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Cultivation of shear stress sensitive microorganisms in disposable bag reactor systems

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ABSTRACT

Technical scale (≥5 l) cultivations of shear stress sensitive microorganisms are often difficult to perform, as common bioreactors are usually designed to maximize the oxygen input into the culture medium. This is achieved by mechanical stirrers, causing high shear stress. Examples for shear stress sensitive microorganisms, for which no specific cultivation systems exist, are many anaerobic bacteria and fungi, such as basidiomycetes. In this work a disposable bag bioreactor developed for cultivation of mammalian cells was investigated to evaluate its potential to cultivate shear stress sensitive anaerobic *Eubacterium ramulus* and shear stress sensitive basidiomycetes *Flammulina velutipes* and *Pleurotus sapidus*. All cultivations were compared with conventional stainless steel stirred tank reactors (STR) cultivations. Good growth of all investigated microorganisms cultivated in the bag reactor was found. *E. ramulus* showed growth rates of $\mu = 0.56\ h^{-1}$ (bag) and $\mu = 0.53\ h^{-1}$ (STR). Differences concerning morphology, enzymatic activities and growth in fungal cultivations were observed. In the bag reactor growth in form of small, independent pellets was observed while STR cultivations showed intense aggregation. *F. velutipes* reached higher biomass concentrations ($21.2\ g\ l^{-1}$ DCW vs. $16.8\ g\ l^{-1}$ DCW) and up to 2-fold higher peptidolytic activities in comparison to cell cultivation in stirred tank reactors.

1. Introduction

Microorganisms show very different reactions when they are exposed to shear stress. Some of them are not affected by shear stress at all, like most *Escherichia coli* strains, while others show very intensive reactions, like much slower growth rates or possibly even death of the organism. This is mainly caused by fluid-mechanical stress from bursting gas bubbles or direct shear stress by stirring devices (Chisti, 2000; Duddridge et al., 1982; Garcia-Briones and Chalmers, 1992; Murhammer and Goochee, 1990). Shear stress affects cells in different ways and shows different effects depending on the type of cells. Shear stress shows an impact on the disruption of cell membranes, the release of intracellular compounds, alternation of aggregate size, disruption of aggregates and hyphae and changes in the cell wall composition (dosSantos et al., 1997; Esfandabadi et al., 2012; Hooker et al., 1990; Suresh et al., 2009; Takeda et al., 1994; Wucherpfennig et al., 2010).

Disposable bag reactors were introduced in 1996 by WAVE biotech in Switzerland. Since then, they got extensively used for the cultivation of mammalian, plant and insect cells (Eibl et al., 2009b, 2010). Online analytics in the medium is limited. However, measuring of pO_2 and pH is possible, which is achieved by pre-calibrated, fluorescence based optical patches (Glindkamp et al., 2009). The oxygen input into the disposable bag reactor and the medium is achieved by generation of a rolling wave in the reactor. Therefore the bag is fixed on a rocking-motion platform inducing a medium wave which transports the oxygen from the headspace into the culture broth. The intensity of the wave can be regulated by the angle of the platform and the rocking rate (rocks per minute). To ensure that the wave can develop properly, the maximum filling volume (working volume) of the bag is half the total volume (e.g. in this study a 20 l bag was filled with 10 l liquid maximum). The bags are delivered presterilized (γ-ray) by the manufacturer and are filled through a sterile filter, no autoclaving is needed. A disadvantage of these wave systems are the lower k_La-values compared to stirred tank reactors (Eibl et al., 2009a). But due to this wave-induced oxygen entry, where no mechanical stirring device is used, the system should be appropriate also for cultivations of other shear stress sensitive microorganisms beyond the mammalian cell culture. Different shear stress sensitive organisms were chosen for the evaluation of the system: strict anaerobic bacteria and fungi (resp. basidiomycetes).

