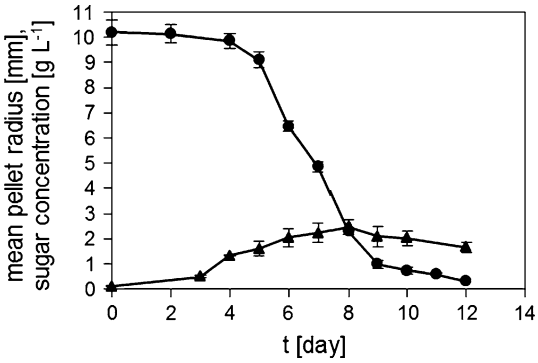

Fig. 1 *D. squalens* pellet growth in shaken submerged cultures at 28°C and 150 rpm in growth medium with 0.125–0.3 mm NLI. Pellet radius (*filled triangle*) and sugar decrease (*filled circle*) with time. The maximum mean pellet radius of 2.5 mm was obtained after 8 days of cultivation

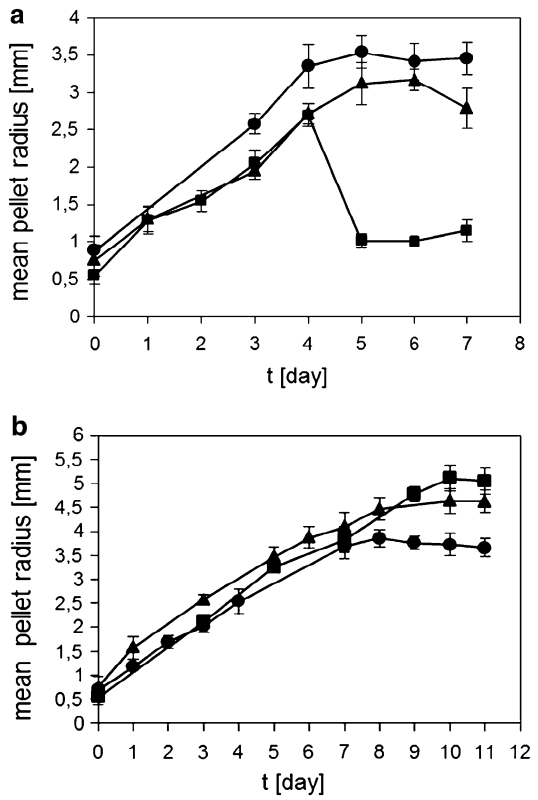

Fig. 2 Time course of *D. squalens* pellet growth in laboratory bioreactors, performed at 28°C in growth medium with 0.125–0.3 mm NLI, inoculated with fungal pellets obtained in shaken cultures. The change in pellet radius versus time in **a** STR at 175 rpm, 75 l h^{-1} (*filled circle*); 250 rpm, 75 l h^{-1} (*filled triangle*) and 350 rpm, 75 l h^{-1} (*filled square*) and **b** BCR at aeration 50 (*filled circle*), 75 (*filled triangle*), and 100 (*filled square*) l h^{-1} is shown. Linear growth in the STR lasted 4 days while in the BCR it lasted 7–10 days. In the STR at an agitation speed (*N*) of 350 rpm, growth was reduced due to pellet breakdown to a pellet size of 1.0 mm

lations, taking into account the operating conditions during our experiments, and are presented in Table 2. Lower shear rates and power inputs were obtained in the BCR compared to the STR. The pellets were composed of two layers, i.e., the inner layer, where the limitation of nutrients increased with pellet size and the external layer, where the nutrients could diffuse constantly. In the linear phase, pellets grew as a result of good diffusion of nutrients into both pellet layers. As soon as one of the essential nutrients was exhausted, the pellets stopped growing and passed over through the stationary phase to the dying phase [13, 39, 40]. The pellets were morphologically similar at t_{max} in the shaken submerged cultures and in the STR—they were rounded and smooth (Fig. 3a, b). In the BCR, the pellets were smooth at the beginning of the experiment and fluffy at t_{max} (Fig. 3c). It was observed that the initial increase in the pellet concentration in the fungal cultures was followed by a rapid decrease, which coincided with a decrease in the specific growth rate [16]. This breakup was caused by cell lysis within the pellets, whereby the stability of the pellet was lost, and it become more susceptible to damage by mechanical forces [19]. We assume that this phenomenon occurred during our cultivation in the STR on the fifth day (cf. Fig. 2a), where the shear forces produced by the stirrer negatively influenced pellet growth and even caused fragmentation of pellets during their lysis. The pellets with a radius of 2.6 mm were fragmented into smaller pellets with a 1.0-mm radius, which represented new centers for further growth [19].

According to the results in Table 2 and the experimental observations, it seems that for successful pellet cultivation with addition of beech wood particles in a STR, the average shear rate (γ) and power input (*P*/*V*) must not exceed 46 s^{-1} and 0.06 W/l, respectively. On the other hand, a lower stirrer speed (*N*) does not ensure homogeneous pellet distribution in the fermentation broth. In the BCR, sedimentation occurs at the lower aeration rates used in our experiments, while at higher aeration rates foaming could be a problem.

Enzyme activities and productivity in BCR and STR with the addition of beech wood particles

The basidiomycete fungus *D. squalens* is able to produce two ligninolytic enzymes, Lac and MnP, but not LiP. The production and activity of these two enzymes can be improved by the addition of natural inducers, e.g., beech wood and straw [21, 32]. Figures 5 and 6 show the results of our investigation with beech wood particles in two types of bioreactors compared to those from shaken cultures (Fig. 4), where the maximum Lac (1,113.8 U l^{-1}) and MnP (201.9 U l^{-1}) activities were achieved after 8 days of cultivation. In Fig. 5a, MnP activities during the experiment in the STR are presented. Extracellular MnP activities show two peaks at all three aeration and agitation conditions. The first peak is detectable between the 4th and the 10th day, while a maximum occurred between the 12th and the 13th day. Extracellular

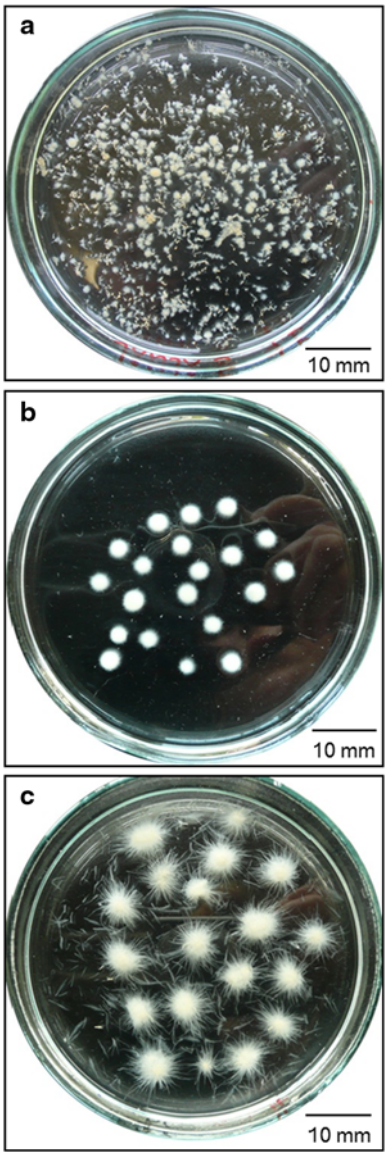

Fig. 3 Maximum mean pellet radius (r_{max}) of the fungus *D. squalens* at t_{max} in different submerged cultivations. **a** Shaken culture cultivation at 150 rpm, 28°C. Maximum mean pellet radius at t_{max} was 2.5 mm. Pellets were round and smooth. **b** STR cultivation at an aeration rate of 75 l h^{-1}, 28°C and agitation speed of 250 rpm. Maximum mean pellet radius at t_{max} was 3.5 mm. Pellets were round and smooth. **c** BCR cultivation at an aeration rate of 75 l h^{-1}, 28°C. Maximum mean pellet radius at t_{max} was 5.1 mm. Pellets were fluffy. *Scale bar* 10 mm

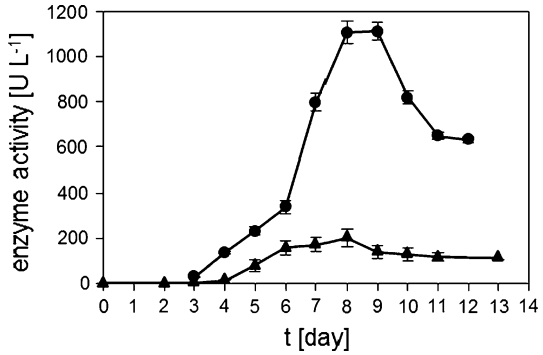

Fig. 4 Enzyme activities of Lac (*filled circle*) and MnP (*filled triangle*) versus time in shaken submerged cultures at 28°C and 150 rpm in growth medium with 0.125–0.3 mm NLI. The maximum Lac (1,113.8 U l^{-1}) and MnP (201.9 U l^{-1}) activities were achieved after 8 days of cultivation

Lac activity reached its maximum between the 10th and the 15th day and then declined (Fig. 5b). In BCR, MnP expression with two peaks was similar to that in STR and occurred only at an aeration rate 50 l h^{-1}. The first peak appeared on the 8th day and the maximum activity on the 18th day. At aeration rates of 75 and 100 l h^{-1}, the maximum MnP activities were found between the 9th and 12th day (Fig. 6a), while the maximal Lac activities appeared between the 10th and 14th day (Fig. 6b). We have no additional experimental data to explain the two peaks in MnP activity expression. Probably the nutrient conditions in the media containing wooden particles changed (depletion of some easily accessible inductor) and forced the fungus to adapt to new conditions. This was expressed as a drop of MnP activity for a short time and after adaptation synthesis recovered and leads to higher MnP activities. A similar pattern for Lac and MnP expression with two peaks in *D. squalens* was shown by Perie and Gold [22] in their study, but they offered no explanation for this.

Maximum enzyme productivity was used as a parameter for comparison of the enzyme production in the two reactors. Here it was determined as the maximum enzyme activity divided by the process time from inoculation. Experiments in the BCR were performed at aeration rates of 50, 75, and 100 l h^{-1} to study the effect of mixing on enzyme activity and productivity. The maximum Lac activity (1,460 U l^{-1}) and Lac productivity (146 U l^{-1} day^{-1}) were obtained after 10 days (t_{max} Lac), while the maximum MnP activity (449.8 U l^{-1}) and MnP productivity (25 U l^{-1} day^{-1}) occurred after 18 days (t_{max} MnP) of cultivation at an aeration rate of 50 l h^{-1} (Table 2). At higher aeration rates, pellet growth rate increased but enzyme activities and enzyme productivities were lower.

STR experiments were performed at an aeration rate of 75 l h^{-1} and agitator speeds (N) of 175, 250, and 350 rpm. It has been reported that an increase in the stirrer speed reduces the productivity in some cases, depending on the organism cultivated [8]. This reduction was also observed in our study. The maximum Lac activity (1,882 U l^{-1}) was obtained after 12 days (t_{max} Lac), and maximum MnP activity (189 U l^{-1}) 1 day after that. Lac productivity (157 U l^{-1} day^{-1}) was the highest at 175 and 250 rpm, while the highest MnP productivity (14.5 U l^{-1} day^{-1}) occurred at 250 rpm on the 12th and 13th day, respectively (Table 2). At the highest agitator speed used in our experiments, maximum enzyme activities and productivities were lower.

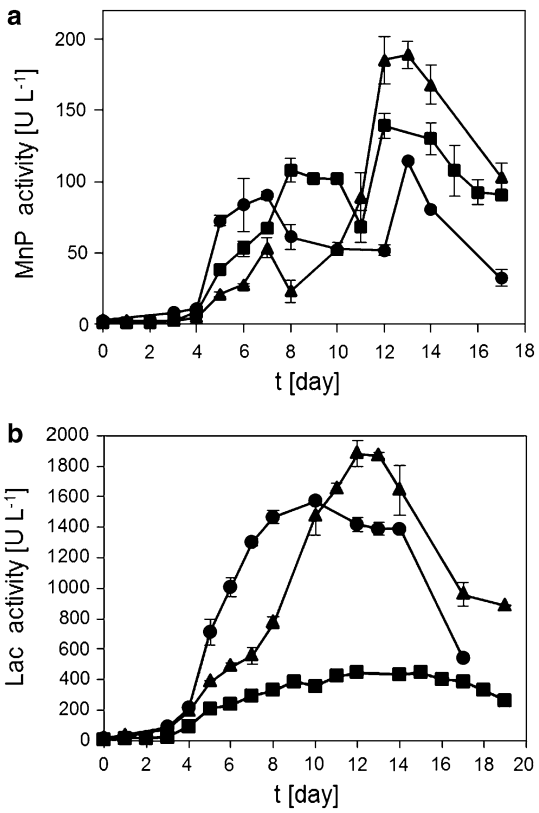

Fig. 5 Time course of *D. squalens* ligninolytic extracellular enzyme activities in the STR, performed at 28°C in growth medium with 0.125–0.3 mm NLI, inoculated with fungal pellets, obtained in shaken cultures. The change in **a** MnP and **b** Lac activities with time at agitation speeds of 175 rpm, 75 l h^{-1} (*filled circle*), 250, 75 l h^{-1} (*filled triangle*) and 350, 75 l h^{-1} (*filled square*) is shown. Maximum MnP activity (188.8 U l^{-1}) occurred on 13th day at 250 rpm (**a**), while Lac activity reached its maximum (1,882.3 U l^{-1}) on the 12th day also at 250 rpm (**b**)

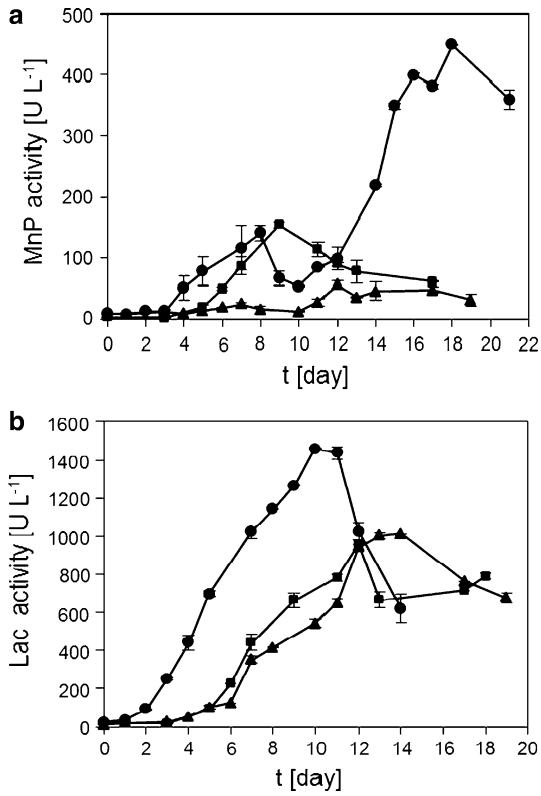

Fig. 6 Time course of *D. squalens* ligninolytic enzyme activities in BCR, performed at 28°C in growth medium with 0.125–0.3 mm NLI, inoculated fungal pellets, obtained in shaken cultures. The change in **a** MnP and **b** Lac activities with time at aeration rates of 50 l h^{-1} (*filled circle*), 75 l h^{-1} (*filled triangle*) and 100 l h^{-1} (*filled square*) is shown. Maximum MnP activity (449.8 U l^{-1}) was found on 18th day (**a**), while maximum Lac activity (1,460.0 U l^{-1}) appeared on the 10th day (**b**), both at an aeration rate of 50 l h^{-1}

Our results show that maximal productivity of a particular enzyme is found under the experimental conditions where maximal activity also occurs. There is a correlation between those two parameters and pellet growth, morphology and size, therefore controlling of pellet morphology and size by cultivation conditions can be used for optimal production of a particular enzyme [28]. Maximal Lac production was obtained in the STR with smaller, smooth and round pellets, while maximal MnP production occurred in the BCR with larger and smooth pellets. The choice of enzyme production process should depend on the use of a particular enzyme or enzyme mixture for the degradation of the selected target material.

The results of the study with basidiomycetes in pellet form from SmF in bioreactors with or without a natural inducer are presented elsewhere [1–3, 6, 33]. Maximal Lac activities were around 1,200–1,400 U l^{-1}. Addition of NLI in our investigation enhanced Lac activity in the BCR by 20% and in the STR by 60%. This shows that not only *Trametes* but also other basidiomycete species like *Dichomitus* display potential for ligninolytic enzyme production in pellet form in reactors. Of course, additional research on the cultivation conditions in laboratory bioreactors with this less studied fungus is necessary to optimize the enzyme production process. For example, some improvements in Lac production with *Trametes* by using air pulses in a reactor system have already been presented [2].

Conclusions

The results of this investigation demonstrate that the non-spore growing fungus *D. squalens* can be successfully cultivated in pellet form in bubble-column and stirred-tank bioreactors. At appropriate mixing and aeration conditions, and by adding beech particles to the liquid medium, the production of extracellular Lac and MnP was substantially increased. The levels of enzyme activities obtained were high and comparable to the results with other industrially important basidiomycetes, so *D. squalens* is a promising

fungus for practical application. Furthermore, the addition of a natural inducer in the form of small particles like sawdust can be applied in other industrial processes to increase enzyme production. The important role of an engineering approach to research on the microbial cultivation process was also demonstrated. Optimization of enzyme production was made via several experiments at various mixing intensities in two laboratory bioreactors. Considering the parameters such as shear rate, power input, and enzyme productivity gave better insight into the microbial growth and enzyme production process and lead to successful optimization. Aeration in the BCR and agitation in the STR have a significant effect on growth, morphology, and enzyme production in both reactors. Higher Lac activities and productivities were achieved in the STR, while MnP activities and productivities were better in the BCR. The necessary enzyme activities to degrade a particular material depend on its chemical structure. Therefore, control of pellet morphology and pellet size can be used through choice of reactor type and cultivation conditions to achieve the optimal production of the necessary Lac or MnP activities, or their ratio in the enzyme mixture.

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