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# Validation of the transferability of membrane-based fed-batch shake flask cultivations to stirred-tank reactor using three different protease producing *Bacillus* strains

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**Most industrial fermentation processes are operated in fed-batch mode to overcome catabolite repression, undesired by-product formation and oxygen limitation. To maintain comparable process conditions during screening of optimal production strains, the implementation of a fed-batch mode at small scale is crucial. In this study, three different protease producing *Bacillus* species, *Bacillus aeolius*, *B. licheniformis* and *B. pumilus*, were cultivated using the previously described membrane-based fed-batch shake flasks. Under carbon-limited conditions, catabolite repression was avoided, so that proteases were produced in all strains. Protease yields of *B. aeolius* and *B. licheniformis* increased 1.5-fold relative to batch cultivations. To validate process scalability between shake flasks and stirred tank reactors, membrane-based fed-batch shake flask cultivations were transferred to laboratory-scale stirred tank reactors with equal feeding rates. Despite inevitable differences between the scales such as pH control, feed supply and feed start, comparable results were achieved. Oxygen transfer rates of *B. licheniformis* and *B. pumilus* measured with the respiration activity monitoring system (RAMOS) in shake flasks and in stirred tank reactor with an off-gas analyzer were almost identical in both cultivation systems. The protease activities referring to the total consumed glucose were also mostly comparable. A slight decrease from shake flask to stirred tank reactor could be observed, which is presumably due to differences in pH control. This study successfully demonstrates the transferability of membrane-based fed-batch shake flask cultivations to laboratory-scale stirred tank reactors.**



[**Key words:** Fed-batch; Membrane-based fed-batch shake flask; Respiration activity monitoring system; Scale-up; *Bacillus*; Protease]

Most biotechnological large-scale production processes rely on a fed-batch cultivation strategy with carbon representing the limiting nutrient (1). However, screening and characterization studies are generally conducted in batch mode (2–4). Although this is associated with less technical complexity, the established batch screening set up can significantly affect strain selection (5). Strains that would show high productivities under production conditions may not perform well in batch screenings. Selecting a suboptimal strain during screening implies productivity losses that may not be compensated during process optimization. To ensure reliable results, small-scale screening systems should ideally represent the commercial production environment.

In recent years various fed-batch screening systems have been developed and are now commercially available. Panula-Perälä et al. (6) presented the small-scale EnBase system (BioSilta Oy, Oulu, Finland) based on the enzymatic degradation of the complex carbon source starch, continually releasing glucose into the culture medium. Bearing a similar work principle, Hemmerich et al. (7) developed the Feed-in-Time system (m2p-labs, Baesweiler, Germany), a convenient fed-batch cultivation media series, which provides enzymes as part of the media mix. An enzyme independent release system was introduced to the market as the so-called FeedBeads and FeedPlates (Kuhner Shaker, Birsfelden, Switzerland). The glucose embedded in a silicone matrix is continuously released upon contact with aqueous solutions. Bähr et al. (8) established a membrane-based fed-batch shake flask system. This system presents a rather simple and very adaptable operating platform. Different membrane types with various characteristics are available. Membrane-based fed-batch shake flasks enable the feeding of various substances exceeding the sole release of a single carbon source (8). The set-up and operating principle of the membrane-based fed-batch shake flask is illustrated in Fig. 1A.

The limiting nutrient, such as glucose, is provided as a highly concentrated feed solution in a screw-on glass reservoir installed in the center of the shake flask. The reservoir has a flexible tube extension at the bottom, which contains a diffusion tip equipped with, e.g., a cellulose membrane. While the culture is shaken, the diffusion tip rotates in-phase with the bulk liquid so that the membrane is constantly in contact with the culture broth. The concentration gradient between the feed solution and the culture broth triggers a diffusion-driven nutrient release into the culture broth. While small nutrient molecules can pass the membrane in

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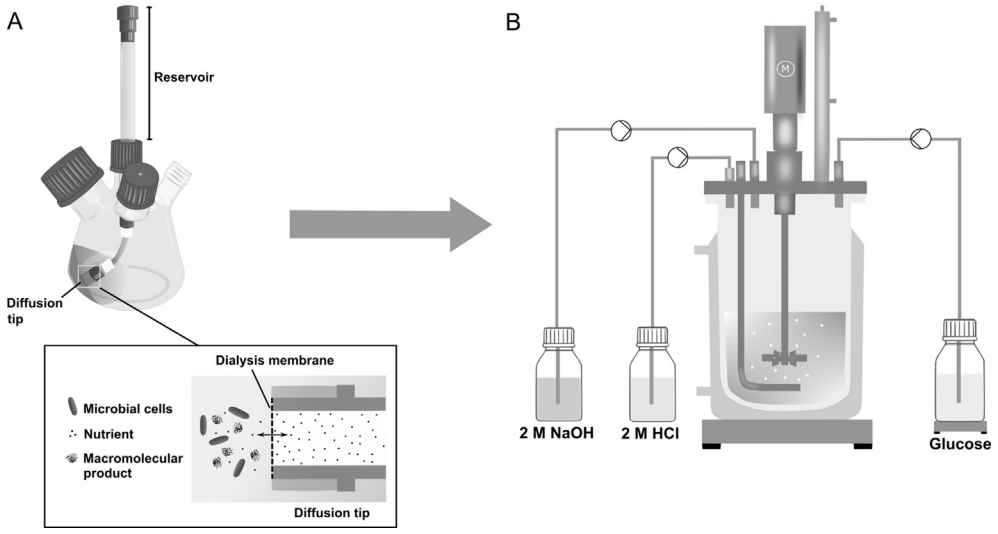


FIG. 1. Applied fed-batch cultivation systems. Fed-batch cultivations in small-scale were performed with (A) membrane-based fed-batch RAMOS shake flasks. The system contains a feed reservoir in the center of the flask. A diffusion tip is connected via a flexible tube, enabling an in-phase rotation with the bulk liquid. A cellulose membrane separates reservoir feed solution and culture medium. While small molecules like the limiting nutrient pass through the cellulose membrane (molecular cut-off of 10–20 kDa) driven by a concentration gradient, microbial cells and secreted macromolecular products are kept from entering the reservoir. This small scale process was transferred to (B) a 3 L stirred tank reactor using the same glucose release rates obtained in membrane-based fed-batch shake flask experiments.

both directions, cells as well as secreted macromolecular products like proteases are unable to enter the reservoir due to the molecular cut-off of 10–20 kDa. Once the nutrient becomes limiting, the culture turns into the fed-batch phase with the metabolic activity restricted by the nutrient flow across the membrane.

This membrane-based fed-batch shake flask system was combined with the respiration activity monitoring system (RAMOS) (9,10) to monitor oxygen and carbon dioxide transfer rates online, which provide information on various metabolic phenomena as well as limitations of oxygen or secondary substrates. Numerous successful applications of the RAMOS device for batch cultivations have already been published such as the detection of protein production (11,12), elucidation of auxotrophies (13), testing of yeast extract quality (14), evaluation of cellulose digestibility (15) and determination of polymer biocompatibility (16). Membrane-based fed-batch shake flasks connected with RAMOS devices allow an easy adjustment of feeding rates, feeding of various substrates, parallel feeding of substrates and pH stabilizing agents (8,17–19). The adjustment of various feeding rates can easily be achieved by adapting the feed concentration within the reservoir, as well as by varying the diffusion area of the membrane on the diffusion tip. Increasing one of these parameters leads to an increase of the feeding rate. Moreover, various types of membranes with different permeability can be applied. Philip et al. (17) described these correlations in detail. In addition, a high degree of parallelization as well as feeding without external pumps and electronic equipment becomes possible. However, the applicability as a new screening tool is only reasonable, if the transferability to laboratory and larger scale stirred tank reactors is given. Since there are important differences of operating conditions between shake flasks and stirred tank reactors, transferability cannot be simply assumed (20).

Due to simplicity, buffers are traditionally used for pH stabilization in small-scale cultivations (21–23). In industrial production processes, these agents are usually omitted due to their cost and increase in osmotic pressure (24). Instead, the pH is controlled by titration. Moreover, the selection of unsuitable operating conditions during shake flask cultivations often cause oxygen limitations (25), which are prevented in stirred tank reactors by automatic control of dissolved oxygen tension (DOT) (1). All these differences can lead to drastically diverging physiological conditions for the cultured microorganisms between shake flask and fermenter scale.

Therefore, the aim of this study was to scale up membrane-based fed-batch shake flask cultivations to laboratory-scale stirred tank reactors to evaluate the transferability (Fig. 1). For this purpose, three different protease producing *Bacillus* species, *Bacillus aeolius*, *B. licheniformis* and *B. pumilus*, were chosen as model organisms. Most large-scale industrial protease production processes with *Bacilli* are performed in fed-batch operation mode to avoid a repression of protease production by glucose (26). Consequently, a glucose feed ensuring carbon-limited fed-batch conditions was selected in this study. In addition, it was investigated whether or not membrane-based fed-batch shake flasks are able to mimic fed-batch production processes.

## MATERIALS AND METHODS

**Microbial strains** The protease producing strains *B. aeolius* (DSM 15084) and *B. pumilus* (DSM 18097) were used. *B. licheniformis* H215 was provided by Henkel AG & Co. KGaA (Düsseldorf, Germany). All strains carried the same plasmid, which contained a constitutive P43 promoter (27,28), a gene encoding a protease and a kanamycin resistance. A map of the used plasmid is shown in Fig. S1. In *B. licheniformis* H215, genes for poly(γ-glutamic acid) (γ-PGA) synthesis were deleted.

**Media preparation** For cryoculture preparation, strains were cultivated in terrific broth (TB) medium with yeast extract 24 g/L (Roth, 275225976), tryptone 12 g/L (Roth, 395234974), glycerol 5 g/L, $K_2HPO_4$ 12.54 g/L, $KH_2PO_4$ 2.31 g/L, and kanamycin 0.05 g/L. After harvesting during the exponential growth phase, the cells were stored in 2 mL vials with a final concentration of 100 g/L glycerol at −80°C.

Cells from the glycerol stock were plated on lysogeny broth (LB) agar plates (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L, agar 15 g/L, and kanamycin 0.05 g/L) supplemented with 10 g/L skim milk (Merck, skim milk powder for microbiology) and incubated at 37°C over night. For preculture, a complex medium (sucrose 4.5 g/L, yeast extract 7.4 g/L, plant protein 25 g/L, $K_2HPO_4$ 17.4 g/L, $KH_2PO_4$ 13.6 g/L, NaCl 5.6 g/L, and kanamycin 0.05 g/L) was inoculated with a single colony from the LB agar plate. After reaching the exponential growth phase, cells were harvested and stored with a final concentration of 100 g/L glycerol at −80°C. The main culture was inoculated with 2.5 % (v/v) defrosted preculture.