Many anaerobic bacteria belong to these shear stress sensitive microorganisms. Furthermore anaerobic cultivation generally

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requires special techniques. Mostly used is the Hungate technique or modifications of this technique (Hungate and Macy, 1973; Miller and Wolin, 1974). As soon as the culture volumes increase above shake flask scale, no commonly accepted cultivation systems exist. Usually systems for aerobic cultivations are modified and adapted, leading to long downtimes for upstream unit operations as sterilization and qualification of the systems.

Eubacterium ramulus is a gram positive, non-sporulating, non-flagellated, obligate anaerobically growing, rod-shaped and chain building bacterium. Originated from the human intestinal tract, it was first described in 1976 and isolated from human faeces in 1999 (Moore et al., 1976; Schneider, 1999). When cultivated in small anaerobic flasks it shows a very intensive reaction to shear stress which manifests itself in a strong inhibition of growth (Schmidt, 2011). Beside the strictly oxygen free conditions, it is also necessary to prevent shear stress. *E. ramulus* shows potentials of transforming flavonoids originated from plant material that could be economically interesting, e.g. as antioxidants (Graf et al., 2005; Schneider and Blaut, 2000).

Other microorganisms showing sensitive reactions to shear stress are various basidiomycetes. In comparison to ascomycetes, yeast or bacteria they show low growth rates. Thus, cultivations run up several days. Basidiomycetes show large potential for the production of technical enzymes for the white biotechnology (Lindequist et al., 2005). From particular interest are peptidases and esterases which can be used e.g. in the food, leather and detergent industry (Rao et al., 1998). Furthermore many basidiomycetes show interesting antibacterial and antioxidant effects (Karaman et al., 2010; Suay et al., 2000).

Their morphology during the growth often depends on the culture conditions. Aggregation to pellets and growing in form of mycelium as well as intermediate morphologies were observed. One factor to influence the morphology as well as the growth rate could be the mechanical stress level the fungi are exposed to (Kelly et al., 2006). The morphology (e.g. aggregation and pellet formation alter the surface area exposed to the medium) is extremely important in regard to the proteins produced and their activity. Thus, different morphologies should be achieved during the cultivation process (Cho et al., 2006; Kelly et al., 2006; Krull et al., 2013; Wang et al., 2005; Wucherpfennig et al., 2010). This can influence the supply of the cells with nutrients and oxygen (Cui et al., 1998). Therefore it is interesting to investigate different bioreactors for their impact on the growth behaviour, resulting morphologies and the influence on the enzymatic activities. Regarding the larger-scale cultivation of basidiomycetes, usually bioreactors were used that were optimized for bacteria and yeasts.

In this study it is demonstrated that a disposable bag bioreactor (BIOSTAT Cultibag RM optical, Sartorius Stedim Biotech, Germany) is useful for the cultivation of shear stress sensitive microorganisms. First, strict anaerobic, shear stress sensitive *E. ramulus* was cultivated in batch and fed-batch mode and compared to STR cultivation. To proof the suitability of the system for other shear stress sensitive microorganisms, cultivations of the basidiomycetes *Pleurotus sapidus* and *Flammulina velutipes* were carried out and compared to common cultivations in stirred tank reactors regarding the growth behaviour, the morphology and the enzymatic activities (peptidolytic, respectively esterolytic activity).

2. Materials and methods

2.1. Anaerobic cultivations

All media and solutions were, unless otherwise stated, prepared with fully desalted water (Arium 611, Sartorius Stedim Biotech, Germany). Chemicals were purchased from Carl Roth GmbH & Co KG (Germany), Sigma–Aldrich (Germany), Fluka Chemie AG (Switzerland) or Merck KGaA (Germany) in purum p.a. purity grade or higher.

The bags (20 l Cultibag optical, Sartorius Stedim Biotech, Germany) were prepared anaerobic by filling them to capacity first with an oxygen-free gas mixture (N_2/CO_2 80:20, Linde AG, Germany). After that the gas was depressed mechanically out. This procedure was repeated until dissolved oxygen value was below 1%. Then 9.2 l anoxic medium was filled into the bag through a sterile filter (0.2 μm). Anoxic preparation was performed by using a modified Hungate technique (Miller and Wolin, 1974).

