INFORS HT

Application note

Cultivation of extreme halophiles in corrosive conditions with Labfors PEEK bioreactor

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1. Introduction

Halophile bacteria are special types of extremophile species, since they can survive in hypersaline environments: halophile strains are able to grow in habitats with the presence of sodium chloride from 2% to 30%.

Halophiles can be categorised by the percentage of sodium chloride which they tolerate and grow at. Slight halophiles grow best in concentrations of salt from 2% to 5% which range corresponds to the average salt concentration of seawater (3–5%). Moderate halophiles can be cultivated in concentrations of sodium chloride between 5% and 15%. Extreme halophiles grow best in concentrations of salt above 20% up to 30%. The higher the sodium chloride concentration gets, the more difficult it is to cultivate halophiles due to the corrosivity issues of the processing equipment.

Regarding the extremely high salt concentrations that only extreme halophiles and no other species can tolerate, there can be a big potential in future bioprocesses to cultivate them for different purposes, e.g. recombinant protein production because the laborious sterilisation issues can be avoided and upstream and downstream bioprocess costs can be reduced as well. However, most probably due to the corrosive character of the process, there is very little activity to develop processes in this promising field.

In order to characterise bioprocesses with halophiles, production needs systems which allow reproducible cultivation and quantitative bioprocess development under corrosion-resistant circumstances. In Labfors PEEK bioreactor (INFORS HT, CH-Bottmingen, Fig. 1), which was made of corrosion-resistant parts like borosilicate glass and PEEK polymer, the cultivation of extreme halophiles could be quantitatively established.

This document aims at demonstrating the reproducibility of the process with halophiles as well as showing the potential for quantitative process development using the above-mentioned equipment for batch and continuous culture.

2. Material and Methods

2.1 Technical specifications

- Borosilicate glass culture vessel: 2 L total volume
- Borosilicate glass exhaust gas cooling
- Special corrosion resistant Polymer (PEEK) bioreactor top lid and thermometer holder
- Borosilicate glass sampling tube and gas inlet tube
- Glass coating on the agitator
- Borosilicate glass jacket on the bioreactor vessel

Online Analytics:

- Exhaust gas CO_2 and O_2
- Glass probe pH
- Hastelloy Clark pO_2
- Thermal mass flow controller for air

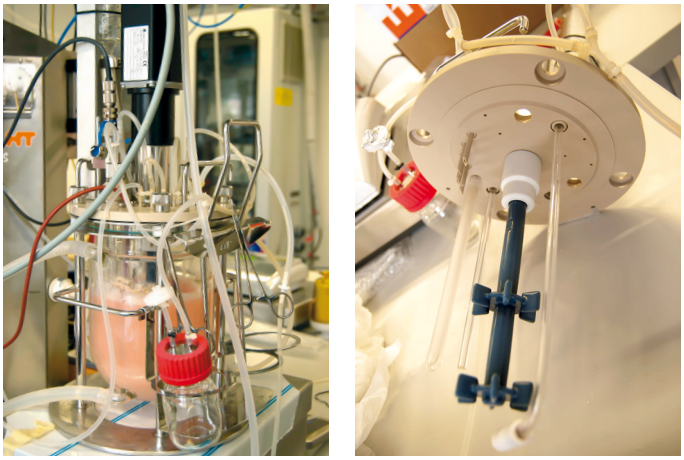

Fig. 1: Labfors PEEK bioreactor

2.2 Experimental specifications

2.2.1 Strain

The extreme halophilic strain *Haloferax mediterranei* (DSMZ No. 1411, ATCC No. 33500, CCM No. 3361; abbreviated as "HFX" further on) was utilised for the cultivation experiments. According to previous literature studies, this strain has the ability of growing on different single-carbon sources.

2.2.2 Medium

For the first experiments to cultivate the strain HFX, 1L synthetic defined medium (named as Halomedium) was utilised with the following composition:

Halophiles – Halomedium (g/L)	
C-Source	10
NH_4Cl	2
KH_2PO_4	0.3
$FeCl_3$	0.005
NaCl	194
$MgCl_2.6H_2O$	16
$MgSO_4.7H_2O$	24
$CaCl_2.6H_2O$	1
KCl	5
$NaHCO_3$	0.2
KBr	0.5
Trace element solution (Fe, Cu, Mn, Zn)	1 mL
Distilled water to 1 L, pH 7.2	

2.2.3 Parameter settings in Labfors PEEK bioreactor

In order to obtain the ideal growing conditions of halophiles, with Labfors PEEK bioreactor the following parameters were monitored as well as controlled:

- Temperature: 38°C
- pH: 7.2
- Agitation: 400 rpm
- Air inlet: 0,2 NL/min

2.3 Cultivation

The aim of the study was to quantify the uptake of carbohydrates as the C-source with halophiles in defined medium and develop C- limited cultivation conditions for process development for further possible applications.

