

Article

## Dynamic Experiments for Bioprocess Parameter Optimization with Extreme Halophilic Archaea

Bettina Lorantfy, Bernhard Seyer and Christoph Herwig \*

Research Division Biochemical Engineering, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorferstrasse 1A 166-4, A-1060 Vienna, Austria;

E-Mails: bettina.lorantfy@tuwien.ac.at (B.L.); bernhard.seyer@chello.at (B.S.)

\* Author to whom correspondence should be addressed; E-Mail: christoph.herwig@tuwien.ac.at; Tel.: +43-(1)-58801-166-400; Fax: +43-(1)-58801-166-980.

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**Abstract:** The to-date studies on extreme halophiles were focused on shake flask cultivations. Bioreactor technology with quantitative approaches can offer a wide variety of biotechnological applications to exploit the special biochemical features of halophiles. Enabling industrial use of *Haloferax mediterranei*, finding the optima of cultivation parameters is of high interest. In general, process parameter optimizations were mainly carried out with laborious and time-consuming chemostat cultures. This work offers a faster alternative for process parameter optimization by applying temperature ramps and pH shifts on a halophilic continuous bioreactor culture. Although the hydraulic equilibrium in continuous culture is not reached along the ramps, the main effects on the activity from the dynamic studies can still be concluded. The results revealed that the optimal temperature range may be limited at the lower end by the activity of the primary metabolism pathways. At the higher end, the mass transfer of oxygen between the gaseous and the liquid phase can be limiting for microbial growth. pH was also shown to be a key parameter for avoiding overflow metabolism. The obtained experimental data were evaluated by clustering with multivariate data analyses. Showing the feasibility on a halophilic example, the presented dynamic methodology offers a tool for accelerating bioprocess development.

**Keywords:** dynamic ramps; process parameter optimization; bioprocess with extreme halophiles; acceleration bioprocess development

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## 1. Introduction

### 1.1. Halophiles

There is an emerging interest on using halophilic microorganisms for new and alternative bioprocesses with industrial biotechnological relevance [1]. They have been widely used for waste water treatment with different purposes and origins [2–4], especially with the archaeon *Haloferax mediterranei* (HFX) [5]. The production of biopolymers, namely polyhydroxyalkanoates (PHAs), is also a valuable feature of these organisms [6]. In addition, there are examples for engineering halophilic microorganisms for valuable product productions [7]. Extreme halophilic Archaea can thrive in hypersaline environments with up to 3–5 M sodium chloride concentrations and, therefore, have a number of still unexploited characteristics [8,9]. The representatives of halophilic Archaea are typically aerobic or methanogenic. Although anaerobic halophilic Archaea are rare, numerous examples exist for anaerobic halophilic bacteria [10,11]. The archaeon HFX is known as a denitrifier and assimilates the present nitrate or nitrite [12]. Halophilic Archaea are capable of growing aerobically on a wide variety of carbon sources. They are reported to grow on different organic acids [13–15], diverse sugars [16], the sugar alcohol, glycerol [17], and even aromatic compounds [18]. In the case of halophilic Archaea, the metabolic pathways are slightly different from the conventional microbial metabolism [19,20]. Depending on the substrate, different glycolytic pathways coupled to the glyoxylate cycle play the main role in intermediary carbon metabolism [21]. Due to the fact that the high osmotic pressure of hypersaline environments ensures a low risk of contamination, the capacity for cost-effective non-sterile cultivation can make extreme halophilic Archaea potentially valuable host organisms for future biotechnological applications, as inherent selection towards non-extreme halophilic microorganisms occurs. The feasibility of the ease of downstream operations can be also reasoned by the simple lysis of extreme halophiles if the salt concentration falls below 10% w/w.

### 1.2. Bioreactor Technology with Extreme Halophiles

Operating industrially relevant bioprocesses, with optimal process parameter sets, is a prerequisite from physiological and also from economical aspects [22]. We have established bioreactor technology concepts for physiological characterization, process scale-up, and exploiting economically feasible operating ranges for bioproducts in laboratory scales [23–25]. The bioreactor setups are equipped along above mentioned quantitative approaches. Well-defined and controlled cultivation conditions are also ensured. These concepts need to be implemented for the bioreactor cultivation of extreme halophiles as well. Though, due to the high salt requirement of their cultivation, a special corrosion resistant environment is required. Therefore, we have recently constituted a methodological basis for physiological characterization of extreme halophile Archaea in a corrosion resistant bioreactor [26]. There are other examples of using bioreactors with *Haloferax mediterranei* for different purposes [27,28], these works, however, do not assess quantitative approaches.

### 1.3. Optimal Process Parameters for Extreme Halophilic Archaea

There is little known about the optimal and defined cultivation conditions of extreme halophilic Archaea in bioreactors, as the majority of the published studies are only made with shake-flask cultures [29,30]. It is well-known that growth affecting limitations occur in shake-flask cultivations [31], especially with halophilic Archaea's own aerobic metabolism. It should also be added that, at elevated temperatures and at high salt concentrations, the dissolved oxygen concentration is drastically affected, and as low as 0.5 mg/L. Therefore, to avoid oxygen limited culturing, oxygen is provided and the dissolved oxygen level is monitored by a dissolved oxygen electrode. More details about the experimental setup are available elsewhere [26].

