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Oxygen uptake rate measurements to monitor the activity of terpene transforming fungi

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Abstract

The application of a simple system for measuring the oxygen uptake rate (OUR) to determine the activity of pellet-forming fungi is described. A non-invasive easy-to-use respirometric system was used to measure the OUR as an activity parameter. The model sensor system was optimized in shake flask experiments with *Pleurotus sapidus* (DSMZ 8266) and *Chaetomium globosum* (DSMZ 1962). The toxicity of (+)-limonene and (+)-valencene to submerged fungal cultures and the influence of solvents on *C. globosum* were investigated. Finally, the new respirometric method was used for the determination of oxygen uptake rates in small scale bioreactors to control the activity of terpene transforming fungi.

Keywords: Activity; Biotransformation; Fungi; Oxygen uptake rate; Process-monitoring; Terpene

1. Introduction

Oxyfunctionalized derivatives of mono- and sesquiterpenes play a major role in the flavor and fragrance industry as they are often found as character impact compounds of essential oils from natural resources [1]. Many endeavors have been made to produce these flavors and fragrances biocatalytically as, very often, the extraction from natural sources is uneconomical [2].

Due to their great arsenal of enzymes fungi are known to have the ability to transform terpenes into valuable flavor and aroma compounds [3,4]. Basidio- and ascomycetes in particular show a great variety of interesting oxyfunctionalisations which have led to valuable natural fragrance compounds [5–7].

Regarding the suitability of fungi for biotransformations, the toxicity of the terpenes impedes the commercial application of biotransformations [8], since this is a major problem for a bioprocess on an industrial scale. Due to their lipophility terpenes interfere with the membrane system of the cell and this results in a decrease in activity [9]. Biotransformation is an action of self-defense. Therefore, the concentration of the substrate has to reach a specific concentration to induce the mycelia to perform it. To induce the transformation and to avoid overdosing, the feeding rate of terpenes should be dependent on the activity of the batch, which needs to be monitored throughout the process.

Fungi have different morphologies (filamentous type and pellet type), e.g. concentration and volume of spore inoculum and its ratio to the total culture volume are factors concerned in pellet formation by *Penicillium chrysogenum* [10]. For this reason the cell dry weight (CDW) is not a parameter of activity or growth, because the centre of pellets is inactive. Microprobe measurements of oxygen concentrations in mycelia pellets show that the centre of the pellet is very low in dissolved oxygen [11], so activity in terms of oxygen consumption must be located on the surface of the pellet.

For bioprocess monitoring oxygen is measured as it is mandatory and often a limiting substrate for aerobic cell growth. Oxygen can be measured in the exhaust gas to monitor the oxygen uptake rate (OUR) of the culture, but dissolved oxygen in the broth is also an important parameter [12]. Even though the dissolved oxygen concentration is commonly used to monitor fermentations with bacteria and yeasts, fermentations with higher fungi are slow

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and the mycelia often cover the membranes of the commonly used oxygen electrodes within 2 weeks of fermentation, so that oxygen measurement is not possible in situ. Therefore, in the research work described we developed an off-line measurement system. Respirometric measurements are independent of CDW and pellet form, which makes it a perfect tool to determine the activity of a fungal batch as it is more sensitive than measuring the respiration coefficient.

In previous work, we showed that the principle of oxygen uptake measurements is best to determine the activity of submerged fungal cultures. This work focuses on the toxicity of terpenes in conjunction with biotransformation of terpenes. The change in activity of *Chaetomium globosum* in the presence of solvents was also investigated with respect to the potential use of low-water media in the final bioprocess.

2. Materials and methods

2.1. Chemicals

Agar agar, L-asparagine monohydrate, D-(+)-glucose monohydrate, yeast extract, KH_2PO_4, $MnSO_4{\cdot}H_2O$, Titriplex III were obtained from Merck KGaA, Darmstadt, Germany. (+)-Limonene, (+)-valencene, $CuSO_4{\cdot}5H_2O$, $FeCl_3{\cdot}6H_2O$, $MgSO_4{\cdot}H_2O$, $MnSO_4{\cdot}H_2O$, $ZnSO_4{\cdot}7H_2O$, ethanol, ethylene glycol, *n*-decan-1-ol, isooctane, dimethyl formamide, dimethyl sulfoxide, and oleyl alcohol were supplied by Sigma–Aldrich, Taufkirchen, Germany. Miglyol was obtained from Sasol Germany GmbH, Witten, Germany.

2.2. Cultivation of fungal strains

Buffer was mixed by adding citric acid solution (0.1 M) to di-sodium hydrogen phosphate solution (0.2 M) until the pH was 6. SNLH medium contained 30 g l^{-1} D-glucose monohydrate, 4.5 g l^{-1} L-asparagine monohydrate, 1.5 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4{\cdot}H_2O$, 1 ml l^{-1} solution of trace elements ($FeCl_3{\cdot}6H_2O$, 80 mg l^{-1}; $ZnSO_4{\cdot}7H_2O$, 90 mg l^{-1}; $MnSO_4{\cdot}H_2O$, 30 mg l^{-1}; $CuSO_4{\cdot}5H_2O$, 5 mg l^{-1}; titriplex III, 0.4 g l^{-1}), and yeast extract 3 g l^{-1} (SNLH-agar: as SNLH, but additionally 15 g l^{-1} agar agar).

Medium ingredients were dissolved in demineralized water, pH was adjusted to 6.0 prior to autoclaving. Erlenmeyer flasks were filled with 90 ml (in 300 ml flasks) or 180 ml medium (in 500 ml flasks), the flasks were sealed using cellulose stoppers. Finally, the flasks were steam sterilized (20 min, 121 °C, 1 bar). A 1 cm^2 fully grown piece of mycelia fungi agar plate was inoculated into 100 ml of SNLH, incubated for 48 h on an orbital shaker, and then homogenized using Ultraturrax treatment (TP 18/10, 170 W, maximum 20 000 rpm (level 6), Janke and Kunkel) for 30 s. 10 ml of this preculture was inoculated into 90 ml SNLH medium in a 300 ml Erlenmeyer flask. Standard conditions used for submerged cultivations were 25 °C (standard incubator, Heraeus) and 150 rpm (standard orbital shaker, Certomat). Culture medium was regularly checked for contamination using a light microscope.

2.3. Strain maintenance on agar plates

Pleurotus sapidus (DSMZ 8266) and *C. lobosum* (DSMZ 1962) were maintained on SNLH agar plates. Approximately 1 cm^2 of the original slant or plate was inoculated into a new agar plate, incubated at 25 °C until half the surface was covered with fungal mycelium. Afterwards the plates were sealed with Parafilm® and stored at 4–7 °C.

2.4. Fermentation of C. lobosum

An Infors HT-ISF 100 bench fermenter with 1.5 l SNLH medium was inoculated with 250 ml of a *C. lobosum* preculture and cultivated at 25 °C, 250 rpm, and 9 l h^{-1} aeration. The 5 ml samples of the cultures were directly analyzed in an external respirometer. Sample supernatants were used for glucose determination (YSI 2700 Select, Yellowsprings). Direct supplementation of liquid (+)-valencene (13.5 mM) was conducted at 96 and 126 h and (+)-valencene (4.5 mM) at 149 h after inoculation.

2.5. Respirometric toxicity measurement

The oxygen uptake rates of submerged cultures of *P. sapidus* and *C. globosum* were determined using a two-channel dissolved oxygen electrode (Dual Digital Model 20, Rank Brothers Ltd., UK, oxygen membrane polarographic detector). The samples were filled into the water-jacketed vessel with the electrode placed at the bottom, aerated and sealed from atmospheric oxygen by adjusting a plunger to the sample surface before measuring the dissolved oxygen with 1 Hz at 25 °C.

2.6. Solvent screening for process design

Solvent screening with *C. globosum* was conducted with ethanol, ethylene glycol, *n*-decan-1-ol, isooctane, dimethyl formamide, dimethyl sulphoxide, miglyol 812, and oleyl alcohol. Experimental cultures were prepared with a concentration of 5, 10, and 30 vol.% of each solvent and a blank value cultivated at 25 °C with 150 rpm. Samples of 5 ml for respirometric measurements were taken daily (up to 72 h), in the case of two-phase systems in the liquid the probe was taken from the aqueous phase.

2.7. Long-term toxicity experiments (biotransformations)

Dosage of terpenes was started 24 h after inoculation by adding the terpene precursor directly to the culture flasks. For long-term toxicity four subsequent additions were performed every 24 h.

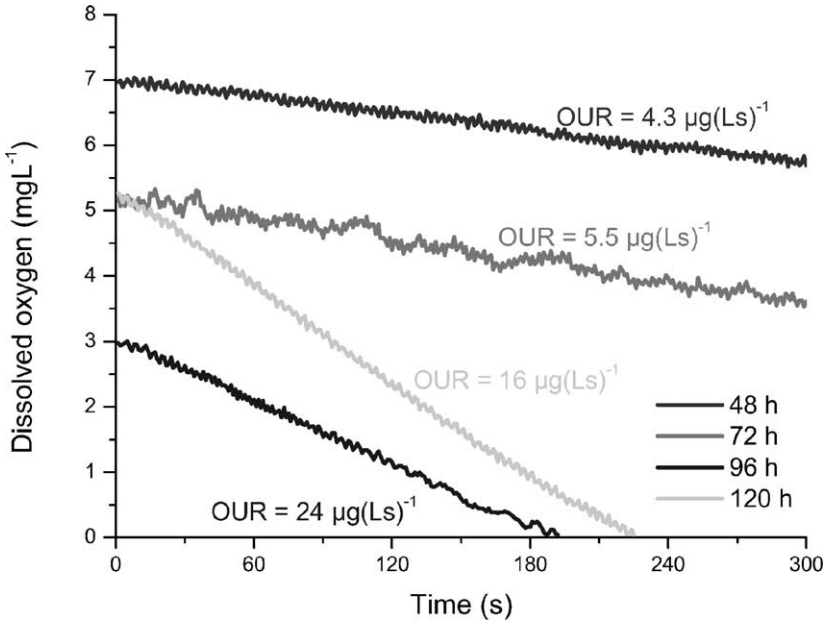

Fig. 1. Four samples of a cultivation of *Pleurotus sapidus* were analyzed with a dissolved oxygen electrode which led to the above plots of dissolved oxygen concentration as a function of time; OUR is calculated from the linear regression of the plots and given in $\mu g\,(l\,s)^{-1}$.

2.8. Calculation of the results

The OUR is always given as the first absolute derivation of a dissolved oxygen/time plot and calculated to $\mu g\,(l\,s)^{-1}$. Fig. 1 illustrates four samples taken from a cultivation of *P. sapidus* with the derived OUR. The OUR increased as active biomass increased. The higher the value of the OUR the higher the fungal activity.

3. Results and discussion

3.1. Respirometric measurements without dosage

In order to show the correlation between OUR and CDW, the OUR was determined in samples from a culture which was diluted with SNLH medium in concentration steps of 20%. Provided that the pellet size and form in each dilution

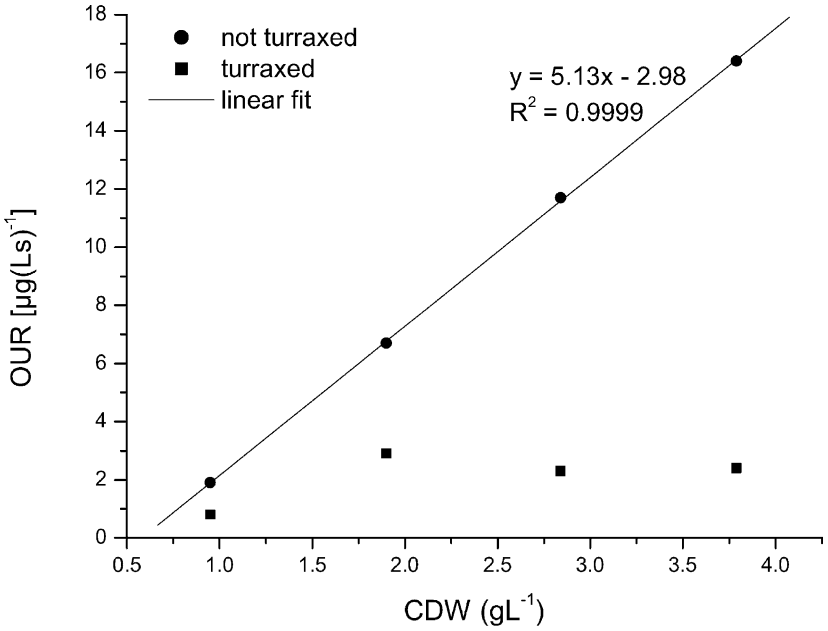

Fig. 2. Linear correlation of OUR and CDW after dilution of a certain culture of *Chaetomium globosum* (closed circle). After turraxing the different solutions (closed square) no correlation between OUR and CDW could be found.

was the same a proportional correlation between the OUR and the CDW could be observed (Fig. 2).

In the case of different pellet forms or different status of a culture no proportion could be stated. This is evident as fungal pellets are inactive in the centre, therefore activity is located at the outer surface of the pellet [13]. To homogenize a preculture for further inoculation, ultraturrax treatment is used to obtain mycelia, which being a homogeneous viscous liquid can easily be pipetted. The ability to sprout in pellets remains as turraxing does not kill all cells. Direct turraxing before respirometric activity measurement could be useful to obtain easily manageable probes, and furthermore the possibility of unrepresentative sampling could be lowered. It was observed that after direct turraxing the OUR did not show a proportional relation even if the same culture was used, this inhibitory effect concerning the OUR is not reproducible nor is it proportional to CDW (Fig. 2). Therefore, all experiments were carried out by direct sampling from the pellet culture, always attempting to obtain representative samples in the case of different pellet sizes.

Due to the formation of pellets, the activity is located in the outer surface of the pellets, and therefore activity is not correlated with the CDW but it is with the diameter of the pellets formed. In a certain culture if pellet sizes are uniformly distributed, there is a linear correlation between OUR and CDW. With regard to the basic aim of the experiments conducted, namely to create an activity sensor for fungal biotransformations, the CDW is a very unsuitable parameter as the pellets themselves can be completely inactive on account of the toxicity of the precursors. Glucose determination is not significant enough due to slow metabolism. With respirometry it is possible to determine the metabolic activity of the sample, which can be caused by a multitude of not very active pellets or by a small quantity of very active pellets. Control of the activity of a batch of transforming fungi is necessary to conduct a feeding strategy to avoid lethal doses of terpenes and also to initiate biotransformation by the fungi.

3.2. Dosage experiments

It was observed that *C. globosum* remained active under glucose-free cultivation in buffer within approximately 1 month (data not shown). Dosage experiments with D-glucose, (+)-valencene, and *m*-kresol after 120 h of cultivation (glucose-free after 72 h) showed significant changes in OUR. After a starvation period, dosage of D-glucose and (+)-valencene enhanced the OUR while *m*-kresol led to a decrease in OUR activity as it is a deactivating agent (Table 1).

The higher OUR after dosage of D-glucose and (+)-valencene is initiated by metabolism as the added substances can act as a substrate for the *C. globosum* stored under nutrient-free conditions.

As the fungus *C. globosum* remains active even when stored in buffer this may be due to storage substances within the cells or it may back up the theory that the outer active zone of the pellets can be nourished from the inner part of inactive (dead) cells of mycelia [10].

Table 1
Comparison of the OUR of a *Chaetomium globosum* culture (120 h cultivation, after 72 h glucose-free) before and after a dose of D-glucose, (+)-valencene, and *m*-kresol

Dosage	mM	OUR ($\mu g\,(l\,s)^{-1}$)	
		Before dosage	After dosage
D-Glucose	167	4.5	10.3
(+)-Valencene	0.8	8.8	14.8
m-Kresol	191	15.3	2.1

The dosage experiments with D-glucose, (+)-valencene, and *m*-kresol showed the suitability of OUR measurements for determining changes in the OUR after the direct addition of substrates or toxins.

Under normal conditions a supplement of (+)-limonene and (+)-valencene to cultures of *P. sapidus* and *C. globosum* has a toxic effect. A daily addition of (+)-limonene to *P. sapidus* and to *C. globosum* led to a complete inhibition of OUR while CDW and glucose concentration remained at the same level. In contrast, a daily addition of (+)-valencene to *P. sapidus* first decreased the OUR, but after 72 h of cultivation in the presence of (+)-valencene the OUR increased (Table 2) whilst CDW remained almost constant.

This enhancement of respirometric activity during daily supplementation of (+)-valencene may be caused by adaptation effects of *P. sapidus*. A daily addition of (+)-valencene to *C. globosum* lowered the OUR compared to the OUR without supplement, but an increasing CDW and degradation of D-glucose was observed (Fig. 3).

These respirometric experiments were carried out to determine long-term toxicity of terpenes to fungi. Even though fungi show different growing phases, including varying pellet forms and sizes [14], it was possible to determine the toxicity of (+)-limonene and (+)-valencene. Toxicity is firmly associated with the status of the culture, in times of high cell proliferation the effect may be worse than in times of resting cells or spores (cf. [15]). Toxicity is also dependent on the permeability of the membranes which also varies within the growing status [16]. With the aid of respirometric analyses dosing (+)-limonene in amounts optimized for biotransformation for *P. sapidus* [17] was found to cut off

Table 2
OUR of a culture of *Pleurotus sapidus*, daily addition of (+)-valencene (4 mM) started after 24 h of cultivation and without dosage

Time (h)	OUR ($\mu g\,(l\,s)^{-1}$)	
	Daily dosage	Without dosage
48	1.5	7.6
72	1.4	7.6
96	5.7	6.3
120	7.4	7.5

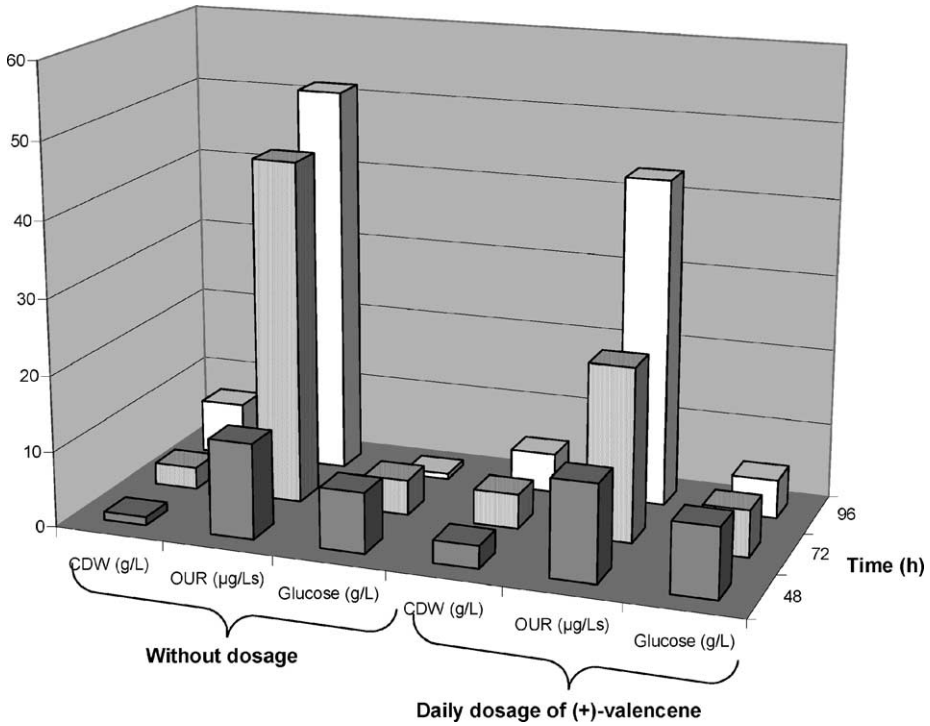

Fig. 3. OUR, CDW, and glucose concentration of a culture of *Chaetomium globosum*, with and without a daily addition of (+)-valencene (0.8 mM).

oxygen consumption completely in various states of growth. The same results were found for *C. globosum*.

Long-term toxicity by means of a daily dosage of (+)-valencene was observed in both cases, but the effect is not so obvious as that caused by a supplement of (+)-limonene. Especially the results of *P. sapidus* may permit the assumption that an adaptation to (+)-valencene is taking place. *C. globosum* remains active in the presence of (+)-valencene but metabolism is reduced.

These results led to further investigations on acute toxicity in contrast to long-term toxicity. To determine acute toxicity, *P. sapidus* and *C. globosum* were supplemented with terpenes and OUR was measured after 120 s. (+)-Valencene does not show acute toxicity either to *P. sapidus* or to *C. globosum*. *P. sapidus* does not show any decline in OUR after a direct addition of (+)-limonene (data not shown). The acute toxicity of (+)-limonene to *C. globosum* is proportional to the concentration of terpene supplemented (Fig. 4).

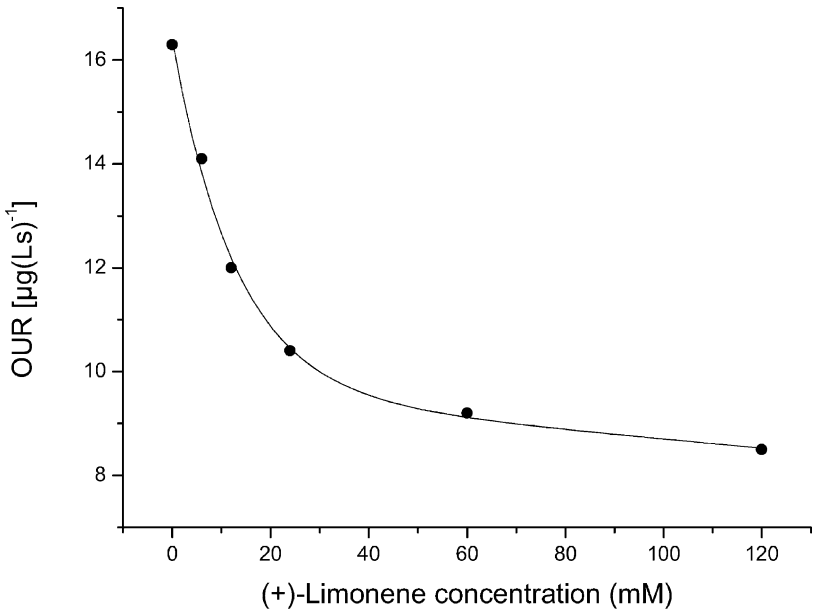

Fig. 4. Acute toxicity of (+)-limonene to *Chaetomium globosum* in various concentrations (0–120 mM).

The OUR decreases with an increasing (+)-limonene concentration, when concentration exceeds 30 mM the OUR remains nearly constant at a low level. These investigations were conducted about 48 h after inoculation of the cultures. Acute toxicity is dependent on the state of a batch culture as the metabolism passes through different activity states (e.g. growth phase, ageing phase). In terms of acute toxicity, the sesquiterpene (+)-valencene does not have any acute toxic effects either on *P. sapidus* or *C. globosum* which may be explained by the size of the precursor or its chemical structure, which may hamper fast incorporation into the membranes.

(+)-Limonene as a monoterpene shows a concentration-correlated acute toxicity to *C. globosum*, the higher the concentration the lower the OUR in the sample. In the case of *P. sapidus*, a significant change in OUR after a dose of (+)-limonene cannot be observed, which may be due to slower metabolism compared to *C. globosum*. The incubation time of 120 s and the sampling time of 900 s may be too short to detect any differences in metabolism. One disadvantage of the measuring principle used to control oxyfunctionalizations by fungi may be that the measured parameter (OUR) is made up of two components: oxygen consumed for the metabolism of the fungi and oxygen required for the biotransformation [18]. Lipophilic toxic substances are often converted into higher water soluble compounds as this enables the microorganisms to get rid of the substances, which have penetrated and which interfere with the membrane system. In consideration of the amount of oxygen required for the biotransformation compared to the oxygen demand for fungal metabolism, the oxygen incorporated can be neglected (in the case of *P. sapidus* and *C. globosum*).

3.3. Solvent screening

With regard to the process design of biotransformations of hydrophobic substances the use of organic solvents to improve the supply of precursors and to recover the products should be tested. Therefore, the toxicity of solvents concerning the activity of *C. globosum* was checked by screening. While ethanol, *n*-decan-1-ol, isooctane, and dimethylformamide inhibited the OUR of *C. globosum* at all times, miglyol 812 and oleyl alcohol did not cause a decrease in activity compared to the blank value. In the case of ethylene glycol and dimethyl sulphoxide, the toxicity was correlated with the applied concentration and exposure time. In Fig. 5, the OUR dependence of *C. globosum* on ethylene glycol concentration after 48 h is shown. Even though the oxygen consumption curves are not linear, a qualitative analysis of the curves is possible. It was found that the higher the concentration of ethylene glycol, the lower the oxygen consumption.

Miglyol 812 and oleyl alcohol were selected by solvent screening to act as the extraction phase in a two-phase cultivation, because a toxic effect could not be found for concentrations from 5 to 30%. The appropriateness of miglyol 812 and oleyl alcohol for application in biotransformations in order to enhance the availability of precursors and to extract products has to be examined in further experiments.

3.4. Fermentation monitoring

With the experimental setup of the OUR measurements monitoring of the activity of a fungal batch during a fermentation of *C. globosum* was carried out. Fig. 6 shows the OUR during fermentation before and after a dosage of

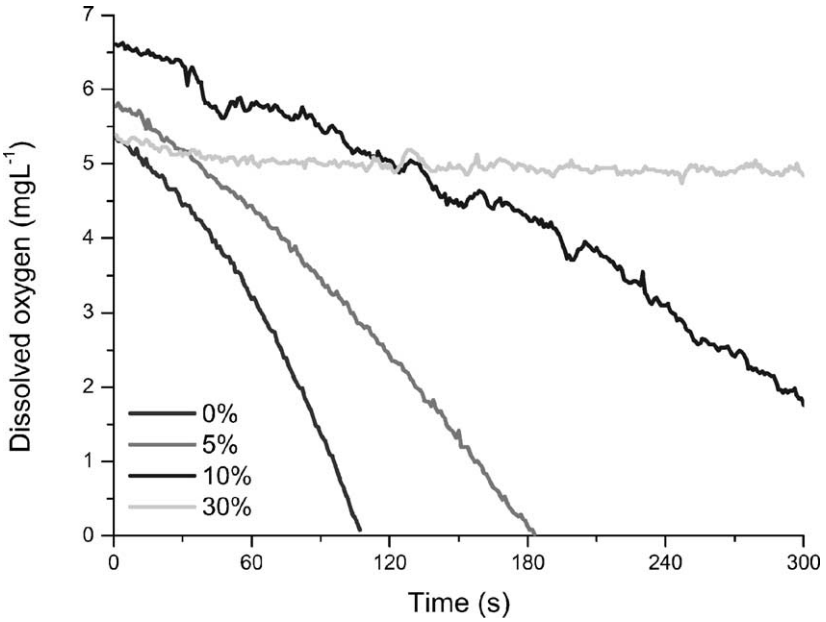

Fig. 5. Oxygen concentration curves of *Chaetomium globosum* (taken after 48 h of cultivation with different concentrations of ethylene glycol).

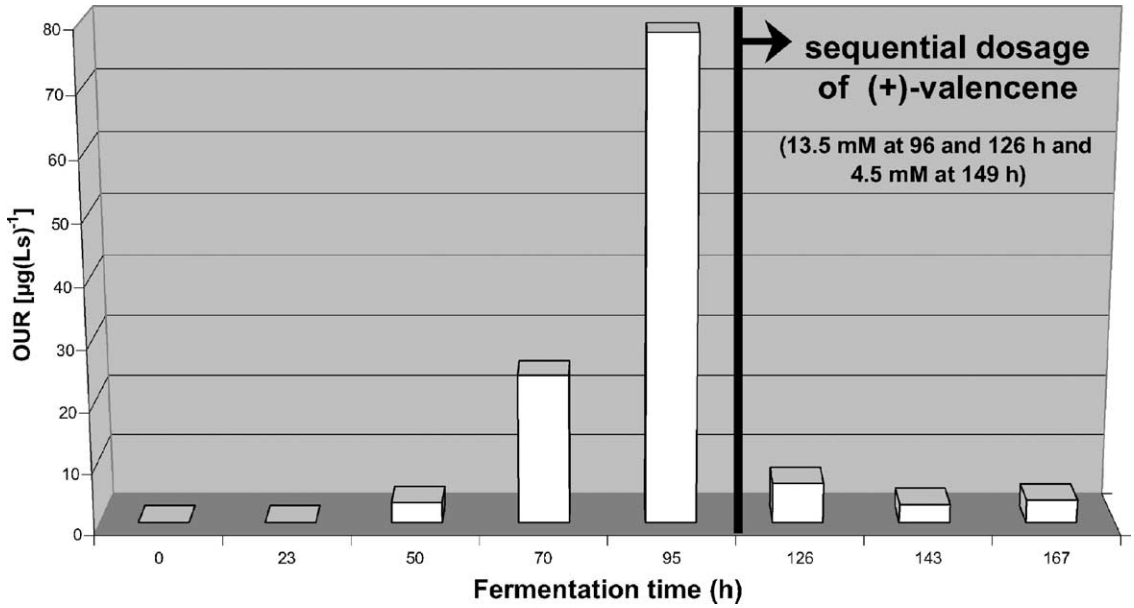

Fig. 6. Fermentation monitoring of *Chaetomium globosum* (1.75 l, 25 °C, 250 rpm, 9 l h^{-1} aeration, and direct supplementation of (+)-valencene at 96, 126, and 149 h) by external OUR measurement (5 ml samples).

(+)-valencene. After 23 h there is no significant activity as oxygen consumption is zero, *C. globosum* has a very low metabolism and needs about 48 h to adapt to the fermentation conditions. From 50 to 95 h, the OUR increased due to metabolism including mycelia augmentation, degradation of glucose, and an increase in CDW could be observed (data not shown). After a dose of terpene precursor, the OUR of the batch is lower as (+)-valencene has a toxic effect on *C. globosum* which results in less oxygen consumption (Fig. 6). The rate of glucose degradation is also lower compared to the rate without a dose of (+)-valencene.

With the newly devised activity measurement it was possible to monitor the activity of a batch (1.5 l) of *C. globosum* on the one hand during growing and on the other hand in the presence of (+)-valencene. Before an addition of (+)-valencene an increasing OUR caused by the growing fungus could be detected, while the OUR was lower in the presence of terpene.

As oxygen electrodes are commonly used in fermentation processes to control the oxygen concentration of the batch, it might be assumed that the implementation of the respirometric activity measurements by means of OUR is easily possible. Unfortunately fungal mycelia grow on synthetic material, such as silicone, rubber gaskets, etc. Fermentations with *P. sapidus* and *C. globosum* showed that the mycelia sprouted into the silicone membranes of conventional oxygen electrodes, so that the measurement of dissolved oxygen was either impossible or not representative due to the settlement of fungi on the electrode. Process monitoring of the activity of the batch in a small scale bioreactor (1.5 l) by means of offline measurements of the OUR with the implemented system can show a correlation between the OUR and the activity of the batch. The results of the flask experiments (as concerns toxicity) can be transferred to fermentations, the main problems lie in representative sampling, which is only possible with homogeneous cultures. As pelleting fungi form different shapes and sizes of pellets or mycelia within a 250 h fermentation and even gas phase mycelia can be observed, an online sensor is required for adequate determination of feeding strategies based on activity measurement.

An oxygen sensor with an inert surface to avoid swelling with substrates and to avoid ongrowing fungi is needed. In connection with these demands, the recently developed fibre-optic oxygen meters [19] are a promising tool as the sensors can be coated with various materials. Experiments are currently being conducted in our group to establish the suitability of fibre-optic oxygen meters for terpene transforming fungi, to assess a feeding strategy and to optimize the yield of natural flavors.

Acknowledgements

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