Quantitative bioprocess development may require continuous bioprocesses. Therefore, after the first batch experiments with the strain HFX, when the exponential growth phase of halophiles on the specific substrate could be observed, we aimed at performing continuous investigations only with substrate limitations.

During the batch experiments, it must be taken into consideration that halophiles have low growth rates (approx. 0.04 1/h), furthermore, the continuous operations can only be carried out with low dilution rates (less than 0.04 1/h).

2.4 Analysis

In order to describe the bioprocesses, some off-line analysis steps were also required. The samplings were performed with the help of the sampling tube system of the Labfors PEEK bioreactor.

2.4.1 Biomass quantification

2.4.1.1 Optical density determination of the broth
The optical density of the broth can be determined with fotometer at 600 nm wavelength. The optical density values of the samples correlate with the amount of biomass in the broth.

2.4.1.2 Dry weight determination in test tubes
The amount of dry weight can also be estimated by gravimetrical methods after centrifuging, resuspending and drying biomass samples.

2.4.1.3 Elemental composition of the biomass
The elemental composition of the biomass which was produced by the strain HFX was determined by producing a lyophilised biomass sample and subsequent elemental analysis. According to the results, the elemental composition of biomass during steady state conditions was:

$$CO_{0.537}H_{1,59}N_{0,108}S_{0,004}P_{0,005}$$

2.4.2 Substrate and metabolite concentration analysis

2.4.2.1 HPLC method for the determination of acids, alcohols and other by-products as well as metabolites during fermentation.
Agilent 1100 Series
Column: SUPELCOGEL C-610H (Sigma, 9 µm particle size, 300 x 7.8 mm); 30°C
Eluent: 0.1% H_3PO_4 in dist. Water (traces of NaN_3); 0.5 mL/min
Detector: RI

2.4.2.2 Cubian Enzymatic Robot (Innovatis)
Determination of the C-source by photometric assay

2.4.3 Growth rate calculations

The growth rate was calculated in two different ways:
With the utilisation of the base consumption: the pH of the broth got acidic during growth and the pH was controlled in the bioreactor. Namely, the medium contained NH_4^+ ions and when the cells used this nitrogen source in the form of NH_3, H^+ was produced and neutralised by the OH^- ions from the NaOH base. Therefore, the base consumption curve was in correlation with the growth rate in the exponential growth phase. Moreover, with utilising this principle, the amount of biomass was attempted to be estimated from the online base consumption results.

During the exponential growth, the pO_2 curve also shows exponential characteristics. In case of fitting an exponential curve to the derived pO_2 curve, the exponent of the curve showed the value of the maximum growth rate (μ_{max}).

3. Results and interpretation

3.1 Quantification in batch

3.1.1 Reproducibility of the bioprocesses – Substrate concentration

The initial C-source concentration was always chosen as 10 g/L in every batch experiment. The following graph shows the characteristic of the exponential growth phase of the batch experiments. On Fig. 2 it can be seen how the substrate was consumed during the exponential growth phases of the batch experiments. The trajectory of substrate consumption can also be analysed:

- lag phases at the beginning of the experiments
- subsequent exponential phases, which can be used for the determination of the μ_{max} value
- the exponential growth phases end after approx. 100 h; the periods were dependent of the inoculum

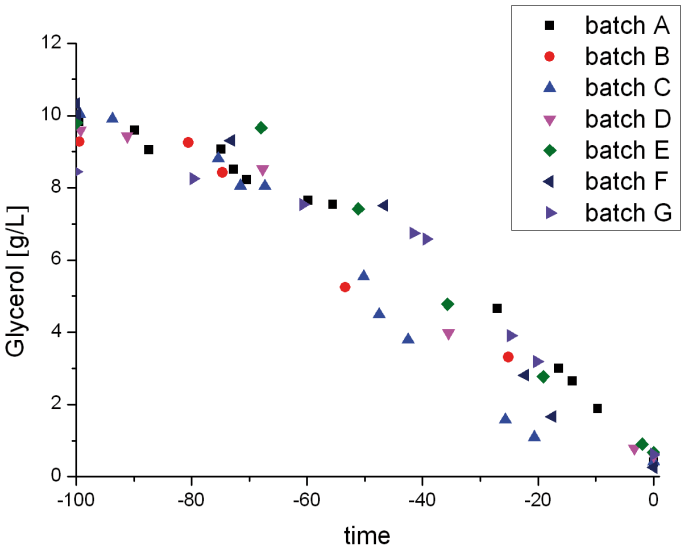

Fig. 2: Substrate uptake in different batch experiments with the strain HFX, normalized to substrate depletion. The datasets of the parallel experiments demonstrate the reproducibility of the cultivation of the strain under the same conditions in Labfors PEEK bioreactor.

3.1.2 Parameters: pH, temperature and agitation

In order to gain better quantification of the bioprocesses and obtain reproducible datasets, it is necessary to maintain constant and stable cultivation parameters in the bioreactor (Fig. 3). The pH value, which is physiologically determined by the strain, is controlled in the bioreactor until the end of the batch phase when the C-source is consumed and the pH begins to increase. At the same time, the bioreactor temperature is kept constant. The agitation was set from 400 rpm to 450 rpm to avoid the limitation on the oxygen supply in the bioreactor shortly before the end of the exponential phase (the switch can also be seen on Fig. 3).

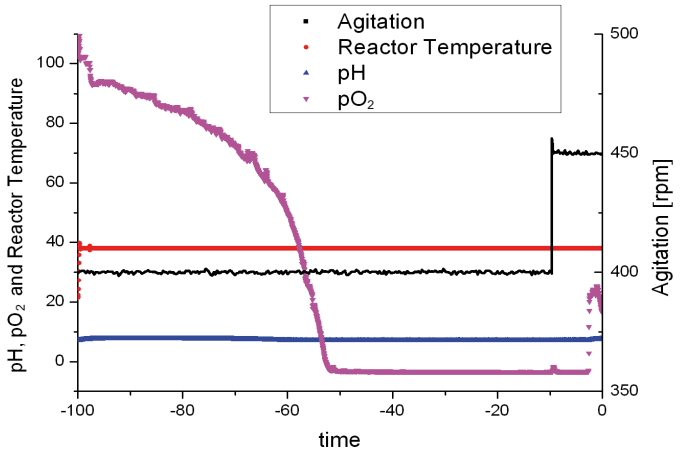

Fig. 3.: The most important cultivation parameters: pH, pO_2 temperature as well as the agitation in the bioreactor in the quantification phase in this batch experiment with the strain HFX.

3.1.3 Determination of the biomass: OD600 and base consumption

Optical density and the base consumption are the markers of the amount of biomass which is produced during the bioprocess (Fig. 4).

The optical density of the broth at 600 nm wavelength correlates with the amount of dry weight which is in the broth.

The base consumption during the batch experiments was used to estimate biomass concentrations in the bioreactor with the knowledge of the elemental composition of the biomass. However, compared to the amount of biomass which is produced by the halophiles, a lot of base is consumed in every experiment; therefore a correction factor could be derived: A certain ratio of the base consumption is utilised for the biomass production. This value was found to be around 0.3 in our experiments.

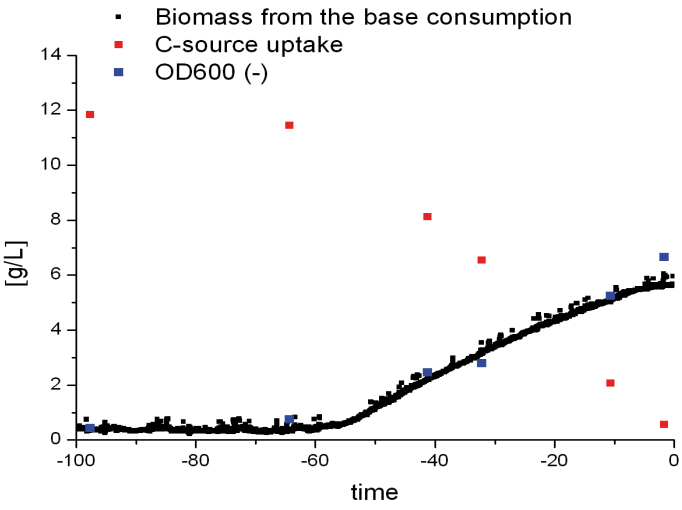

Fig. 4.: Optical density and the estimated biomass concentration from the base consumption as the markers of the amount of biomass produced during one HFX batch experiment. It can be clearly observed that with the introduction of the base correction factor for the base calculations, the biomass data which was derived from the base consumption fits nicely to the OD600 dataset.

3.1.4 OUR, CER; RQ, growth rate

Oxygen uptake rate (OUR, mmol/L/h), Carbon dioxide evolution rate (CER, mmol/L/h) and Respiratory Quotient (RQ, -) also provide information about the characteristics of the batch processes. On Fig. 5 there is a clear example of how to discover if the culture is not only limited by the C-source but also by the oxygen supply. Between process times 30 h and 45 h the culture could grow exponentially because it was only limited by the C-source but thereafter, when the culture was limited by the oxygen supply, the OUR and CER values did not increase further. At process time 72 h, the agitation was changed from 400 rpm to 450 rpm (see also on Fig. 3) in order to avoid limitation by oxygen so that the OUR and CER values also began to increase again.

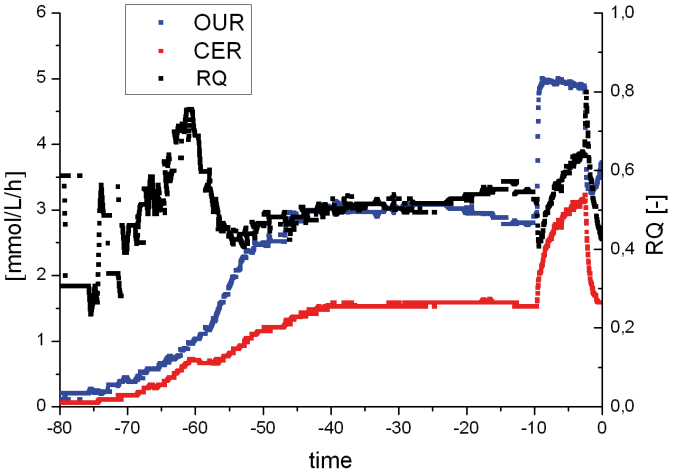

Fig 5.: OUR, CER and RQ values in one HFX batch experiment. The changes of the OUR and CER values clearly show the characteristic of the bioprocess.

3.1.5 Determination of μ_{max}

For the determination of μ_{max}, the derived pO_2 curve was utilised. In the substrate-limited exponential phase of one batch experiment, the pO_2 curve showed the characteristics of the clear exponential growth (Fig. 6). The pO_2 curve had to be derived, multiplied by (-1) in order to meet the requirements of exponential curve fitting.

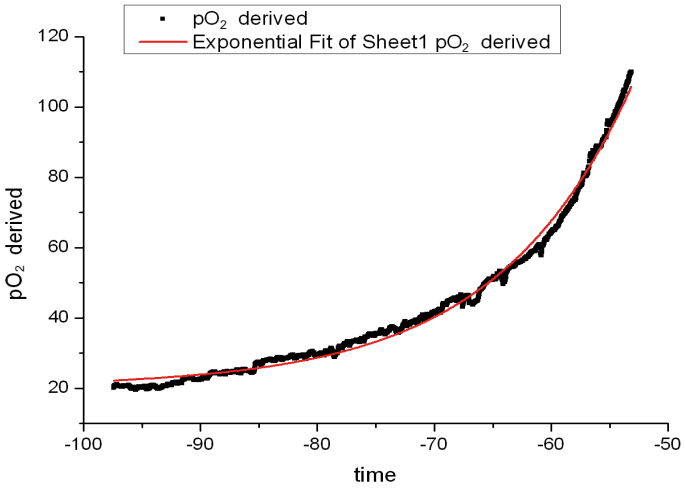

Fig. 6.: The derived pO_2 curve in the substrate-limited exponential phase of one batch experiment. The exponential trendline fitting for the curve can be observed and the maximum growth rate (μ_{max}) can also be seen as the exponent (R0 0.08648, R2 0.99252) of the curve fitting.

3.1.6 Other metabolites

According to the HPLC measurements, there were no other metabolites during the process; the substrate had been converted into biomass and carbon dioxide (purely aerobic process).

3.2 Quantification of the continuous experiments

3.2.1 OUR, CER; RQ

Continuous experiments were carried out with two different dilution rates (0.022 1/h and 0.033 1/h).

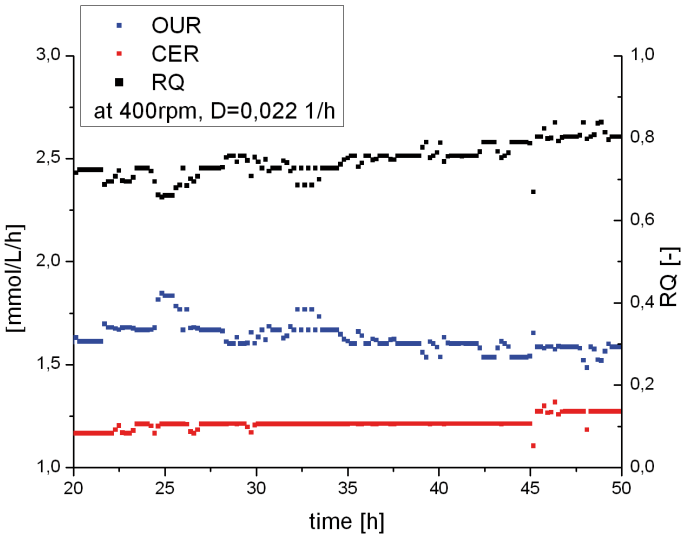

Fig. 7: OUR, CER (mmol/L/h) and RQ (-) values at D=0.022 1/h dilution rate and at 400 rpm agitation in steady-state, HFX continuous experiments.

3.2.2 Yields and balances

Compared to the amount of biomass which is produced by the halophiles, a lot of base is consumed in every experiment; therefore a correction factor could be derived:

A certain ratio of the base consumption is utilised for the biomass production and the other part is consumed for physiological reasons. This value was found to be around 0.3 in our experiments, matching the value from batch cultures.

The other part of the base consumption is utilised for other purposes. Literature survey had shown that this amount of base can be consumed for the compensation of the proton pump activities of the halophiles. Reference: Shiladitya DasSarma, Priya Arora: Halophiles; ENCYCLOPEDIA OF LIFE SCIENCES Nature Publishing Group (ww.els.net)

Regarding these facts, every experimental datasets were re-calculated with the usage of the correction factor of the base consumption.

	D_1=0.022 1/h	D_2=0.033 1/h
Vol rate C-source (mol/h)	0.00185	0.00384
Vol rate C-source (g/h)	0.170	0.354
Rbase (mol/h)	0.00151	0.00193
Base ratio for biomass	0.33	0.21
OUR (mmol/l/h)	1.63	8.099
CER (mmol/l/h)	1.22	4.45
RQ (-)	0.748	0.550
YO2/S (g/g)	0.311	0.733
YO2/S (mol/C-mol)	0.298	0.703
Yco2/s (g/g)	0.319	0.554
Yco2/s (mol/C-mol)	0.223	0.386
rX (mol/h)	0.00462	0.00717
rX (g/h)	0.111	0.179
Yx/s (g/g)	0.654	0.507
Y x/s (mol/C-mol)	0.833	0.621
C-balance (mol/mol)	**1.06**	**1.01**
C-balance (g/g)	**0.974**	**1.06**
DoR balance	**1.00**	**1.40**

Table 1: Calculations at the steady-state intervals of the continuous experiments. The carbon balance and the DoR balance could be also seen.

4. Conclusions

For the cultivation of these halophile strains in bioreactor, more time is required as they grow much slower than for example *E. coli*, and they also have lower growth rates. With the utilisation of Labfors PEEK bioreactor we were able to obtain reproducible results with the cultivation of extreme halophiles under hypersaline and highly corrosive environment.

Due to the experienced reproducible processes, the ability for quantification and quantitative bioprocess development is possible and promising.

With exploiting the advantages of extreme halophiles, the following possible future applications can be suggested:

- Waste water treatment for special industrial effluent water with high salt concentrations
- Novel host organisms for recombinant protein production
- Easier upstream – no sterility required – therefore lower upstream costs can be expected
- Easier downstream – product purification process is easier as extreme halophile cells simply lyse in water – also results in lower downstream cost

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