In order to make extreme halophiles novel host organisms for future biotechnology, more quantitative, as well as optimization studies on their cultivation should be executed under defined and controlled cultivation conditions. It is well-known that applying different cultivation conditions affect bioprocess performance. For example, applying different cultivation temperatures may affect the characteristics of the respiratory electron transfer activity [17]. For the substrate acetate, the acetate incorporation rates showed pH dependency, as acetate can enter the halophilic cell only in an unionized form [13]. The optima for pH and temperature of extreme halophilic microorganisms were published as relatively broad ranges, 7.0 to 7.5 and 35 °C to 45 °C, respectively [29]. To ensure scale-up and the tight specifications of the potential industrially applicable bioprocesses, more accurate process parameter sets for process robustness are required. According to the main requirements of one application of halophiles, namely hypersaline waste water treatment, the residual concentration of the present organic substances (substrate and metabolites) is of higher interest than the production of biomass. The goal of this work was to find the optima for the process parameters pH and temperature aiming at minimizing the residual substrate concentration, and, at the same time, minimize the by-product formation.

### 1.4. Dynamic Experiments for Process Parameter Optimization

One task in bioprocess development is the optimization of cultivation parameters in order to ensure scale-up. Instead of running labour-intensive continuous cultures with diverse parameter sets [32,33], the use of transient and/or dynamic experiments can help decreasing the time-scale required for bioprocess optimization [34]. The simulation of transient states and fast parameter shifts was also shown to speed up process development [35]. At the same time, however, transient experiments can decrease the quality of the extractable process information. Therefore, the extractable information also needs to be differentiated from random noise [36]. In order to extract the appropriate process information from the dynamic experimental set, a careful experimental design and quantification approach is required.

For instance, a dynamic method with applying pulses was recently published as a faster alternative to set up a feeding strategy for recombinant *Pichia pastoris* fed-batch processes [37]. In the case of extreme halophilic microorganisms, the concerns about the dissolved oxygen availability were previously discussed. Thus, fed-batch cultures with high cell densities for bioprocess development should be avoided to circumvent oxygen limited culturing. For instance, aerobic continuous cultures

with lower biomass concentrations are recommended for extreme halophilic microorganisms. Additionally, with lower maximum specific growth rates ( $\mu_{\max} < 0.2 \text{ h}^{-1}$ ), such as extreme halophilic Archaea, avoiding time-consuming continuous cultures for bioprocess optimization is highly proposed by triggering dynamic experimental conditions with shifts and ramps.

### 1.5. Goal and Structure

This contribution advocates a fast alternative for process parameter optimization with applying pH shifts and temperature ramps on an extreme halophilic continuous culture in a corrosion resistant bioreactor. This work was focussing on studying the changes in the biological activity (volumetric rates, (g/L/h)) over temperature and pH, and did not aim at investigating the Monod kinetics for each state, or even at assessing metabolome analysis. The goal of this study was to minimize the residual substrate concentration and to avoid the by-product formation by optimizing temperature and pH process parameters. Continuous cultivation of the extreme halophilic *Haloferax mediterranei* under non-sterile conditions on the substrate glycerol was chosen as a model system for the dynamic study with the, already established, full quantitative approach for extreme halophiles. Temperature ramps were applied in the 25–40 °C temperature range, while pH shifts were accomplished in the broad physiological range 6.8 to 7.6, along with what was found in literature [29]. The quantitative analysis was based on discussing the response of primary metabolism to the dynamic changes and using multivariate techniques. Based on the results of the described halophilic example, the feasibility of the methodology of dynamic experiments was demonstrated as a general tool for the speeding up of bioprocess parameter optimization.

## 2. Experimental Section

### 2.1. Strains and Cultivation Procedures

*Haloferax mediterranei* (HFX, DSMZ 1411) wild type strain was purchased from DSMZ-German collection of microorganisms and cell cultures. Shake flasks for bioreactor inoculation were grown at 170 rpm and 37 °C in laboratory incubator (Infors, Bottmingen, Switzerland) with the following medium composition and time (g L<sup>-1</sup>): 48 h, NaCl 156; MgSO<sub>4</sub> 7H<sub>2</sub>O 20; MgCl<sub>2</sub> 6H<sub>2</sub>O 13; CaCl<sub>2</sub> 6H<sub>2</sub>O 1; KCl 4; Yeast extract 5; NaHCO<sub>3</sub> 0.2; NaBr 0.5; Glucose 1.0; pH 7.0. Inoculation was carried out with 10% inoculum volume. The benefit of using complex medium for inoculation with a shake flask is to obtain higher cell densities in a much shorter time than using a defined medium.

### 2.2. Defined Medium Composition

The following defined medium composition without the compounds nitrate as well as nitrite was used for HFX according to [38] with modifications (g L<sup>-1</sup>): NH<sub>4</sub>Cl 2; KH<sub>2</sub>PO<sub>4</sub> 0.3; FeCl<sub>3</sub> 0.005; NaCl 194, MgCl<sub>2</sub> 6H<sub>2</sub>O 3.2; MgSO<sub>4</sub> 7H<sub>2</sub>O 4.8; CaCl<sub>2</sub> 2H<sub>2</sub>O 1; KCl 5; NaHCO<sub>3</sub> 0.2; KBr 0.5; 1 mL trace elements solution, Struktol J673 Antifoam 0.2 mL. Trace elements solution (1,000× Stock, mg/100 mL): FeSO<sub>4</sub> 7H<sub>2</sub>O 139; CuSO<sub>4</sub> 5H<sub>2</sub>O 100; MnCl<sub>2</sub> 4H<sub>2</sub>O 50; CoCl<sub>2</sub> 2H<sub>2</sub>O 44; ZnSO<sub>4</sub> 7H<sub>2</sub>O 86. The substrate concentration was 3.5 g L<sup>-1</sup>.

### 2.3. Bioreactor Setup, Cultivation Conditions

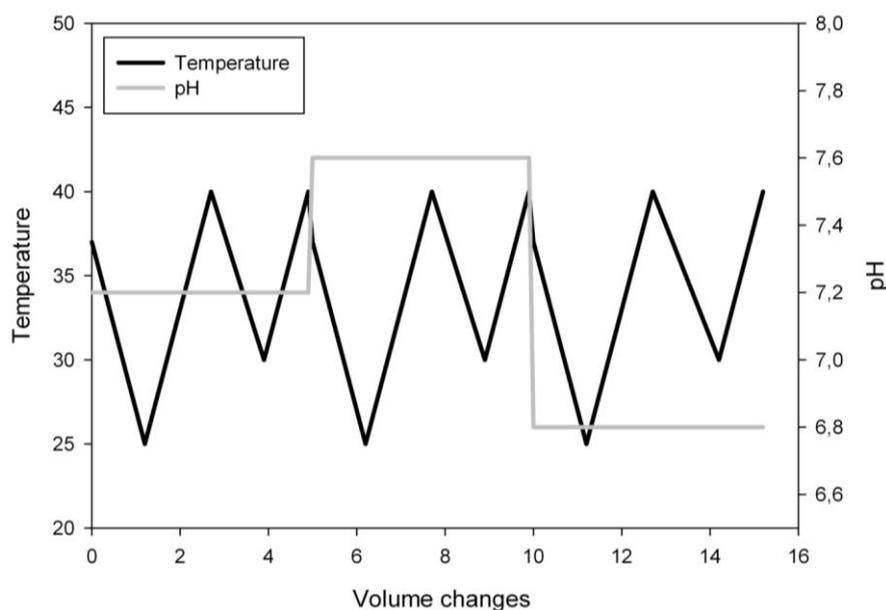
The batch and continuous experiments were carried out in a fully instrumented corrosion resistant 2 L laboratory PEEK Labfors bioreactor (Infors, Bottmingen, Switzerland) with 1 L working volume. The setup was previously described elsewhere [26]. The on-line data monitoring and process control were executed with a Process Information Management System (Lucillus, Biospectra AG, Schlieren, Switzerland). The pH and temperature were controlled by PID controllers. The pH value in the reactor was maintained with the addition of 0.5 M NaOH. The bioprocesses were carried out under non-sterile conditions, due to the high salt requirements of extreme halophiles. The cultivation parameters in the bioreactor were 600 rpm of agitation speed and 1 vvm air inlet flow.

### 2.4. Experimental Design

The chosen dilution rate for the continuous study was  $0.03 \pm 0.005 \text{ h}^{-1}$  which approximately equals to  $\frac{1}{2} \mu_{\max}$ , in accordance with the previously conducted Monod-studies [26]. In the dynamic ramps, the process parameter temperature has been varied by applying temperature ramp down and ramp up, with a slope of  $15 \text{ }^{\circ}\text{C}$  per 1.5 volume change, while maintaining the pH constant (pH control in the bioreactor  $\pm 0.05$ ; T control in the bioreactor  $\pm 0.1 \text{ }^{\circ}\text{C}$ ). Performing continuous culture imposes volume changes in the bioreactor, one volume change is considered as one reactor working volume (1 L) was fed into the reactor and one reactor volume (1 L) was taken out of the reactor at the effluent line.

The concept of the study with synthetic medium is depicted in Figure 1. As it can be seen, the temperature range of  $25 \text{ }^{\circ}\text{C}$  to  $40 \text{ }^{\circ}\text{C}$  was investigated by shifting simultaneously rendering pH values between 6.8, 7.2, and 7.6.

**Figure 1.** The concept of the study with temperature ramps and pH shifts with 1 L bioreactor working volume. On the x-axis, volume changes are depicted. The temperature range was chosen in accordance with meeting the temperature requirements of extreme halophiles and regarding the economical feasibility at the same time. The pH values were chosen to cover the 7.0–7.5 optimal range for growth from the literature.



### 2.5. Off-Line Analytics

Off-line sampling from the bioreactor was executed in regular intervals to determine substrate as well as metabolite concentration and optical density. After sampling, the samples were placed immediately on ice to avoid further reactions. Subsequently, 1 mL of the culture broth was centrifuged in eppendorf tubes at 14,000 rpm for 10 min at 4 °C (Eppendorf centrifuge, Hamburg, Germany). The supernatant was then removed from the top and used for HPLC and enzymatic analysis of the substrate and the metabolite. From the samples, the optical density at 600 nm (OD600) was determined with a photometer (Genesys 20 Photometer, Thermo scientific, Waltham, MA, USA). To prevent the lysis of the samples, 20% NaCl diluent was used for the dilution of the samples for photometric analysis. The substrate and metabolite were quantified by HPLC measurements (Agilent 1100 Series, Santa Clara, CA, USA) with SUPELCOGEL C-610H column (9 µm particle size, 300 × 7.8 mm, Sigma Aldrich, St. Louis, MO, USA) at 30 °C, 0.1% H<sub>3</sub>PO<sub>4</sub> in distilled water (traces of NaN<sub>3</sub>) eluent with 0.5 mL min<sup>-1</sup> flow and with RI detector. Substrates and metabolites cannot be detected under 2 to 5 ppm concentrations, referring to the limit of quantitation of the used analytical methods. Regarding the high salinity of the samples, calibration standards were prepared with the same analytical matrix. For the biomass determination, biomass concentration in the bioreactor can be determined via on-line and off-line methods. The on-line indirect biomass determination, based on the nitrogen balance in the bioreactor, was published elsewhere [26]. For off-line method, there is a linear correlation between OD600 and biomass data of a continuous culture. Therefore, calculating the biomass formulation rates from either OD600 data or biomass data is identical and for the actual biomass concentration calculation, a linear correlation is given.

### 2.6. Data Evaluation

The data evaluation was focussing on the three-dimensional representations of the dependencies on the process parameters pH and temperature. The following dependencies were investigated as the function of pH and temperature, as the biological activity: the volumetric rate of substrate, of by-product, of biomass calculated with using the optical density values. To support the three-dimensional data representation, multivariate data analysis of the obtained dataset of rates was carried out using DataLab software (provided by H. Lohninger, Vienna University of Technology, Vienna, Austria). As the variances, as well as the units of the variables, differed, the data were standardized prior to principal component analysis. The multivariate data analyses were accomplished according to the generally accepted directives for multivariate data treatment.

## 3. Results and Discussion

### 3.1. Ramp Design

The dynamic parameter optimization study on defined medium and glycerol as model substrate was conducted with applying temperature ramps in continuous culture. The dilution rate set point of  $0.03 \pm 0.005 \text{ h}^{-1}$  was applied while maintaining the pH constant in the bioreactor at different pH set points of the shifts. Operating a bioreactor below room temperature with halophilic microorganisms is

unlikely, regarding the elevated temperature requirements of the applied microorganisms [29]. At temperatures higher than 40 °C, however, the extremely low solubility of oxygen becomes critical for conducting aerobic bioprocesses, even with lower biomass concentrations, and not just in the case of bioprocesses with high cell densities, namely fed-batches. The obtained data has been divided into the following four categories: **down 1**: decreasing temperature from 37 °C (40 °C at pH 6.8) to 25 °C with 0.3 °C h<sup>-1</sup>; **down 2**: decreasing temperature from 40 °C to 30 °C with 0.2 °C h<sup>-1</sup>; **up 1**: increasing temperature from 25 °C to 40 °C with 0.3 °C h<sup>-1</sup>; **up 2**: increasing temperature from 30 °C to 40 °C with 0.3 °C h<sup>-1</sup>.

Acetate has been reported as major metabolite by the genus *Haloferax* when they are grown on glycerol [39]. The production of acetate is a result of an overflow metabolism effect on the substrate glycerol, as it was previously analogously described for glucose and ethanol in yeast cultures [40]. When the maximum capacity of the glyoxylate cycle is already reached, by-product formation can be detected. Hence, it is expected that the formation of acetate can show parallel behavior to the accumulation of glycerol in the medium. Acetate is excreted into the medium as metabolite, but can also be taken up again. Therefore, it is also important to minimize or ideally eliminate acetate concentration for a preferably low amount of organic substances in the effluent medium.

### 3.2. Analytics: The Necessity for Off-Line Data

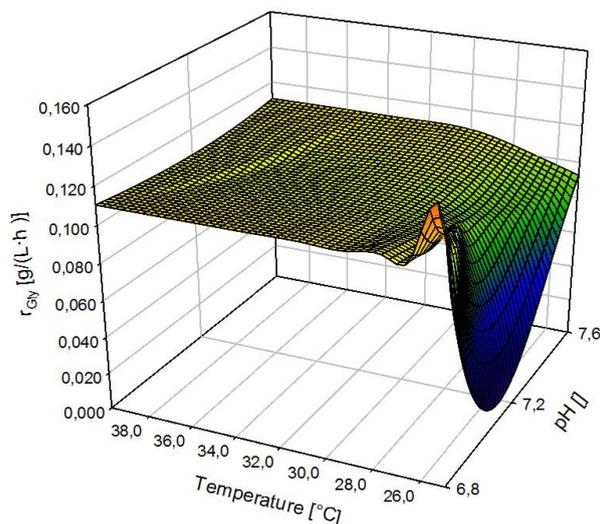
The optical density, residual substrate concentration and by-product formation were measured off-line. A recent study suggests the usage of off-line measurements for a given optimization problem if time and space requirements both allow it [41]. Applying dynamic cultivation conditions, to avoid the superposition of false effects on the measurements, using analytical methods, which offers the least errors, is a prerequisite for differentiating dynamic process information from experimental noises. Moreover, in continuous cultures which offer in general less biological variability than e.g., fed-batches, off-line data with applying the most accurate analytical methods can also help with data evaluation. Therefore, data evaluation, multivariate analyses were always carried out with using the more accurate off-line datasets. For the off-line sampling rate along the dynamic ramps, for an acceptable signal quality, the averaging windows for calculating metabolic rates were much greater (nearly every 5 h) in comparison with the ramp rates (0.2–0.3 °C h<sup>-1</sup>) and the accuracy of the temperature measurements ( $\pm 0.1$  °C).

### 3.3. Ramp Down

As previously discussed in the introduction, the key enzymes in the primary metabolism pathways require elevated temperature for optional functioning. The residual glycerol concentration and the glycerol uptake rate show, therefore, great temperature dependency. The dependency of the latter on pH and temperature is depicted on Figure 2(a). Hence, in order to achieve low residual glycerol concentration, a temperature above 32 °C is recommended. The glycerol uptake rate at biochemically optimal temperatures for halophiles (32–40 °C) shows almost no pH dependency. As glycerol is neither acidic, nor alkaline, the substrate uptake cannot be intensively affected by the pH values in the medium. At the same time, very high operating temperatures are not recommended due to the lower mass transfer between the gaseous and the liquid phase. The acetate by-product formation is therefore

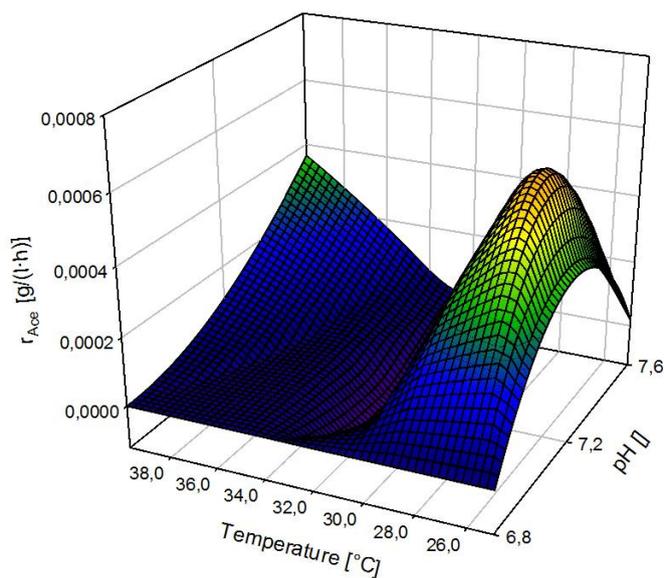
more pronounced at higher temperatures. The reason for that is the adaptation of the metabolic pathways to decreased oxygen supply. Instead of entering the glyoxylate cycle, due to the lower oxygen supply, by-product formation can be observed. The switch between oxidative growth and acetate formation is very sensitive to the state of the oxygen supply. Hence, at higher temperatures, the glycerol which is taken up is also partly metabolized into acetate. As acetate is an acidic by-product, its production can be influenced by the pH in the medium. The dependency of the acetate production rate on both pH and temperature is shown on Figure 2(b). As observed, at higher pH values, more acetate by-product was formulated. Hence, for minimizing by-product formation and maximizing substrate uptake, lower operating pH values are recommended (6.8–7.0), in combination with higher temperatures. Comparing the rates of biomass production, which are depicted on Figure 2(c), with the acetate formation, they clearly show how the switches between different metabolic states are influenced at lower temperatures. This may imply that the activity of the glyoxylate cycle are even more sensitive to temperature than the activities of the metabolic steps, where glycerol is first metabolized to pyruvate and then to acetate. In this case, therefore, the production of acetate indicates less production of biomass. It can be concluded that two different effects have to be counterbalanced regarding the optimal temperature choice. Higher temperatures are better for the biochemical processes and, at the same time, the oxygen mass transfer is decreasing with an increase in the temperature. The optimal pH values are the lower pH values, where the production of the acidic by-product is therefore less pronounced, in accordance with the reported pH dependency of acetate formation [13].

**Figure 2.** (a) The dependency of the glycerol uptake rate on the process parameters pH and temperature for temperature ramp down 1. A significant temperature dependency of the glycerol uptake rate can be observed which is interpreted by the increasing activity of metabolic pathways at elevated temperatures. The glycerol uptake rate is not intensively affected by pH as a consequence of glycerol being neither acidic nor alkaline. (b) The dependency of the acetate production rate on the process parameters pH and temperature for temperature ramp down 1. The acetate production is strongly affected by both pH and temperature. (c) The dependency of the biomass production rate on the process parameters pH and temperature for temperature ramp down 1.

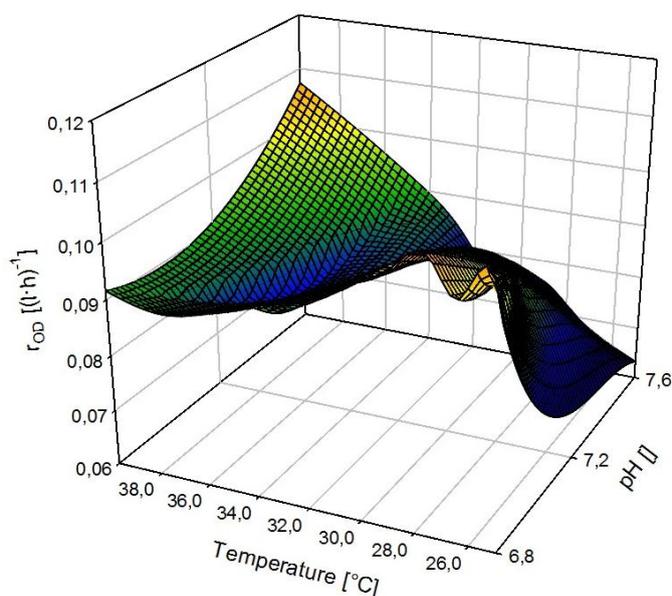


(a)

Figure 2. Cont.



(b)



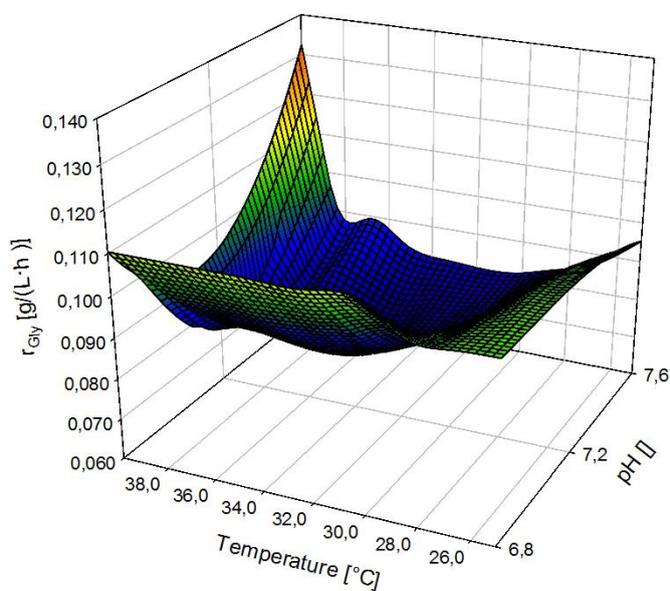
(c)

### 3.4. Ramp Up

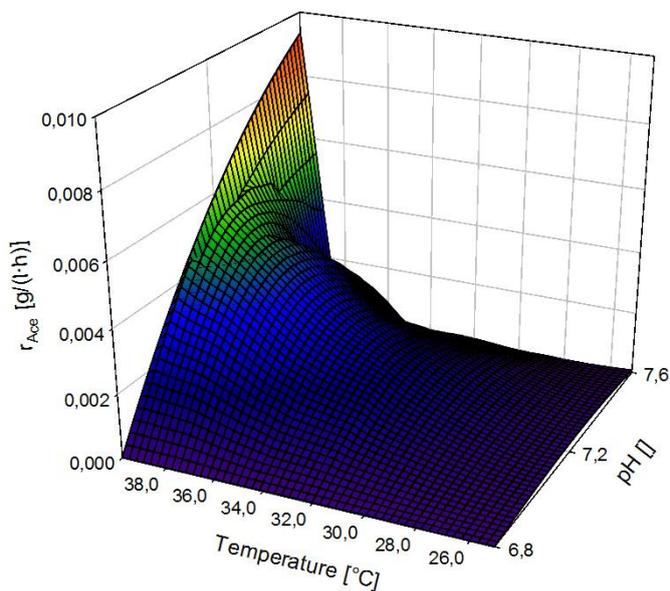
In contrast to the ramp down experiments, in this case increasing temperature ramp was applied. As it was previously presented, the activity of the metabolic paths is negatively affected by the lower temperatures. In the ramp up, the influences of the higher temperature range can be observed; the mass transfer between the gaseous and the liquid phase will be more affected at higher temperatures. For the glycerol uptake, however, as discussed in the ramp down section, higher temperatures are preferred. The changes in the glycerol uptake rate with pH and temperature for ramp up 1 are presented in Figure 3(a). At elevated temperatures, due to mass transfer limitations, glycerol is accumulated. This glycerol accumulation is even more significant at higher pH values. Higher pH values are also more suitable for the production of acetate as it can be seen in Figure 3(b), which shows similar acetate

formation tendency as in Figure 2(c). At the same time, the entering to the glyoxylate cycle is affected, the production of acetate can be observed at higher pH values, and more glycerol is accumulated as well. In accordance with the ramp down observations, the glycerol uptake at biochemically optimal temperatures for halophiles (32–40 °C) shows almost no pH dependency. Biomass production shows the same tendency, as previously observed, higher temperatures at lower pH values are more favorable for microbial growth. Figure 3(c) shows the dependency of biomass production on pH and temperature for ramp up 1.

**Figure 3.** (a) The dependency of the glycerol uptake rate on the process parameters pH and temperature for temperature ramp up 1. (b) The dependency of the acetate production rate on the process parameters pH and temperature for temperature ramp up 1. (c) The dependency of the biomass production rate on the process parameters pH and temperature for temperature ramp up 1.

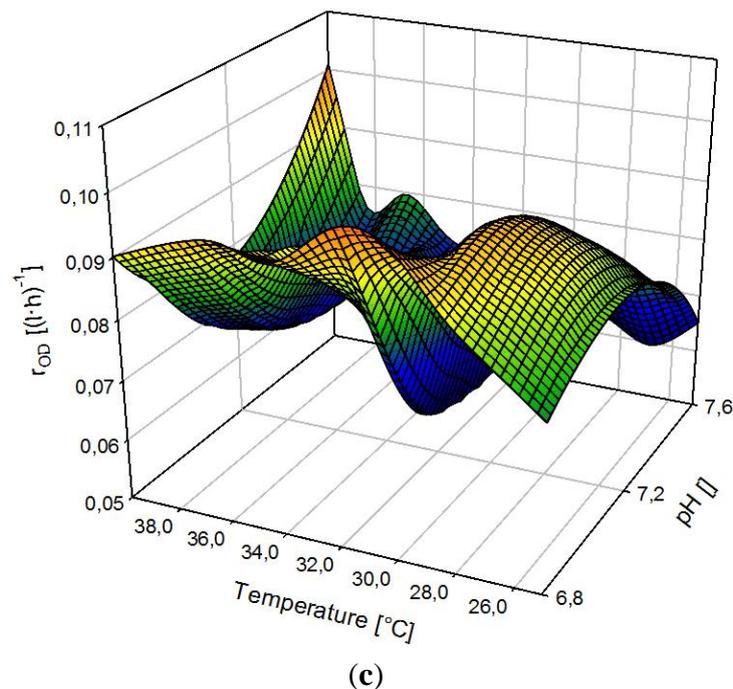


(a)



(b)

Figure 3. Cont.

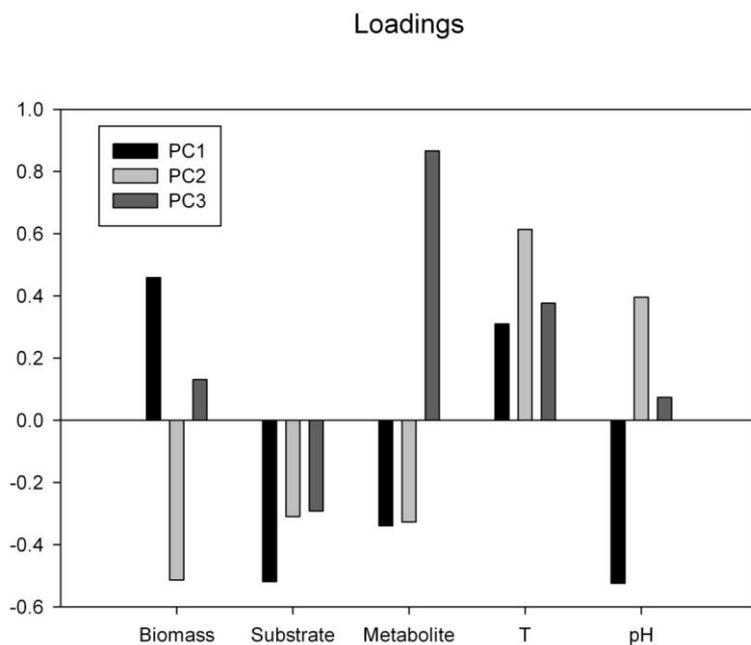


### 3.5. Multivariate Data Analysis: Supporting the Findings of Temperature and pH Optima

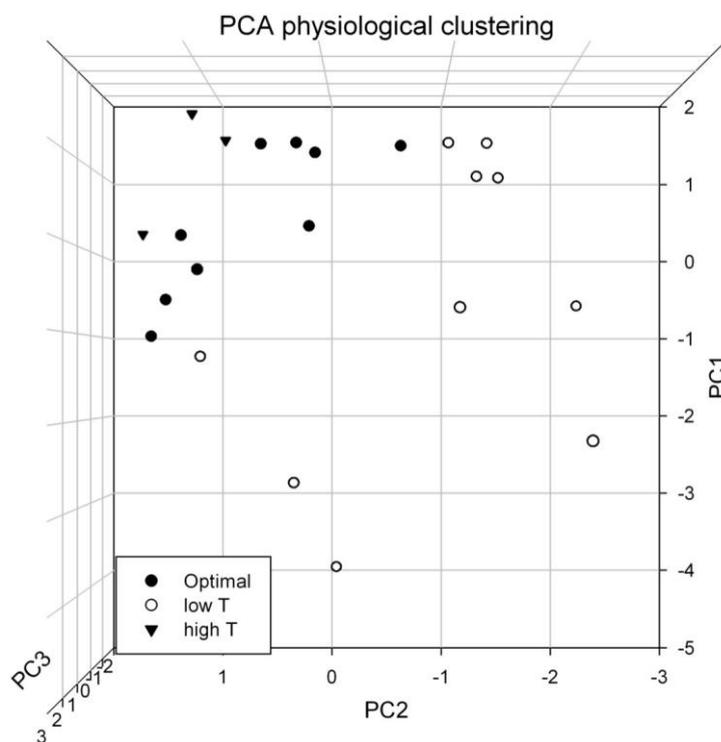
Experimental data clustering is commonly made by using the tools of multivariate data analysis [42]. Principal component analysis has been already reported as a tool for the differentiation among different physiological states of the culture and for recognizing process affecting physiological excursions [43]. Principal component analysis was carried out using standardized variables, the process parameters pH and temperature and the off-line volumetric rates of biomass, substrate as well as the metabolite acetate, including every ramp up and ramp down experiment.

The obtained results are depicted in Figure 4. The first three principal components could cover more than 90% of the variance, and therefore were used for data representation. Figure 4(a) summarizes the loadings of the variables on the principal components. It can be observed how the loadings of the variables vary on the first two principal components which can rather help with data separation by temperature. The loadings on the first and third principal components, especially the loading of the variable acetate on the third principal component, is more pronounced. As previously discussed, the formation of acetate as by-product shows strong pH dependency. Figure 4(b) indicates how the experimental data could be separated by the first two principal components in order to cluster by the physiological differences reasoned by the process parameter temperature. Experimental points at higher temperatures were slightly different from the optimal temperature points, which can be reasoned by the limitation on the oxygen mass transfer at higher temperatures. The experimental points at lower temperatures could be also differentiated due to the previously discussed biochemical limitations on the activity of the metabolic pathways. Taking all of the experimental points into consideration, they clearly show how the data fit to a trend along high temperature and optimal temperature to low temperature. Results from the multivariate data analysis were shown to remarkably support the observations of the three-dimensional data representations.