The STR (BIOSTATC, Sartorius Stedim Biotech, Germany) was prepared anaerobic by autoclaving (121 °C for 21 min) and followed by N_2 gassing (1 l min^{-1}) for the whole cultivation time. The working volume was also 9.2 l.

Cultivations with *E. ramulus* (DSM 16296) were performed in media containing meat peptone 9 g l^{-1}, proteose peptone 1 g l^{-1}, meat extract 3 g l^{-1}, yeast extract 4 g l^{-1}, sodium chloride 3 g l^{-1}, tween 80 0.5 ml l^{-1}, cystine 0.25 g l^{-1}, disodiumhydrogenphosphate 0.25 g l^{-1}, cysteine*HCl 0.25 g l^{-1}, glucose 12 g l^{-1}, and 2.5 ml l^{-1} salt solution. Salt solution concentrations were: 160 mM $MgSO_4$*7 H_2O, 7 mM $FeSO_4$*7 H_2O, 34 mM NaCl, 7 mM $MnSO_4$*2 H_2O. 3 ml l^{-1} resazurin solution was added as oxygen indicator. Cultivation parameters for the bag bioreactor were a platform angle of 3.9°, 8 rpm, 37 °C, pH control at 7 (1 M HCl and 4 M NaOH) and no gassing except for the very beginning when the bag was filled to capacity with N_2/CO_2 (80:20) after inoculation. Parameters for the STR cultivation were 37 °C, stirrer speed between 18 and 25 rpm (3-blade marine-type impeller) and pH control at 7 (1 M HCl and 4 M NaOH). Precultures grew overnight in 1 l pressure-resistant bottles filled with 400 ml anoxic media, inoculation with 800 ml preculture was performed as described above.

2.2. Cultivation of fungi

For cultivation of the basidiomycetes smaller bags (10 l Cultibag optical, Sartorius Stedim Biotech, Germany) were used to compare to STR cultivations of 2.5 l and 5 l. The bags were filled with 4.5 l medium through sterile filter (0.2 μm). Cultivation parameters were a temperature of 24 °C, gassing 2 l min^{-1} with air, platform angle of 9.3° and a pO_2-cascade regulation at 50% with a minimum of 30 rpm (max. 42 rpm). To prevent off gas filter from being blocked by moisture and biomass the bags were modified by lengthening the hose and adding a flask filled with a porous material (to ease condensation) between bag and filter.

STR cultivations were performed in a 5 l reactor (KG5000, Medorex AG, Germany) with marine-type stirrer and a 5 l reactor (ISF100, Infors HT AG, Switzerland) with inclined blade stirrer. Reactors were autoclaved for 30 min at 121 °C. Medium was filled in through sterile filter (0.2 μm).

For *P. sapidus* (DMSZ 8266) cultivations the temperature was 24 °C, gassing was 2 l min^{-1} with air. Agitation in ISF100 STR was 80 rpm and in KG5000 STR 100 rpm.

F. velutipes (DSMZ 1658) was cultivated at 24 °C, agitation 120 rpm and gassing of 5 l min^{-1} with air.

Unless otherwise stated, SNL media (Sprecher, 1959) adjusted to pH 6 containing glucose 30 g l^{-1}, asparagine monohydrate 4.5 g l^{-1}, yeast extract 3 g l^{-1}, KH_2PO_4 1.5 g l^{-1}, $MgSO_4$ 0.5 g l^{-1} and 1 ml l^{-1} trace element solution, was used. Trace element solution was prepared using $CuSO_4 \times 5$ H_2O 5 mg l^{-1}, $FeCl_3 \times 6$ H_2O 80 mg l^{-1}, $MnSO_4 \times 1$ H_2O 30 mg l^{-1}, $ZnSO_4 \times 7$ H_2O 90 mg l^{-1} and EDTA (Titriplex III) 400 mg l^{-1}. The added amount of antifoam is mentioned in the text.

F. velutipes precultures were grown in 250 ml SNL media using a 500 ml shaker flask for 7 d at 24 °C and 150 rpm. Then the precultures were homogenized and 500 ml added into the reactor.

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