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Process optimization of integrated ectoine production and secretion under osmotic stress in a cascade of continuously operated bioreactors

Claas Fallet, Peter Rohe, Ezequiel Franco-Lara*
Institute of Biochemical Engineering, Technische Universität Braunschweig
Gaußstr. 17, 38106 Braunschweig, Germany
*Corresponding author: e.franco-lara@tu-bs.de

Process conditions for the synthesis and subsequent secretion of ectoine and hydroxyectoine were optimized in a cascade of two continuously operated bioreactors. In the first bioreactor the halophile bacterium *Chromohalobacter salexigens* was grown under continuous hyperosmotic and thermophilic conditions and therefore under constant stress driving the cells to accumulate large amounts of ectoines. Crossflow ultrafiltration was employed to concentrate biomass and therefore enhance high cell density cultivation. In the coupled second bioreactor the concentrated cell broth was osmotically down-shocked by addition of distilled water, forcing the cells to secrete the accumulated intracellular ectoines into the medium in order to avoid bursting.
The cultivation conditions in the first bioreactor were optimized with respect to growth temperature and medium salinity to reach the highest productivity; the second bioreactor was optimized using a multi-objective approach for maximal secretion with a simultaneous minimization of cell death and product dilution. Depending on the cultivation conditions, intracellular ectoine and hydroxyectoine contents up to 540 and 400 mg per g cell dry weight were attained, respectively. With a maximum specific growth rate of 0.3 h^{-1} in defined medium, productivities of approximately 2.1 g L^{-1} h^{-1} secreted ectoines in continuous operation were reached.

1. Introduction

In order to survive the osmotic stress in hypersaline environments such as salt lakes or salines, halophile microorganisms have developed highly sophisticated adaptation mechanisms. Two principally different mechanisms can be distinguished: salt-in-strategists adjust their turgor (intracellular pressure) by uptake of charged inorganic molecules (mainly K^+ and Cl^-) from the environment, since their sensitive cellular components are structurally adapted to high intracellular salt concentrations but do not resist low salinities.
Salt-out-strategists on the other hand protect themselves by synthesis and/or import of so called compatible solutes (Csonka and Epstein, 1996) such as ectoine or hydroxyectoine in case of the Gram-negative bacterium *Chromohalobacter salexigens* (Arahal, Garcia et al., 2001). These compatible solutes usually do not interfere with the

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metabolism and – besides providing osmotic activity – have the ability to protect proteins, membranes and even whole cells *in-vivo* and *in-vitro* against denaturation, inactivation and inhibition by heat or hyperosmotic stress (Diamant, 2001; Galinski, 1993) which makes them interesting candidates for biotechnological and especially cosmetical applications (Roberts, 2005).
Halophiles excel in containing high mass fractions of compatible solutes in hyperosmotic media (10-20 % w/v) (Lentzen and Schwarz, 2005), therefore they are convenient producer strains for production of compatible solutes. As the demand for ectoines as stabilizers for biocatalytic processes with enzymes and whole cells will undoubtedly rise in the future, the optimization of its production is most desirable. Although large scale production of ectoine and hydroxyectoine is commercially established applying batch (Sauer and Galinski, 1998; Schiraldi, Maresca et al., 2006) and continuous cultivation (Schubert, Maskow et al., 2007), the potential of productivity increases remains either by constructing recombinant producer strains or by the application of goal-oriented process development as the one approach presented here.

2. Material and Methods

2.1 Organism and medium

Chromohalobacter salexigens DSM 3043 (Arahal et al., 2001) was grown in defined minimal medium containing 5.56 g L^{-1} $(NH_4)_2SO_4$, 15.86 g L^{-1} K_2HPO_4, 1.4 g L^{-1} $MgSO_4 \cdot H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 10 g L^{-1} glucose as the only carbon and energy source and 108.1 g L^{-1} NaCl for continuous cultivation feed and 12.12 g L^{-1} $(NH_4)_2SO_4$, 15.86 g L^{-1} K_2HPO_4, 1.4 g L^{-1} $MgSO_4 \cdot H_2O$, 0.01 g L^{-1} $FeSO_4 \cdot 7H_2O$, 5 g L^{-1} glucose and varying NaCl concentrations for batch cultivation. Precultures for all bioreactor cultivations were grown to mid-exponential growth phase in 1 L baffled shake flasks (containing 0.2 L medium as used for continuous cultivation) at 37°C and 110 rpm.

2.2 Bioreactor system

Cultivation of *C. salexigens* took place in a cascade of two continuous stirred tank reactors that were connected in series (Figure 1). The two reactors are referred to as system 01 (production unit) and system 02 (secretion unit). In continuous mode sterile fresh medium was pumped from the feed tank into system 01, distilled water was pumped from the water tank into system 02 and culture broth was pumped from system 01 into system 02 and from system 02 into the waste tank. Both systems were 3 liter glass bioreactors (Applikon, the Netherlands) provided with pH and temperature control, 3 baffles, 2 six-bladed impellers, sampling tube, air sparger, pO_2-probe and exhaust gas analysis (Bluesens, Germany). The pH was set to 7.5 and controlled by addition of 6 M NaOH (+ NaCl as in the medium) or 1 M HCl (+ NaCl as in the medium). