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Optimized bioreactor setup for scale-up studies of extreme halophilic cultures

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ABSTRACT

Adaptation to highly saline conditions required halophilic organisms to develop unique features. The mounting interest in those features has resulted in a number of potential applications for moderate and extreme halophiles. However, industrial exploitation has been reported only for few instances. With the aim to bring concepts from laboratory to pilot-scale a customized bioreactor for halophilic cultures was developed. The setup combined a bubble column reactor (BCR) with a membrane module for cell retention. The BCR was compared to a conventional stirred tank reactor (STR) in terms of physiological and hydro-dynamical parameters. The results showed that the BCR is preferable in terms of energy efficiency. The BCR reached a maximum k_La of 84 h^{-1} at ambient pressure, which equals an oxygen transfer rate (OTR) of 6 mmol/(L*h) in medium with 150 g/L NaCl. To reach this mass transfer a STR required more than 3-fold the amount of energy. Cultivation of the extreme halophilic archaeon *Haloferax mediterranei* showed suitability of the BCR for continuous halophilic processes. To reduce cost and ecological impact of this process a highly saline industrial waste stream from a chemical production process was used as source for NaCl. The BCR presented in this study allows continuous and competitive cultivation of halophilic organisms at high volumetric rates and high biomass productivities as required for large scale industrial applications.

1. Introduction

Halophilic archaea and halophilic bacteria can be deployed for diverse industrial and environmental applications as remediation and treatment of waste water [1], production of carotenoids [2], formation of biodegradable polyhydroxyalkanoates (PHA), e.g. polyhydroxybutyrate (PHB) [3] or production of bacteriorhodopsin [4]. Common to all these applications is that competitive utilization is only possible if halophilic microorganisms can be cultivated cost-effectively in large scale. However, scale-up studies are challenging at these extreme conditions and therefore rarely reported for halophilic processes. The highly saline culture condition demands corrosion resistant equipment as well as custom-made solutions for process monitoring or analytics. Bioreactors for extreme halophilic processes are niche products that are manufactured from certain corrosion resistant materials, like PEEK or Teflon [5,6]. Schiraldi and De Rosa see this constrain as main reason for the slow progress in industrial exploitation of halophiles [7].

So far, studies on extreme halophilic cultures are limited to shake flasks [8,9] and bioreactors with a volume of 1–10 L [5,6,10–16]. Studies on halophilic processes at higher scales can hardly be found in literature with the only exception presented by Koller, who showed PHA production by *Haloferax mediterranei* in a 300 L bioreactor [17]. Koller investigated utilization of different waste streams with the objective to reduce costs and to decrease the ecological impact of the process. The author showed 1) that surplus whey, as waste from dairy production, can be used as substrate; 2) that cell debris from prior cultivations can replace expensive yeast extract in the medium as source for nitrogen and phosphate and 3) that spent fermentation broth can be used as source for NaCl. Koller's work revealed that recycling waste streams is a promising approach for competitive extreme halophilic cultures.

Utilization of halophilic organisms and their products can be a disruptive innovation for fields like saline waste water treatment,

Abbreviations: BCR, bubble column reactor; c^*, oxygen solubility in the medium ($g\,L^{-1}$); c_L, dissolved oxygen in the medium ($g\,L^{-1}$); D, dilution rate (h^{-1}); DO, dissolved oxygen; F_a, air flow ($m^3\,min^{-1}$); k_La, volumetric mass transfer coefficient (h^{-1}); n, stirrer speed; OTR, oxygen transfer rate ($mol\,L^{-1}\,h^{-1}$); p_1, manifold pressure for the compressor (bar); p_2, reactor pressure (bar); P/V, volumetric energy consumption ($W\,m^{-3}$); R, retention rate; STR, stirred tank reactor; V_R, reactor working volume (L); X, biomass concentration ($g\,L^{-1}$); Y, yield (Cmol/Cmol); η, efficiency; μ, specific growth rate (h^{-1}); ρ, density of the fluid ($g\,m^{-3}$).

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biodegradable polymers or photovoltaic technologies. For example, *Halomonas* strains have been shown to degrade toxic azo dyes from effluents of the textile industry [18]. The halophilic proteobacterium *Halomonas campaniensis* was shown to form PHB from kitchen-waste-like mixed substrate, thus enabling low-cost production of biopolymers [19]. In addition, biosensitized solar cells containing the halophilic membrane protein bacteriorhodopsin have been suggested as a low-cost and environmental friendly biomaterial for photovoltaic devices [20]. As a prerequisite for commercial success those new technologies have to be economically competitive compared to established processes. Hence, both capital expenditures (CapEx) and especially operational expenditures (OpEx) have to be kept low for those advanced biotechnological approaches.

The goal of this study was to design a reactor setup that allows an up-scale of laboratory results to a pilot scale with the objective to develop competitive halophilic processes. The customized reactor was characterized concerning its oxygen transfer, energy input and suitability for continuous halophilic cultures. Quantitative studies were carried out using the extreme halophilic archaeon *H. mediterranei*. For this example process a saline waste water from chemical industry was used as source for NaCl. Results of the hydrodynamical and physiological assessment of the BCR were compared to a conventional STR. The development of an optimized bioreactor setup aims to close the gap between laboratory scale experimenting and industrial application of extreme halophilic archaea and bacteria.

2. Material and methods

2.1. BCR setup

The cultivations were carried out in a custom-made bioreactor with 15 L working volume (Diameter 13.4 cm; Height 1.1 m; Möstl Anlagenbau, Arzberg, Austria). The reactor vessel was made from the corrosion resistant material Hastelloy. The bioreactor was equipped with probes for reactor pressure (Signet 2450, Georg Fischer, USA), reactor temperature (Onigrad TR88, Endress + Hauser, Reinach, Switzerland), dissolved oxygen (DO) in the reactor and in the loop (Visiferm DO Arc 120, Hamilton, Bonaduz, Switzerland), pH (InPro3250i, Mettler Toledo, Columbus, USA), liquid level (Liquifant M FTL50, Endress + Hauser, Reinach, Switzerland) and concentration of CO_2 and O_2 in the offgas (Müller Systems AG, Egg, Switzerland). The online data monitoring and process control were executed with a process information management system (Lucullus, SecureCell, Schlieren, Switzerland).

The reactor was designed as bubble column with a cell retention unit in an external loop (see Fig. 1). A 4-piston diaphragm pump (Quattroflow, ALMATEC Maschinenbau, Germany) with a polypropylene pump head was used to circulate the cell suspension at a flow rate of 105 L/h. No oxygen limitation could be observed at his flow rate. A DO probe was positioned in the loop to ensure sufficient supply of oxygen for the halophilic culture.

The tangential flow microfiltration unit retains the cells inside the reactor, while the cell-free harvest permeates the filter. A polysulfone membrane with an area of 8400 cm^2 was used (CFP- 2-E-9A, GE Healthcare, Chalfont St Giles, Great Britain). The size of the membrane was chosen in a way that membrane fouling is prevented and high harvest flow rates can be achieved.

Harvest and bleed flow were driven by overpressure in the BCR. The flow rates were regulated by PID controllers via operating a digital valve. Feed and base were pumped into the reactor vessel using magnetically actuated membrane pumps (Magdos, Lutz-Jesco GmbH, Wedemark, Germany). All flow rates were monitored gravimetrically using laboratory scales with 0.1 (bleed and base) or 10 g (feed and harvest) resolution (Mettler Toledo, Columbus, USA).

2.2. STR setup

The laboratory scale bioreactor used as reference was a STR with Rushton turbines and a total volume of 2.3 L (working volume 1 L; Labfors bioreactor, Infors, Bottmingen, Switzerland). The vessel of this corrosion resistant bioreactor is made of a glass while stirrer and lid are manufactured from PEEK. The STR setup was equipped equivalent to the BCR with a microfiltration unit (GE Healthcare, Germany, model: CFP-2-E-4A, polysulfone membrane, area: 420 cm^2) and peristaltic pumps for loop, feed, bleed and harvest (Lambda Preciflow, Lambda instruments, Baar, Switzerland). Setpoints for dilution rate, retention rate, pH and temperature were the same for BCR and STR.

2.3. k_La determination

The volumetric mass transfer coefficient (k_La) is a parameter that determines the oxygen transfer from gas bubbles to the liquid phase, according to Eq. (1):

$$\frac{dc_{O_2}}{dt} = k_L a \cdot (c^* - c_L) \quad (1)$$

where c^* is the saturated O_2 concentration in the liquid phase and c_L is the concentration of dissolved oxygen in the liquid phase. Consequently, (c^*-c_L) defines the driving force for oxygen mass transfer.

The k_La values were determined using the two-phase dynamic gassing-out method. To investigate the influence of high NaCl concentrations on the k_La, that procedure was carried out with deionized water and saline industrial medium as described in chapter 2.4 (150 g/L NaCl). The bioreactor was filled to the working volume and heated up to 37 °C. In phase one the reactor was flushed with nitrogen, until the DO was stable at 0%. In the second phase, the bioreactor was gassed with air until the water was saturated with oxygen (DO_{max}). This procedure was repeated with several flow rates and in case of the STR with different agitator speed. The k_La-values were determined by fitting the DO measurements according to Eq. (2):

$$DO = DO_{\max} \cdot (1 - e^{-k_L a * (t - t_0)}) \quad (2)$$

where DO is the dissolved oxygen at a time t and t_0 is the start time of the DO measurement.

2.4. OTR determination

OTR describes the oxygen transfer rate from gas to liquid phase. The parameter is determined using Eq. (1) under the assumption dc_{O2}/dt = OTR. Oxygen solubility was taken from the literature [21]. Values were partially computed by linear interpolation. Solubility of oxygen in saline medium at 37 °C is c^* = 2.89 mg/L. Residual dissolved oxygen concentration was declared 20%, as this is the DO limit that was set for the cultivations performed in this study. No oxygen limitation could be detected at those conditions. Accordingly the driving force for the oxygen transfer (c^*-cL) equals 2.31 mg/L.

OTRs under pressure conditions were calculated using Henry's Law according to Eq. (3) and with the assumption Henry constant H_i = const for constant temperature.

$$c_i^* = \frac{p_i^*}{H_i} \quad (3)$$

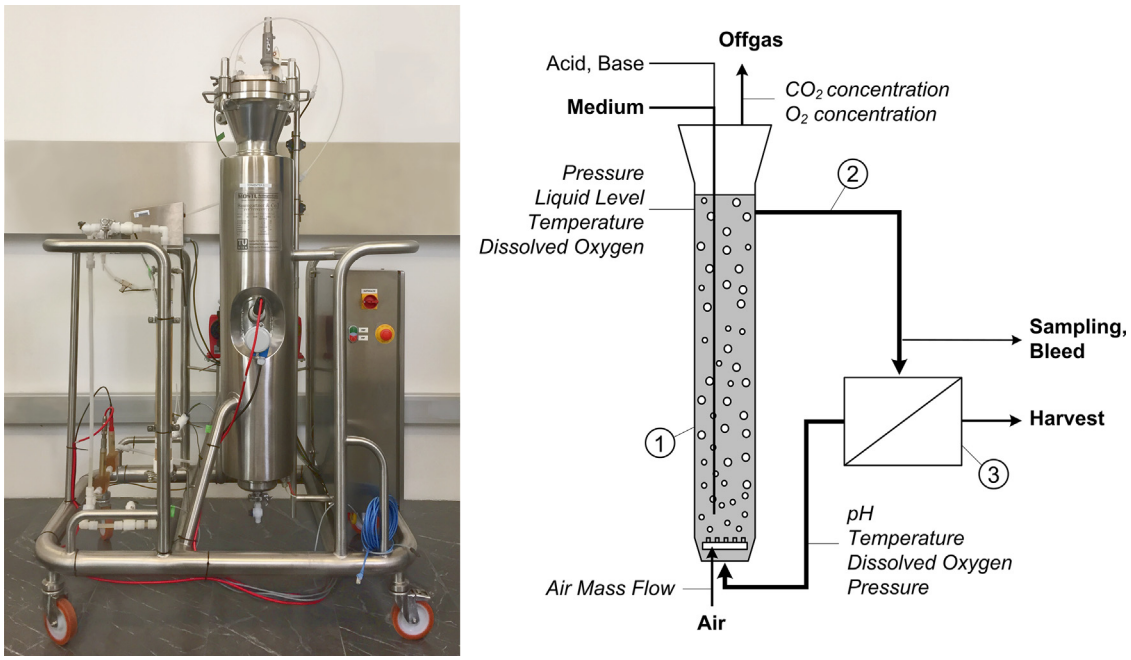

Fig. 1. Schematic diagram of the BCR: 1) Pressure resistant bubble column vessel, 2) continuously circulating loop driven by a diaphragm pump and 3) microfiltration unit for cell retention. Probes indicated in italic, continuous fluid flows indicated in bold.

2.5. Energy input

The volumetric energy consumption of the reactor is the electric energy required to run the reactor at a given performance. The volumetric energy consumption P/V is the sum of energy required for stirring, for compression and for circulation of the loop pump (see Eqs. (4)–(7)):

$$\frac{P}{V} = \left(\frac{P}{V}\right)_{Stirring} + \left(\frac{P}{V}\right)_{Compressor} + \left(\frac{P}{V}\right)_{Loop\ pump} \quad (4)$$

$$\left(\frac{P}{V}\right)_{Stirring} = n^3 \cdot d^5 \cdot Ne \cdot \rho \cdot \frac{1}{V_{Reactor}} \cdot \frac{1}{\eta_{Motor}} \quad (5)$$

$$\left(\frac{P}{V}\right)_{Compressor} = p_1 \cdot \ln\left(\frac{p_2}{p_1}\right) \cdot \frac{F_a}{V_R} \cdot \frac{1}{\eta_{Compressor}} \quad (6)$$

$$\left(\frac{P}{V}\right)_{Loop\ pump} = \frac{\dot{V}_{Loop} \cdot \Delta p}{\eta_{Motor}} \cdot \frac{1}{V_{Reactor}} \quad (7)$$

where n is the stirrer speed, d the stirrer diameter, Ne the Newton's number, ρ the density of the fluid, V_R the reactor working volume, η the efficiency, p_1 the manifold pressure for the compressor, p_2 the reactor pressure, F_a the air flow rate and $(P/V)_{Looppump}$ is the volumetric power consumption of the loop pump. For the BCR is $(P/V)_{Stirring} = 0$. The motor efficiency used in these calculations was $\eta_{motor} = 0.95$ and $\eta_{compressor} = 0.67$ vvm. The values were taken from the literature and describe values for electric motors and multistage compressors with low flow resistance caused by piping, valves and ports [22]. Newton number was determined as described elsewhere [23], using values for the dynamic viscosity from the literature [24].

2.6. Strain and medium

H. mediterranei DSM 1411 was purchased from DSMZ – German collection of microorganisms and cell cultures. The cell concentration was estimated by the measurement of the optical density at 600 nm (OD_{600}). In case absorption exceeded OD_{600} 0.5 the samples were diluted with a saline solution (150 g/L NaCl) to prevent lysis of the cells. Correlation of OD_{600} with biomass concentration was the following: biomass (g/L) = 0.7863·OD_{600} + 0.1562. Biomass concentrations for this correlation were estimated using the base consumption, as reported elsewhere [5].

In all experiments process water from an industrial partner was used as salt source. It contained 150 g/L NaCl and organic residuals equivalent to a TOC of 80–90 mg/L. These residuals are mainly organic acids and aromatic compounds. Due to variations in the production process, composition of residuals in different waste water batches can change. The process water was supplemented with glycerol as C-source and mineral media components. The final media contained (g/L): Glycerol 0.27; NH_4Cl 1.5; KH_2PO_4 0.15; $FeCl_3$ 0.005; $MgCl_2 \cdot 6H_2O$ 1.3; $MgSO_4 \cdot 7H_2O$ 1.1; $CaCl_2 \cdot 2H_2O$ 0.55; KCl 1.66; KBr 0.5; Trace elements solution 1 mL [(mg/100 mL): $FeSO_4 \cdot 7H_2O$ 139; $CuSO_4 \cdot 5H_2O$ 100; $MnCl_2 \cdot 7H_2O$ 120; $CoCl_2 \cdot 2H_2O$ 44; $ZnSO_4 \cdot 7H_2O$ 86]; pH 7.0.

2.7. Continuous culture

Cultivation of *H. mediterranei* was carried out in the two described reactor systems under the same conditions at 37 °C. The pH value in the reactor was maintained at 6.98 ± 0.06 (BCR) or 7.00 ± 0.05 (STR) using 0.5 M NaOH. The process was carried out under non-sterile conditions.

The steady state condition in biological systems with cell retention is determined by dilution rate D and retention rate R. The parameters are calculated as follows:

$$D = \frac{\dot{Feed}}{V_R} \quad (8)$$

$$R = \frac{\dot{Feed} - \dot{Bleed}}{\dot{Feed}} \quad (9)$$

$$\dot{Feed} = \dot{Harvest} + \dot{Bleed} + \dot{Base} \quad (10)$$

The dilution rate was set to 0.37 h^{-1} and the retention rate was 0.93. Cell suspension was removed constantly as bleed stream. The STR was aerated with 0.1 vvm at ambient pressure while the BCR was aerated with 0.67 vvm at 2 bar reactor pressure. Elevated pressure is necessary for outflow of harvest and bleed.

Prior to the continuous process a batch cultivation was carried out in order to reach an initial cell concentration of 3 g/L. Initial substrate concentration for the preculture and batch duration were calculated assuming a yield of 0.55 g biomass per g glycerol and a maximum specific growth rate of 0.070 h^{-1} stated in literature. [5] Specific growth rate was calculated by Lorantfy et al. by using exponential curve fitting of biomass concentration in a batch on the substrate glycerol.

2.8. Carbon and degree of reduction balancing

Using both online and off-line data, the volumetric rates and yields were calculated for the following components: oxygen ($Y_{O2/S}$), carbon dioxide ($Y_{CO2/S}$) and biomass ($Y_{X/S}$). To test consistency of the results, carbon and degree of reduction (DoR) balances were examined according to described methods [25]. Substrate concentration in the culture supernatant was measured using HPLC. Quantification was performed with a HPLC (Thermo-Fisher) using an Aminex HPX-87H column from Bio-Rad at 50 °C, an isocratic eluent of 4 mM sulfuric acid in Milli-Q water with a flow of 0.5 mL/min followed by RI detector and UV detection at 210 nm. The limit of quantification with injection volume of 20 μL was 5 mg/L for glycerol, lactate and acetate. The process was analysed for residual substrate concentration and formation of organic acids to check C-limitation during the continuous culture.

3. Results and discussion

3.1. Customized bioreactor design

The design of the reactor was customized for scale-up studies of extreme halophilic processes from laboratory experimenting to pilot scale. In the design phase of the reactor both scalability and low OpEx for the eventual process had to be considered. The advantages of the chosen bubble column design includes low operating costs, reduced effort for maintenance as a results of the absence of moving parts and reduced shear stress for the cells [26].

Fig. 1 shows a schematic diagram of the setup and a picture of the BCR that was engineered for an optimized oxygen transfer. The slim design ensures a long residence time of the bubbles in the liquid. The height of 1.1 m causes significant hydraulic pressure in the lower part of the reactor thus increases the solubility of oxygen in the liquid. To further increase driving force of the oxygen transfer the BCR can be pressurized to a maximum of 3 bar. A diaphragm pump continuously circulates the cell suspension via the cell loop and supplies the tangential flow membrane with cell suspension.

Bubble columns have been suggested earlier for cultivation of halophilic archaea. Cheng-Kang Lee et al. used an illuminated bubble column photo bioreactor for production of the membrane protein bacteriorhodopsin with *Halobacterium salinarum* [15]. However, to reach high product formation the authors performed a repeated-batch culture to maximize the cell concentration in the reactor. The loading, unloading and cleaning for batch and repeated batch cultures reduce the time-space yield of the process. On the contrary, continuous processing shows advantages, such as high-volumetric productivity, reduced equipment size and reduced capital costs [27]. The BCR presented here enables continuous cultivations with cell retention. Lee et al. used a polysulfone membrane module retaining *H. salinarum* in a common STR [12]. The cells were continuously supplied with fresh medium in a system with total cell retention (R = 1). In contrast to that, the reactor presented in this study should allow continuous growth of the cells, by R < 1. The extreme halophilic organism *H. mediterranei* was cultivated in continuous mode with R = 0.93.

3.2. Volumetric mass transfer coefficient (k_La) in highly saline medium

Sufficient oxygen transfer is a critical parameter for halophilic process development, as oxygen solubility is reduced in high salt concentrations. The solubility of oxygen in the saline medium used in this study is 104.32 μmol/[kg H_2O] at 37 °C. This is less than half the amount of oxygen that can be dissolved in deionized water at the same temperature (212 μmol/[kg H_2O]) [21]. Despite this low solubility a sufficient supply with oxygen has to be achieved in the bioreactor to ensure aerobic growth conditions.

It is known that due to lower oxygen solubility in the medium, the driving force for oxygen transfer is highly reduced for halophilic conditions. However, what is yet unknown is the influence of the high salt concentrations on the k_La value.

Fig. 2A–C shows a comparison of the k_La values in two different media: deionized water and saline medium with 150 g/L NaCl. The k_La values were determined in the STR at low agitator speed (A), in the same STR at high agitator speed (B) and in the BCR (C). The results show that the effect of high NaCl concentrations on the k_La value is low. At low stirrer speed the k_La value in saline medium is up to 12% higher than in non-saline medium. Same shows for the STR at higher agitator speeds where the k_La is increased up to 18%. This increase might be connected to an effect that has been reported earlier: NaCl inhibits coalescence of bubbles [28]. Smaller bubbles cause a higher specific gas-liquid surface and thus a higher k_La. Fig. 2C, however, suggests that the effect of NaCl on the k_La value is not solely positive. For aeration rates >1 vvm k_La values in saline medium are lower than in non-saline medium. Influence factors for the k_La value are manifold, e.g. surface tension or viscosity, and reasons for effects on the k_La are difficult to determine. Even though small differences of k_La values can be seen in Fig. 2A–C, taken into account the 95% confidence bands of the curve fit, it can be stated that the difference of the k_La is not significant. Hence, low solubility is the main reason for low oxygen transfer rates in halophilic cultures. The pressurization of the reactor as measure to increase oxygen transfer is discussed in chapter 3.4.

3.3. Oxygen transfer in the BCR

As bubble columns and airlift bioreactors are not equipped with mechanical stirring devices transfer rates are often found to be limited in comparison to conventional STRs [29]. The BCR presented in this study was characterized concerning its volumetric mass transfer coefficient k_La to assess its oxygen transfer capabilities. Comparison of a bubble column with a conventional STR should determine the hydrodynamic limits of this reactor design. Extreme halophilic cultures in STRs are commonly operated in the range of 100–800 rpm [5,11–14]. Previous cultivations in a 1 L STR suggested a stirrer speed of 420–800 rpm and an aeration rate of 0.1 vvm for cultivations with *H. mediterranei* to ensure aerobic cultivation conditions by maintaining dissolved oxygen concentration above 20% (data not shown). With the k_La determined in saline medium, this would be equivalent to an OTR of 3.3–4.5 mmol/[L*h]. Suitability of the BCR for its purpose of extreme halophilic cultures can be shown when these OTRs can be reached or if OTRs are exceeded.

In order to characterize the gas transfer in the BCR the k_La was determined and compared with values from a 1 L STR at 420 and 800 rpm. Fig. 2A shows the k_La values in saline medium for the two different reactor systems. It can be seen that k_La values in the BCR are equal to k_La values reached in a STR at 420 rpm for aeration rates from 0.1 to 0.5 vvm. However, for higher aeration rates k_La values in the STR (420 rpm) are up to 8.5% higher compared to the BCR at same aeration rates. In a biological process the low k_La values had to be compensated with increased aeration or increased reactor pressure.

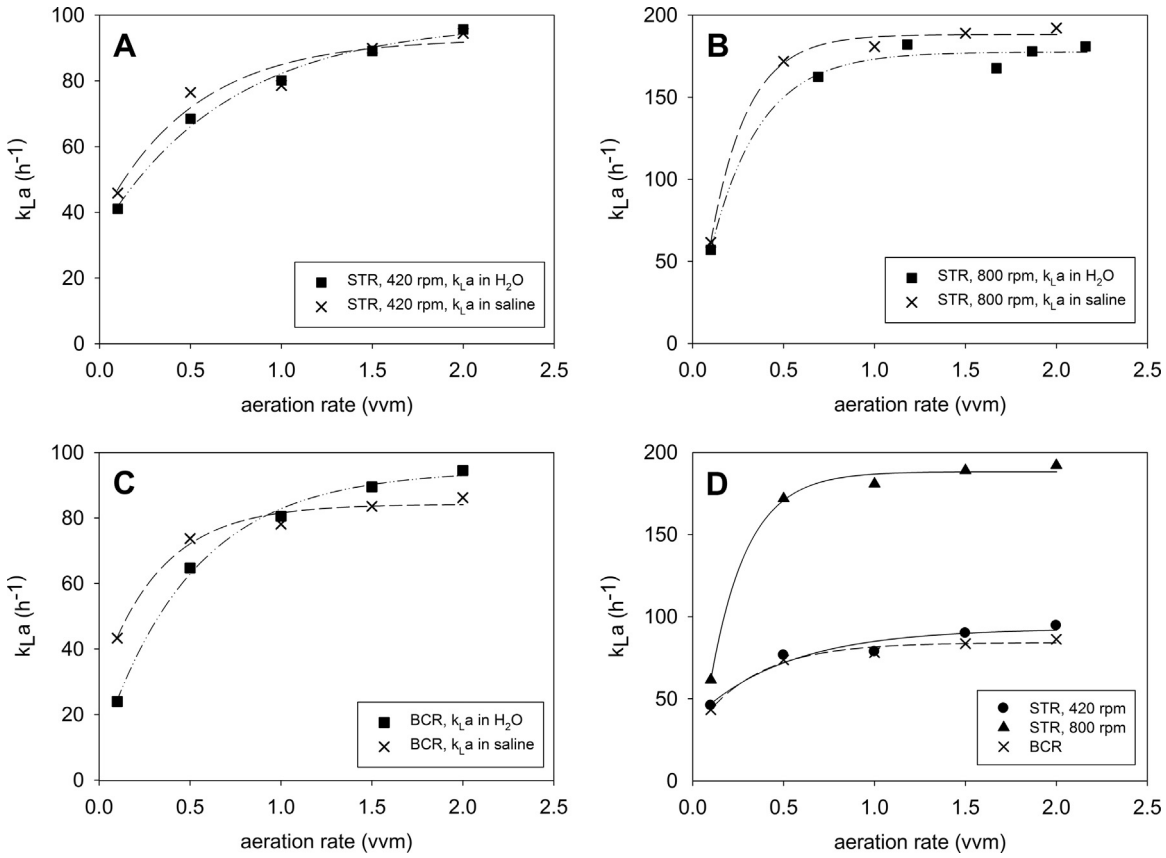

Fig. 2. Volumetric mass transfer coefficient k_La depending on liquid, reactor type and stirrer speed. (A), (B) and (C): Comparison of k_La values in saline medium and deionized water in STR and BCR. (D): The effect of the reactor type and stirrer speed on k_La values in saline medium (150 g/L NaCl).

In STRs k_La values can easily be increased by a higher stirrer speed. Increased mixing results in smaller bubbles, higher turbulences, longer residence times and thus higher k_La values [30]. In case of the 1 L STR the k_La could be more than doubled after increasing the stirrer speed from 420 rpm to 800 rpm and a maximum of 192 h^{-1} could be reached. The results shown in Fig. 2 clearly show the restricted oxygen transfer in bubble column reactors compared to STRs. Achieving k_La values >85 h^{-1} exceeds the hydrodynamic limits of the BCR presented here. However, Eq. (1) shows that oxygen transfer is a function of k_La and driving force. As former is limited by the reactor design the oxygen transfer rate can be increased by higher driving forces. Measures to increase the driving force include increasing the oxygen partial pressure (e.g. by aeration with pure oxygen or elevated reactor pressure) or increasing the gas solubility (e.g. by decrease of temperature). An alternative way for rising the oxygen transfer is decreasing the surface tension of the medium, e.g. by addition of surfactants [31]. Among these methods elevated reactor pressure has been described as both an energy and cost efficient measure [32]. The results showed that the BCR can reach a maximum k_La of 84 h^{-1}, which equals a maximum OTR of 6 mmol/[L*h] in saline medium at ambient pressure. This meets the requirements for a halophilic culture. Nevertheless, to ensure enough supply of oxygen even for high cell density cultures the BCR was constructed for reactor pressures up to 3 bar. Modeling of the OTR at elevated pressures and the estimation of the corresponding energy input shall assess the energy efficiency of the BCR reactor.

3.4. Comparison of the energy input

Oxygen supply is an important part of the OpEx [32]. For industrial scale cultivations it is therefore crucial to ensure an energy efficient oxygen mass transfer. By using the measured k_La values OTR values and the according energy input are calculated. At equivalent OTRs the BCR requires 700 to 1100 W/m^3 less energy input (see Fig. 3A). For an OTR of 4.5 mmol/(L*h) the 14 fold amount of energy is required for operating a STR at 0 bar compared to the more energy efficient BCR. Increase of stirrer speed results in a vast increase of energy consumption, as can be seen in Fig. 3B. While OTR is doubled, volumetric energy input is increased almost 7 fold. OTRs achieved in a STR at a stirrer speed of 800 rpm can only be reached by elevated reactor pressure with the BCR. An OTR of 12 mmol/(L*h) in the STR at ambient pressure thereby required the 2.4 fold amount of energy compared to the BCR. However, when an elevated reactor pressure is applied to the STR oxygen transfer in terms of energy input is comparably efficient. Nevertheless the STR would require a slightly higher reactor pressure compared to the BCR, which is linked to higher equipment costs and larger compressors.

The results of the energy consumption model agree with the results from literature [26]. De Jesus et al. not only showed lower energy demands for pneumatic bioreactors but also proved lower shear rates for airlift reactors and bubble columns. For highly viscous cell suspensions bubble columns were found to be limited compared to airlift reactors. However, due to low specific growth rates OTR requirements of halophilic cultures are low and can be achieved using BCRs.

Energy efficiency for the presented BCR design strongly depends on the efficiency of the compressor. In this example an efficiency of $\eta_{compressor} = 0.75$ was assumed. This efficiency is suitable for large turbo compressors that are customized for application. Smaller compressors that are used for laboratory experimenting usually have a lower efficiency. However, it has to be mentioned that efficiency may vary depending on compressor type and operating point. Values are in the range of 0.5-0.6 for one-step screw

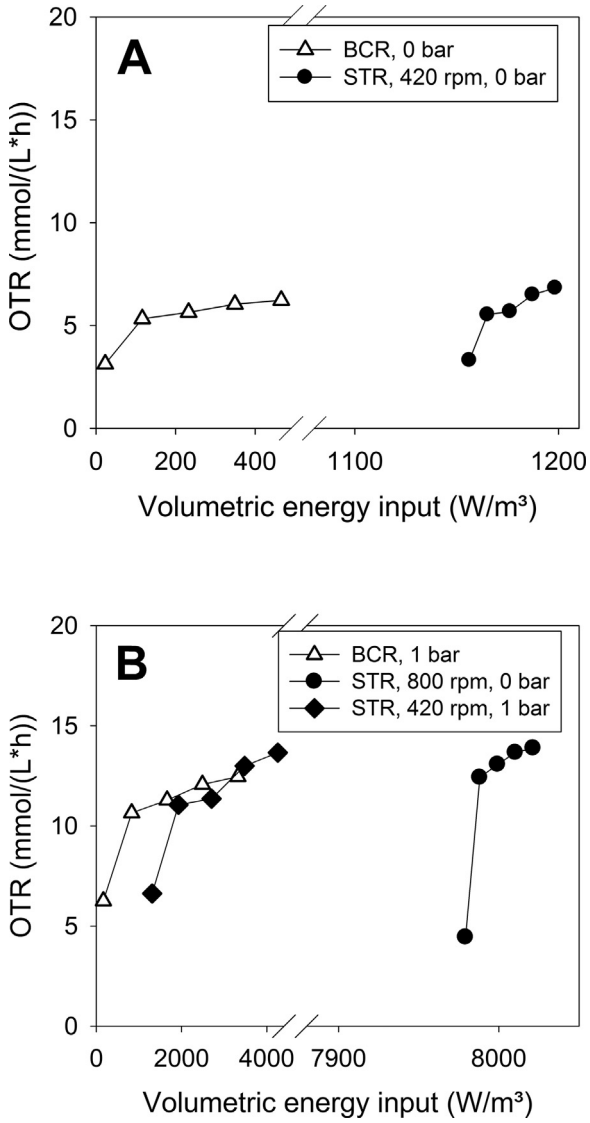

Fig. 3. Oxygen transfer rate depending on the volumetric energy input for the two reactor systems STR and BCR at ambient pressure (A) and elevated reactor pressure (B).

compressors to 0.6-0.8 for built-to-order multi-step compressors [22].

3.5. *Continuous cultivation of* H. mediterranei *in the BCR*

Halophiles have recently been called the 'coming stars for industrial biotechnology' [33]. Among other unique selling points utilization of waste streams as low cost substrates has been proposed. Extreme halophilic cells have been shown to use rice bran, olive mill waste water or vinasse (a highly polluting waste of the ethanol industry) as substrate for the production of PHA [8,9,11]. Those waste streams usually show low concentrations of utilizable carbon. A highly productive waste-to-value process has to perform at high substrate-feeding rates. The low carbon concentrations in the feed therefore would require high dilution rates. The cultivation described here mimics a process that uses brine with low substrate concentrations (0.27 g/L glycerol) at high dilution rates (0.37 h^{-1}) to achieve high productivities.

With the customized BCR setup the flows could be hold at a constant level for more than 12 residence times at a retention rate of 0.93 (see Fig. 4A), resulting in constant levels for biomass concentration (see Fig. 4B). HPLC analytics showed C-limited conditions during the entire cultivation. The physiological parameters specific growth rate μ and the yields $Y_{CO2/S}$, $Y_{X/S}$ and $Y_{O2/S}$ showed constant levels (see Fig. 4C). The integrity of the calculated parameters was proven by C- and DoR Balancing. As Fig. 4D shows the balances close with a deviation of less than 10%.

On time points marked by an arrow the pressure in the reactor had to be reduced to atmospheric condition. Reason was the addition of antifoam via one of the ports. The pressure drop led to a sudden change in offgas composition and accordingly to deviations in the yield values.

In the presented BCR gas transfer rates could be achieved that ensured sufficient oxygen supply for the halophilic culture. However, cultivations with higher cell densities require higher oxygen transfer rates [34]. For up-scaling to large high cell density reactors the bubble column might be exchanged by an airlift reactor design. This reactor type is described to be beneficial for highly viscous fluids [26]. In order to lower the CapEx, alternative materials for large scale bioreactors might be considered. Compared to the high-cost Hastelloy, glass fiber reinforced plastic is a less expensive material with equivalent corrosion resistance.

A cell retention system allows continuous cultivation with dilution rates that exceed the growth rate of the organism. The continuous culture in this study was performed at a dilution rate of 0.37 h^{-1}, which is more than 5 fold the specific growth rate of *H. mediterranei* under these conditions (0.026 h^{-1}). By applying higher dilution rates, higher growth rates are possible. Limitation of that increase would be the membrane module. Although the harvest flow can be enhanced by an increase of the reactor pressure, the possibility to increase the dilution rate is limited by the size of the membrane and the pressure resistance of the whole setup. For the membrane chosen for the BCR no problems with membrane fouling or clogging could be detected.

Exploitation of highly saline waste streams as source for NaCl is an important argument for competitive halophilic processes [33]. Yue et al. showed PHA production by *Halomonas campaniensis* in artificial sea water, containing less than 3% (m/m) NaCl [19]. In the study presented here saline waste water from a chemical process, containing 150 g/L NaCl, was used for salt recycling. There are numerous industries producing highly saline waste streams, like chemical, textile, food and petroleum industries [35]. Some of those waste streams have been successfully treated by halophilic organisms [36–41]. The results of this study show that utilization of saline waste streams for NaCl is possible without any pretreatment. Reuse of waste water as source for NaCl is beneficial for both ecological and economical reasons. However the following three factors have to be considered in case of waste water recycling: (1) Inhibition effects on cellular growth caused by impurities in the brine. (2) Interactions of waste water components with the down streaming of the product. (3) Effect of salinity fluctuations on the activity of the cells. Nevertheless, with *H. mediterranei* an extreme halophilic organism was chosen that tolerates a wide range of salt concentration, reaching from 60 to 300 g/L with an optimum at 170 g/L [42].

3.6. *Comparison of the continuous cultures in BCR and STR*

Physiological parameters like growth rate and yields are common criteria for comparing biological activity. Table 1 shows a direct comparison of two cultivations of *H. mediterranei* with comparable process parameters: in a 1 L STR and in the 15 L BCR. For both cultivations industrial brine was used as source for NaCl. The comparison should detect differences in the physiology of the cells that could be caused by (1) high shear stress that leads to decreased viability of the cells or (2) limited oxygen transfer rates that cause anaerobic conditions. The results show no significant difference for