**Figure 4.** The results of the principal component analysis with the chosen standardized off-line variables performing on all experimental data along the ramps. **(a)** The loadings of the chosen variables on the principal components. It can be seen which variable has significantly greater loading on which principal component. **(b)** In order to obtain clustering by the physiological differences reasoned by the process parameter temperature, the first principal component vs. the second principal component is depicted. The variance along the third principal component can help with the clustering based on the pH values (data not shown).



(a)



(b)

### 3.6. Verification of the Dynamic Experimental Results

The specific rates on biomass are commonly used for the extraction of relevant process information on biological activity [44]. Under dynamic conditions, the specific substrate uptake rate on biomass can offer information on the changes in the metabolic state. The cornerstones for Monod kinetics for halophilic microorganisms were already shown in chemostat continuous cultures [26]. Applying dynamic temperature ramps and pH shifts, however, the equilibrium with the hydraulic dilution in continuous cultures is not reached. Therefore, the results of the dynamic study were verified with comparing the specific substrate uptake rates with a suitable steady-state of a continuous culture and also with a batch culture. Table 1 summarizes the comparable rates of the four different ramps with pH 7.2 and the optimal temperature range. Using ANOVA, applying 10% as the level of significance, it was proved that there are no significant differences in the specific substrate uptake rates among the dynamic and the steady-state processes.

**Table 1.** Comparison of the different process modes for the verification of the dynamic experiments. The specific substrate uptake rates of dynamic ramps, steady state continuous conditions and batch results were compared with the same pH value, in the optimum temperature range and with comparable dilution rates. In accordance with the three-dimensional ramp representations, for better overview, the volumetric rates are also included in each case. Investigating the specific substrate uptake rates, ANOVA results revealed that no significant difference was obtained among the dynamic experiments and the steady state continuous culture.

Experiment	dynamic conditions				steady state	
	down 1	down 2	up 1	up 2	continuous culture	batch culture
T (°C)	37–36	38–36	38–39	36–37	37	37
pH	7.2	7.2	7.2	7.2	7.2	7.2
D = $\mu$ (h <sup>-1</sup> )	0.030 ± 0.005				0.035 ± 0.003	$\mu_{max}$
rGly (g/L/h)	0.104	0.071	0.076	0.057	0.120	0.108
rAce (g/L/h)	0.003	0.008	0.005	0.004	0.002	0.002
rOD (g/L/h)	0.080	0.052	0.067	0.049	0.070	0.083
qS (g/g/h)	0.056 ± 0.005	0.050 ± 0.005	0.051 ± 0.005	0.053 ± 0.005	0.047 ± 0.005	0.054 ± 0.005

### 3.7. Data Repeatability and Reproducibility

As the aim of the study is to describe the applicability of a novel and general dynamic method for accelerating bioprocess parameter optimization, data repeatability, as well as reproducibility, must be discussed. For the repeatability of the ramps, the following should be considered. The indicated deviations in Table 1 can be reasoned by the slightly different substrate concentrations in the different feed charges. Furthermore, due to the experimental noises, the ramps results can only be obtained with approx. 10% uncertainty. The presence of the adaptation to the environmental perturbations can however impose slight changes, the reproducibility has been tested with repeating particular ramps, both ramps up and down were carried out in duplicates. There were no significant effects found on the relevant experimental results as ANOVA declared no significant differences (qS, Table 1). Hence,

with the accomplished repeatable and reproducible experiments based on dynamic methods, the main effects on process parameters pH and Temperature could be statistically anticipated.

#### 4. Conclusions

The applications of dynamic experiments for process parameter optimization can offer faster alternatives in the early phase of bioprocess development with bioindustrial relevances. Optimizing process parameters temperature and pH is of highest interest for a physiologically robust bioprocess. This study presents a dynamic process parameter optimization study, using temperature ramps and pH shifts in continuous culture, as a novel process development tool, with an extreme halophilic strain as an example in a corrosion resistant bioreactor.

The work focused on the quantitative investigation of the biological activity along the applied ramps and shifts in continuous culture extracting the process relevant information from the experimental data, without providing full kinetic, as well as metabolomic assessment. The three-dimensional representations of the obtained datasets could help with the identification of the optimal ranges for temperature and pH as well. The results were physiologically interpreted and interlinked with the primary carbon metabolism of extreme halophilic Archaea. On one hand, multivariate data analysis could help with supporting the observations of the three-dimensional data evaluations. Data clustering with principal component analysis was shown to be an efficient tool for separating different physiological states reasoned by different temperatures and pH values.

On the other hand, the results of this dynamic study were verified with comparing the obtained biological activities along the ramp studies with results of steady-states of continuous cultures and batch cultivations. The robustness of the dynamic experiments was reflected in the acceptable reproducibility along the ramp downs and ramp ups within 10% uncertainty. Hence, the here presented dynamic methodology can serve as a general process development tool for accelerating bioprocess development.

#### Conflicts of Interest

The authors declare no conflict of interest.

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