The standard V3 mineral medium (29) contained glucose 20 g/L, $(NH_4)_2SO_4$ 15 g/L, $CaCl_2 \cdot 2H_2O$ 0.026 g/L, $FeSO_4 \cdot 7H_2O$ 0.05 g/L, $MgSO_4 \cdot 7H_2O$ 1.01 g/L, $MnCl_2 \cdot 4H_2O$ 0.05 g/L, trace element stock solution 5 mL/L, 3-(*N*-morpholino) propanesulfonic (MOPS) acid 41.85 g/L (0.2 M), $K_2HPO_4$ 3.4 g/L and kanamycin

0.05 g/L. Every component was added separately from sterile stock solutions in the order of appearance. The pH value of the MOPS stock solution was set to 8 with 8 M NaOH. Before adding $K_2HPO_4$, the pH was checked and, if necessary readjusted to pH 8 using 8 M NaOH. $K_2HPO_4$ was added just before inoculation to prevent precipitation. The trace element stock solution contained the following components: $CoCl_2 \cdot 6H_2O$ 0.53 g/L, $ZnCl_2$ 0.26 g/L, $H_3BO_3$ 0.01 g/L, $NiSO_4 \cdot 6H_2O$ 0.66 g/L, $CuSO_4 \cdot 5H_2O$ 0.31 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.65 g/L. This stock solution was diluted 1:5 with distilled water to obtain a 200× stock solution, sterile filtered and used for media preparation. Moreover, the stock solutions of $FeSO_4 \cdot 7H_2O$ and kanamycin were sterile filtered and stored in aliquots at −20°C. The MOPS acid stock solution was also sterile filtered. For all sterile filtrations a 0.2 μm cut-off filter (VWR International, 0.2 μm PES membrane) was used. All other stock solutions were separately heat sterilized at 121°C and 1 bar overpressure for 20 min. In all cultivations with *B. pumilus* the V3 medium was supplemented with 15 g/L yeast extract. For cultivations with pH control by titration, the MOPS buffer was not added to the V3 mineral medium.

Glucose was not provided in the V3 mineral medium of all fed-batch shake flask cultivations. However, the reservoir contained 200 g/L glucose. The assembly and preparation of membrane-based fed-batch shake flasks was based on the description by Habicher et al. (19).

**Shake flask cultivation** All shake flask cultivations were carried out in 250 mL flasks at 37°C with a shaking diameter of 50 mm and a shaking frequency of 350 rpm. The filling volume for batch cultivations was 10 mL and for membrane-based fed-batch shake flask cultivations 12 mL.

In all precultures and shake flask main cultures, the respiration activity was monitored by an in-house manufactured RAMOS. Eight 250 mL flasks are equipped with an oxygen partial pressure sensor and differential pressure sensors to determine the oxygen transfer rate (OTR), the carbon dioxide rate and the respiratory quotient (9,10). Commercial versions of the RAMOS device can be acquired from Kuhner AG (Birsfelden, Switzerland) or HiTec Zang GmbH (Herzogenrath, Germany).

**Fermentation in stirred tank reactor** Fed-batch cultivations were carried out in 3 L stirred tank reactors (Sartorius 2L Biostat Bplus, Sartorius, Goettingen, Germany) with one 6-blade Rushton impeller (5.3 cm diameter) and a working volume of 1 L.

For all cultivations, temperature was controlled at 37°C and an aeration rate of 1 vvm was chosen. To maintain the DOT ≥ 30 %, the stirring rate was automatically controlled between 500 and 2000 rpm. The pH was regulated at pH 7.2 by adding 2 M NaOH and 2 M HCl. To prevent foaming, 1 mL of the antifoam agent Plurafac LF 1300 (BASF, Ludwigshafen, Germany) was added when required.

The exhaust gas composition was monitored by exhaust gas analysis (DASGIP GA4, Eppendorf, Wesseling-Berzdorf, Germany) to calculate the OTR. For fed-batch cultivations, a glucose feeding rate of 0.64 g/L/h was accomplished with peristaltic pumps (ISM830, Ismatec, Zürich, Switzerland). The concentration of the glucose feed stock was 400 g/L. The specific feeding rate was selected to replicate the glucose release from membrane-based fed-batch shake flask in the stirred tank reactor. This glucose release rate was calculated as described by Philip et al. (17). Glucose feeding started after complete consumption of the 8.5 g/L glucose initially provided.

**Sample analytics** The optical density (OD) of the culture broth was measured at a wavelength of 600 nm in standard 1 cm cuvettes in a photometer (Genesys 20, Thermo Scientific, Bonn, Germany). To keep $OD_{600}$ in the linear range of the photometer, samples were diluted with 0.9 % NaCl solution if exceeding an OD of 0.3.

To determine concentrations of glucose, glycerol and acetate, samples were analyzed by HPLC (Ultimate 3000, Dionex, Sunnyvale, CA, USA) equipped with an organic acid-resin column (250 × 8 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany) and a Shodex RI-101 refractometer (Showa Denko Europe, Munich, Germany). The column was eluted with the mobile phase 5 mM $H_2SO_4$ at 60°C at a flow rate of 0.8 mL/min.

For quantitative measurement of the protease activity an enzymatic assay developed by DelMar et al. (30) was used. The detection of enzymatic activity is based on the release of the chromophore para-nitroaniline (pNA) from a synthetic peptidic substrate suc-L-Ala-L-Ala-L-Pro-L-Phe-pNA (AAPF-pNA). Proteases cleave AAPF-pNA to release AAPF and pNA, which leads to an increase of absorption. The experimental procedure was already described by Meissner et al. (31). The assay was performed in a microplate reader (Synergy 4, BioTek, Winooski, VT, USA) with transparent 96-well microtiter plates (Rotilabo microtest plates, flat bottom) at a wavelength of 405 nm. The substrate stock solution was prepared by dissolving 70 mg/mL suc-AAPF-pNA (Bachem AG, Bubendorf, Switzerland) in anhydrous dimethyl sulfoxide and stored at −20°C. For the assay, the substrate solution was freshly diluted 1:20 with 0.1 M Tris HCl buffer, pH 8.6, 0.1 % (w/v) Brij 35. The protease reaction was started by adding 50 μL substrate solution per well. The assay was conducted at a temperature of 30°C for 15 min. The enzymatic activity was calculated from the change of absorption at 405 nm with the extinction coefficient of pNA at 405 nm (8.48 $cm^2 \mu mol^{-1}$).

In membrane-based fed-batch shake flasks, water fluxes due to differences in osmotic pressure lead to an increase of the concentration of the culture broth. Furthermore, evaporation affects the volume of the culture broth. Therefore, protease activities were always corrected relative to the initial volume of 12 mL (19). To enable fairer comparisons of shake flask and stirred tank reactor cultivations, protease activities were also referred to the total consumed glucose.

## RESULTS AND DISCUSSION

**Batch cultivations** Initially, the three different *Bacillus* strains, *B. aeolius*, *B. licheniformis* and *B. pumilus*, were cultivated in shake flasks with V3 mineral medium (29) in batch operation mode. Fig. 2 shows OTR over cultivation time. The measurements were performed in duplicates and the low variations prove their excellent repeatability in one parallel experiment.

After a short lag phase of 4.5 h, the OTR of the cultivation of *B. aeolius* increases exponentially up to a maximum of 61 mmol/L/h (Fig. 2A). During this exponential growth phase, *B. aeolius* consumes the carbon source glucose (Fig. S2). Following the OTR maximum, a sharp drop indicates the depletion of glucose. This drop is followed by an increase in OTR reaching a second maximum of 40 mmol/L/h after 15.5 h. This secondary peak is caused by growth on glycerol, which was transferred from the defrosted preculture. The diauxic behavior can be explained with energy balances for the carbon sources. Ko and Gross (32) showed for *B. licheniformis* ATCC 9945a that the carbon source glucose resulted in a higher growth rate compared to glycerol. Between 17 h and 25 h a slightly elevated OTR level of around 17 mmol/L/h could be observed, probably caused by the metabolization of the polymeric by-product poly(γ-glutamic acid) (γ-PGA) formed during the exponential growth phase. γ-PGA was described to be an undesired

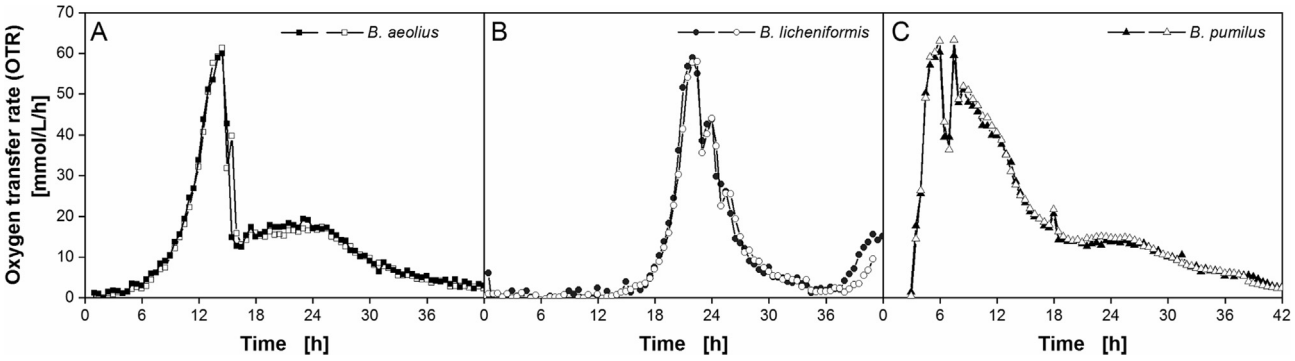


FIG. 2. Batch cultivations of three different *Bacillus* strains in shake flasks. Oxygen transfer rates during the batch cultivation of (A) *B. aeolius* (DSM 15084), (B) *B. licheniformis* H215 and (C) *B. pumilus* (DSM 18097) in V3 mineral medium (20 g/L glucose). *B. pumilus* was grown in V3 medium supplemented with 15 g/L yeast extract. All main cultures were inoculated with 2.5 % (v/v) preculture ($OD_{600} \approx 0.3$) and started with an initial $pH_0 = 8$. For the cultivations biological duplicates are shown. Culture conditions: 250 mL shake flask, 10 mL filling volume, 350 rpm shaking frequency, 50 mm shaking diameter and 37°C temperature.

by-product in industrial protease production processes employing *B. licheniformis* (29,31). Wilming et al. (29) observed a very similar OTR course with a protease producing *B. licheniformis* strain. This strain was analogously cultivated in V3 mineral medium. It produced, reassimilated and degraded γ-PGA. As a result, the viscosity first increased and then decreased. A similar trend of viscosity was visually observable in *B. aeolius* batch cultivations, however, was not quantified in the present study. A protease activity of 352 U/mL and a yield of 17.6 U/mg based on the total consumed glucose were measured in the final samples.

Batch cultivations with *B. licheniformis* exhibited a lag phase of 15.5 h (Fig. 2B). Then, the OTR exponentially increased up to 59 mmol/L/h, then decreased due to the exhaustion of glucose (Fig. 2B). The second peak reaching 44 mmol/L/h at 24 h corresponds again to the consumption of glycerol from the defrosted preculture. After 38 h, a third OTR peak appears to indicate the metabolization of an overflow metabolite formed at an earlier phase of the cultivation. The protease activity was 310 U/mL in the final samples resulting in a protease yield of 15.6 U/mg based on the total consumed glucose.

As shown for *B. pumilus* in previous studies (13), V3 mineral medium did not provide enough nutrients for the strain to exhibit growth. Therefore, 15 g/L yeast extract was provided in the culture medium. The cultivation of *B. pumilus* starts with a steep increase in OTR up to a maximum of 63 mmol/L/h, reached after 6 h (Fig. 2C). Similar to the above described batch cultivations, the first and second peak correspond to the consumption of glucose and glycerol, respectively. Following the glycerol peak, the OTR rises slightly and afterwards decreases slowly. This diauxic behavior appears to be based on the metabolization of amino acids from the supplemented yeast extract and the overflow metabolite acetoin formed during the exponential growth phase (13). Protease activity was detected at 380 U/mL and a protease yield of 19 U/mg was calculated.

All three batch cultivations were oxygen limited for a very short period of time. With the correlation of Meier et al. (33) maximum oxygen transfer capacities ($OTR_{max}$) can be calculated using the osmolality of the medium. According to this correlation, an $OTR_{max}$-value of 62 mmol/L/h ± 5 mmol/L/h was determined for V3 mineral medium and 59.5 mmol/L/h ± 5 mmol/L/h for the medium supplemented with yeast extract. These $OTR_{max}$-values are in the same range as the maximum oxygen transfer rates reached in these experiments. Thus, oxygen limitation is highly probable.

Large-scale processes to produce alkaline proteases are primarily performed in fed-batch mode to avoid undesired by-products, such as acetate or lactate (26). Furthermore, alkaline protease production with *Bacillus* species is sensitive to glucose levels and is only de-repressed when the concentration falls below a specific threshold (34–37). Glucose limitation with a gradual glucose feed can circumvent the repression of alkaline protease expression and extensive overflow metabolism. The supply of a higher total substrate amount is possible, enabling higher final product titers without oxygen limitations. Therefore, *B. aeolius*, *B. licheniformis* as well as *B. pumilus* were cultivated in fed-batch mode.

**Fed-batch cultivations** For membrane-based fed-batch shake flask cultivations, buffered V3 mineral medium was used and no glucose was added to the initial culture broth. Glucose from the reservoir is released into the culture broth due to the high concentration gradient across the membrane and starts as soon as the culture broth gets into contact with the membrane. This mechanistic principle for glucose release is advantageous, because the fed-batch phase automatically follows the batch phase without any intervention. If glucose was added to the initial culture broth for the batch phase, feeding would have to be started manually after glucose depletion. In order to implement the manual feeding start, the shaking of the cultures would have to be interrupted for filling or integrating the reservoir with the feed solution. The interruption of shaking would strongly complicate the experimental procedure and would lead to oxygen limitations possibly affecting growth rates and metabolism (25).

The good reproducibility of the membrane-based fed-batch shake flasks is shown in Fig. S3 for *B. aeolius*. The oxygen transfer rates and the glucose release rates are stoichiometrically related to each other. Therefore, the reproducibility of the OTR also implies the reproducibility of the glucose release rate. These results demonstrate that the whole process is well defined and reproducible. In addition, Habicher et al. (19) also achieved reproducible results of *Bacillus* cultivations using the membrane-based fed-batch shake flasks.

For stirred tank reactors, unbuffered V3 mineral medium initially containing 8.5 g/L glucose was used. The pH was controlled by titration and a glucose feed rate of 0.64 g/L/h was initiated once the initially provided glucose was exhausted. The feed rate of 0.64 g/L/h was calculated based on fed-batch cultivations in shake flasks. The supply of feed solution was realized with an external pump. These stirred tank reactor cultivations allow a verification of the transferability from shake flask to fermenter scale based on the applied glucose feed rate.

Fig. 3 shows a comparison of the two cultivation systems, membrane-based fed-batch shake flasks and stirred tank reactor for all three *Bacillus* strains. Oxygen transfer rates were measured for all cultivations over time (Fig. 3A–C). Offline data were acquired from stirred tank reactor cultivations (Fig. 3D–I).

*B. aeolius* shows an exponential increase in OTR, indicating unlimited growth in the initial batch phase in both, shake flasks and stirred tank reactor (Fig. 3A). For shake flask cultivations, this batch phase is a result of glucose accumulation. Firstly, the glucose release rate is higher than the consumption rate, whereas glucose accumulates in the culture broth. Thus, all substrates are available in excess (8,17,19,23). In contrast, for stirred tank reactor cultivations glucose was initially supplemented to the medium. Despite these differences in operation, the batch phases were almost identical. The three successive OTR peaks developed in cultivations of *B. aeolius* indicate the metabolization of the carbon sources glucose, glycerol and acetate (Fig. 3A, D). This was previously described for batch cultivations (Fig. 2). After depletion of the accumulated glucose, the shake flask cultures automatically turn into fed-batch mode. Under fed-batch conditions, the glucose consumption rate is higher than the glucose release rate (8,17,19,23). For stirred tank reactor cultivations, the feed supply was started by manually switching on the external pump.

A discrepancy in the OTR of shake flasks and stirred tank reactor is observed in the early fed-batch phase after 14 h (Fig. 3A). *B. aeolius* cultivations in the reactor show a steady OTR level at 10 mmol/L/h. In contrast, the OTR fed-batch plateau in shake flasks starts with an elevated level of 25 mmol/L/h. However, the OTR gradually decreases and converges to the OTR levels reached in the stirred tank reactor cultivation. The source of the elevated OTR plateau is yet to be identified. It seems reasonable to assume that it is related to γ-PGA formation as already presumed for batch cultivations (Fig. 2A). In contrast to the pH controlled stirred tank reactor cultivation, V3 mineral medium containing MOPS buffer without pH titration showed similar elevated OTR values in stirred tank reactors at the beginning of the fed-batch phase (data not shown). Therefore, the transition from buffered to a pH regulated system seems to be the decisive factor leading to a difference in the OTR profile. Adding buffers to a medium increases its osmolality. Presumably, this difference in osmolality causes the differences in OTR between the pH controlled and buffered system. Wei et al. (38)

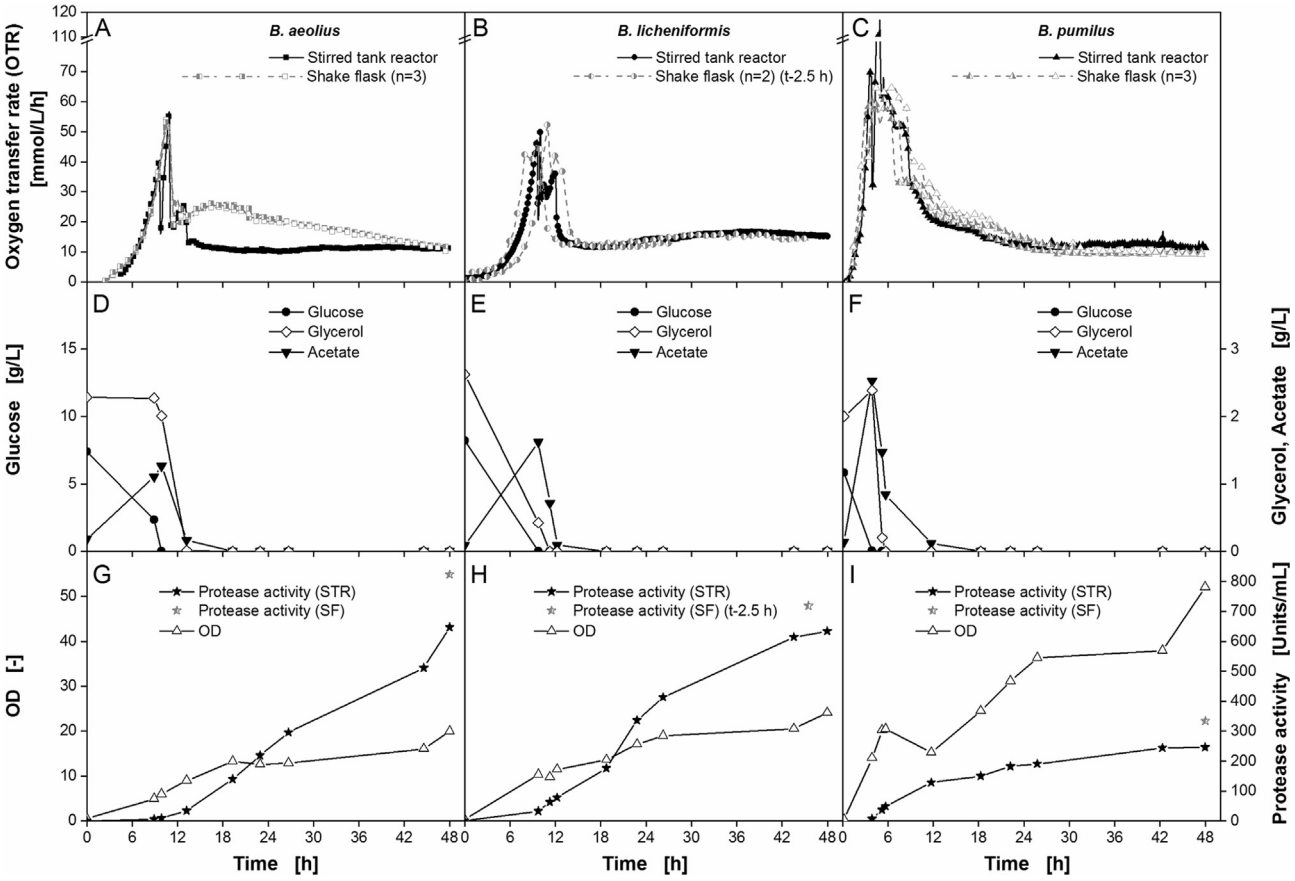


FIG. 3. Fed-batch cultivations of three different *Bacillus* strains in membrane-based fed-batch RAMOS shake flasks and stirred tank reactor. (A, D, G) *B. aeolius* (DSM 15084), (B, E, H) *B. licheniformis* H215 and (C, F, I) *B. pumilus* (DSM 18097) were grown in V3 mineral medium both, in membrane-based fed-batch RAMOS shake flasks (half or complete hollow symbols with dashed lines) and stirred tank reactors (full symbols with solid lines). (C, F, I) *B. pumilus* was grown in V3 medium supplemented with 15 g/L yeast extract. Fed-batch cultivations in stirred tank reactor started with an inital batch phase containing 8.5 g/L glucose. After the depletion, feeding was manually started (0.64 g/L/h). The pH was controlled at pH 7.2 with 2 M NaOH and 2 M HCl. The DOT was maintained above 30 % air saturation by controlling the stirring rate (500–2000 rpm). For shake flask cultivations *Bacillus* strains were cultivated in V3 mineral medium (0.2 M MOPS buffer, 3 mL with 200 g/L glucose in the reservoir, $pH_0 = 8$) without initially provided glucose in culture broth. Oxygen transfer rates are shown in (A–C). For clarity, only every second measuring point over time is represented by a symbol for shake flask cultivations and only every tenth for stirred tank reactor cultivations. For the shake flask cultivations biological duplicates or triplicates are shown. Glucose, glycerol and acetate concentrations are presented in (D–F) as well as protease acitivity and optical density (G–I) for stirred tank reactor cultivations (STR). (G–I) Final protease activites for shake flask cultivations (SF) are also presented. Culture conditions for shake flasks: 250 mL shake flask, 12 mL filling volume, 350 rpm shaking frequency, 50 mm shaking diameter, 3 mL fed-batch reservoir filling volume, Reichelt dialysis membrane RCT-NatureFlex-NP (cellulose), 18.1 $mm^2$ membrane area and 37°C temperature. Culture conditions for stirred tank reactor: 1000 mL reactor working volume, 1 vvm aeration, DOT controlled at 30 % air saturation and 37°C temperature.

have reported an improved γ-PGA formation due to elevated osmotic stress.

The batch phases of *B. licheniformis* cultivations are highly comparable among the two scales investigated (Fig. 3B). Merely, the shake flask batch phase is shifted by approximately 2.5 h due to an elongated lag phase, which is compensated in Fig. 3B. As seen before (Fig. 3A), the three OTR peaks correspond to the metabolization of the carbon sources glucose and glycerol as well as the overflow metabolite acetate (Fig. 3E). Differences in the fed-batch phase between shake flask and stirred tank reactor are not apparent. The OTR level during fed-batch phase is at 11–16 mmol/L/h and well comparable for both scales. All fed-batch cultivations of *B. licheniformis* showed a significantly shorter lag phase compared to the batch cultivations. A possible reason for the elongated lag phase of the batch cultivation could be, for example, a lower inoculum size or a preculture with decreased vitality. However, the lag phase, in general, is still poorly understood and the most sensitive bacterial growth phase and difficult to quantify (39).

For *B. pumilus* cultivations, no lag phase is observed as the medium was supplemented with 15 g/L yeast extract (Fig. 3C) and the OTR increases exponentially. In addition to the consumption of glucose and glycerol as well as the formation and reassimilation of acetate, *B. pumilus* metabolizes complex compounds originating from the supplemented yeast extract. This leads to a higher overall OTR level with OTR peaks that are not distinctly separable, as well as a more gradual decrease of the OTR following the batch phase. Nevertheless, the OTR courses of shake flasks and stirred tank reactor cultivations were highly comparable throughout the entire cultivation process.

The OD of *B. aeolius* and *B. licheniformis* are very similar (Fig. 3G, H). Maximum OD values of 20 for *B. aeolius* and 24.1 for *B. licheniformis* were achieved after 48 h. Due to the addition of yeast extract to the cultivation medium of *B. pumilus*, higher OD values of 52 were obtained (Fig. 3I).

As for OD values, *B. aeolius* and *B. licheniformis* performed similarly concerning the protease formation. After glucose exhaustion, protease activities of stirred tank reactor cultivations increase linearly up to 647 U/mL for *B. aeolius* and to 634 U/mL for *B. licheniformis*. This high similarity in protease activities can be attributed to the genetic disposition. *B. licheniformis* and *B. aeolius* are genetically very similar, as they are both strains of the *B. licheniformis* species (40).

With *B. pumilus*, a final protease activity of only 246 U/mL is achieved, although the highest ODs are measured. After glucose

depletion, no further increase in protease formation was observed as with the other strains. This may be due to the fact that some components of the yeast extract lead to a catabolite repression, thus, reducing protease formation. This inhibition of alkaline protease synthesis due to the presence of yeast extract was also reported by Moon and Parulekar (41) as well as Sharma and Singh (42). The large number of different amino acids leads to this repression (43).