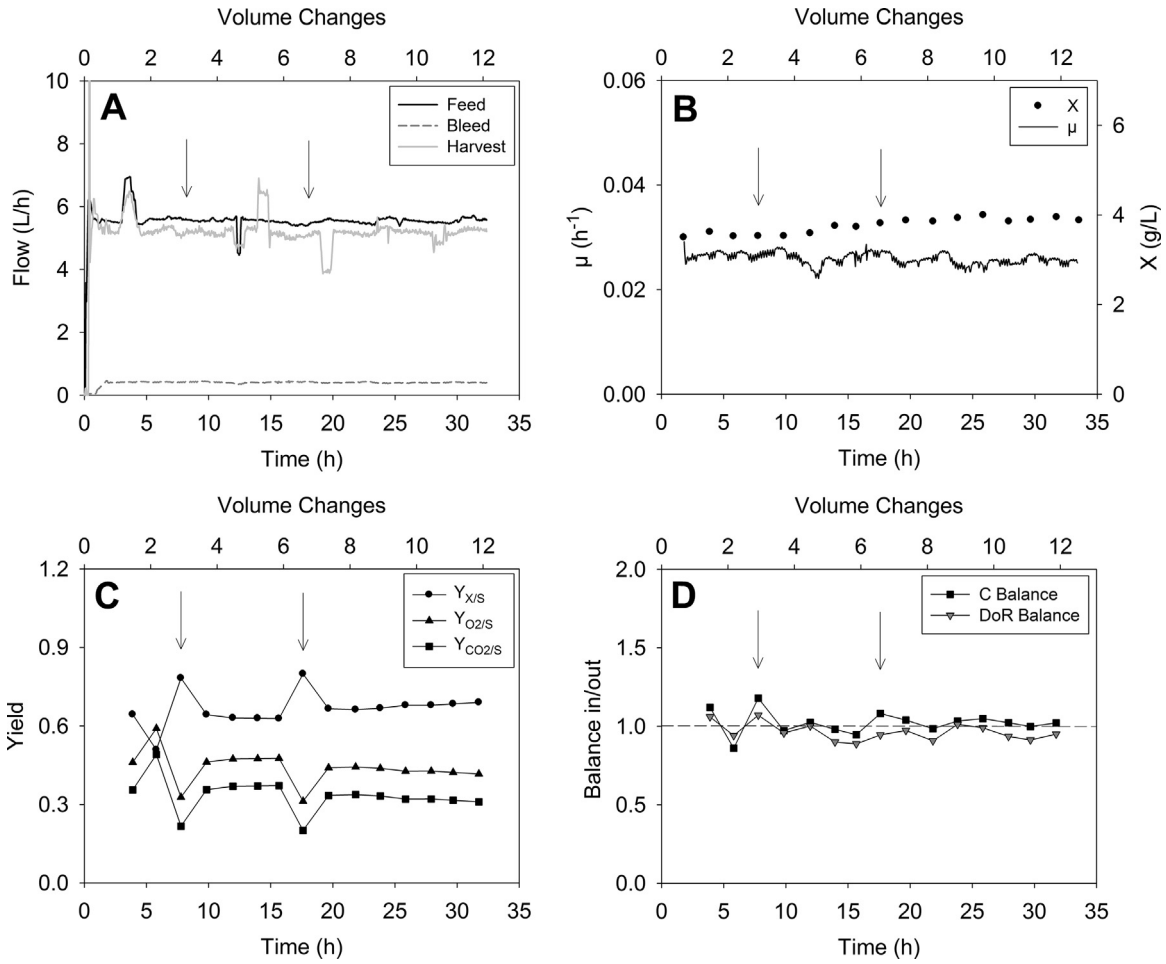

Fig. 4. Time dependent profile of flows (A), yields (B), specific growth rate μ and biomass concentration X (C) and carbon and degree of reduction balance (D). Continuous cultivation of *H. mediterranei* on glycerol in BCR. Arrows indicate pressure reduction due to addition of antifoam.

Table 1
Process variables (reactor volume V_R, dilution rate D, retention rate R) and physiological parameters (biomass concentration X, specific growth rate μ and Yields per substrate glycerol) for comparing continuous cultivation of *H. mediterranei* in laboratory scale STR and pilot scale BCR.

	STR	BCR
	0.1 vvm, 600 rpm, 0 bar	0.67 vvm, 2 bar
V_R (L)	1	15
D (h^{-1})	0.37	0.37 ± 0.02
R	0.93	0.93 ± 0.08
X (g/L)	4.13	3.92 ± 0.06
μ (h^{-1})	0.026	0.026 ± 0.001
$Y_{O2/S}$ (mol/Cmol)	0.49	0.45 ± 0.02
$Y_{X/S}$ (Cmol/Cmol)	0.61	0.64 ± 0.05
$Y_{CO2/S}$ (Cmol/Cmol)	0.38	0.34 ± 0.07

the specific growth rate μ or the yields $Y_{CO2/S}$ and $Y_{X/S}$ and therefore an equivalent metabolism in STR and BCR. However, $Y_{O2/S}$ in the BCR was shown to be 8% lower compared with the results from the STR. We assume that reasons for this drift are the two different batches of process water that were used for NaCl recycling. As a waste product from chemical production the process water can change in composition and residual TOC. An altered composition would result in different yields for oxygen consumption as the oxidation of the components requires additional oxygen.

The customized BCR was designed to enable up-scale studies for extreme halophilic cultures. The sample process presented in this study was transferred from 1 L to 15 L pilot scale with equivalent physiological parameters. However, even though the experiments showed that growth of *H. mediterranei* was not influenced by scale or bioreactor type, this conclusion might not be extended to all applications. It has been shown previously that the reactor type can influence the productivity of a biological process. Yen and Shih described the production of coenzyme Q10 with purple bacterium *Rhodobacter sphaeroides* in two 5 L reactor setups: an airlift and a STR [29]. Q10 concentration was found to be more than 38% higher in the airlift reactor. The authors explain that effect by alternating zones of high and low DO concentrations. The accumulation of CoQ in *R. sphaeroides* is known to be stimulated by an environment that is low in oxygen. In other cases product formation in a STR reactor was superior compared to an airlift reactor [30] because of increased biomass growth. The effect of the reactor design is therefore highly depending on the bioprocess.

4. Conclusion

In this study a customized bioreactor for continuous bioprocessing of extreme halophilic organisms was evaluated to assess its applicability for up-scale studies. Detailed investigation of the volumetric mass transfer coefficient k_La and the oxygen transfer rate OTR showed that the BCR is eminently suitable for halophilic cultures. With respect to the lower energy input the stirrer-less BCR approach is preferable compared to a conventional STR. A continuous cultivation of *H. mediterranei* showed that the reactor is suitable for extreme halophilic production processes with high productivi-

ties. The cell retention enabled cultivation with a dilution rate that was 5 times the maximum specific growth rate of the organism. The sample process showed the feasibility of a continuous cultivation with low concentrated feed streams (0.27 g/L glycerol) and high volumetric productivities ($D = 0.37\ h^{-1}$). The OpEx can thereby be reduced by recycling of saline industrial waste water as source for NaCl. The performance of the cells in the BCR was not different to a conventional STR when growth characteristics and yields for CO_2 and biomass were compared.

Based on our findings we believe in a strong future of competitive extreme halophilic bioprocessing in unsterile continuous cultivations with the option of utilizing waste streams as substrates and highly saline process wasters as source for NaCl. This approach might be an important step in the direction of efficient and economic biological processes using extreme halophilic archaea and bacteria.

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