Apart from comparing the metabolic behavior of the microorganisms over time during the culture, the final protease yields are of major interest. Therefore, final protease activities of membrane-based fed-batch shake flask cultivations are also presented in Fig. 3G–I. Protease activities in shake flasks are higher than in stirred tank reactors. With *B. aeolius*, a final activity of 823 U/mL was achieved for shake flask cultivations. The final protease activities of *B. licheniformis* and *B. pumilus* were 719 U/mL and 334 U/mL, respectively. However, it is important to compare final protease activities based on the amount of metabolized glucose. The final protease yields of shake flask and stirred tank reactor fed-batch cultivations are shown in Fig. 4.

Transferring the fed-batch process from shake flasks to the stirred tank reactor results in a slight decrease in protease yield for all strains. Presumably, the reason for this activity loss was the difference in pH range. In stirred tank reactor cultivations the pH was controlled at 7.2, whereas in shake flasks pH was kept in a range from 7.2 to 8 by buffering. Applying pH buffers instead of pH stabilization agents HCl and NaOH seems to have enhancing effects on the protease productivity. This correlation was also previously observed for final protease activities reached in *B. licheniformis* batch cultivations in V3 mineral medium (31). Whether differences in osmolality or the non-controlled pH course in contrast to a constant pH causes this phenomenon is under further investigation. *B. aeolius* and *B. licheniformis* show similar protease yields of 21 U/mg and 19 U/mg for stirred tank reactor cultivations. Despite the addition of the complex compound yeast extract, protease yields of *B. pumilus* cultivations are less than half of the other two strains in both scales. Considering the very different pH control between the scales, the results are in good agreement. The strains perform almost identically in terms of OTR and protease yield in stirred tank reactor and in shake flasks. Only *B. aeolius* shows some larger differences. This may be due to its property to form the polymeric by-product γ-PGA. In addition, when comparing protease yields between batch and fed-batch shake flask a 1.5-fold increase relative to batch was determined for *B. aeolius* and *B. licheniformis*. A repression of protease production by glucose was thereby avoided. This positive effect of the fed-batch operation mode on protease production was not observed for *B. pumilus*.

In the batch cultivation, *B. pumilus* exhibited the highest protease yield of 19 U/mg, followed by *B. aeolius* (17.6 U/mg) and *B. licheniformis* (15.6 U/mg). In the fed-batch cultivations, however, the ranking of the strains regarding their protease yield was different. Fed-batch cultivation of *B. pumilus* resulted in the lowest protease yields among the three tested strains and, above all, it was observed in both fed-batch cultivation systems, in shake flasks and fermenter. These observations demonstrate the applicability of the membrane-based fed-batch shake flasks as a suitable high-throughput screening tool for high-producing strains in fed-batch operating mode.

Membrane-based fed-batch shake flasks are a powerful screening tool (8,17,18) especially in combination with the RAMOS technology. However, production processes take place in stirred tank reactors so that a successful scale-up from shake flask to fermenter scale is indispensable in order to reproduce culture characteristics also on a large scale. In this study, the transferability of fed-batch cultivations from shake flasks to laboratory-scale stirred tank reactors has successfully been demonstrated. The membrane-based fed-batch shake flasks mimicked the larger scale process environment for fed-batch operation mode.

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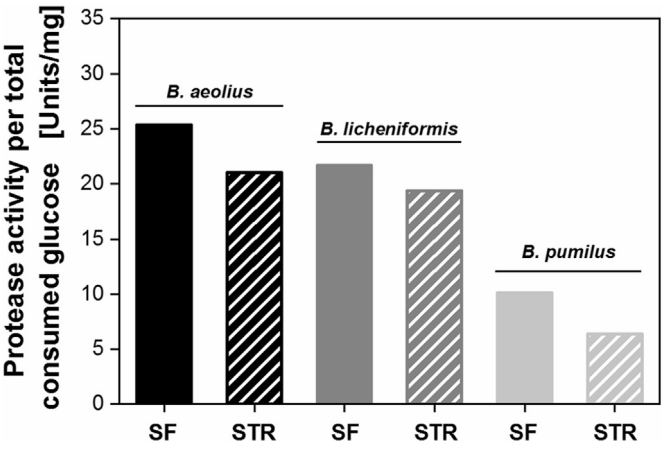


FIG. 4. Protease activities related to the total consumed glucose of *B. aeolius*, *B. licheniformis* and *B. pumilus* cultivations. *B. aeolius* (DSM 15084), *B. licheniformis* H215 and *B. pumilus* (DSM 18097) were cultivated in membrane-based fed-batch RAMOS shake flasks (SF) and in stirred tank reactor (STR) in fed-batch mode according to Fig. 3. Protease activities were measured in samples taken at 48 h culture time. For the shake flask cultivations 0.2 M MOPS was used for pH control. For fed-batch cultivation in the stirred tank reactor pH was controlled at pH 7.2 by titration. Feeding of glucose in shake flasks started at the beginning. No inital glucose was provided. The medium for stirred tank reactor cultivations initially contained 8.5 g/L glucose, feeding was started manually when the inital glucose was exhausted. The glucose feeding rate of 0.64 g/L/h was the same for both systems.

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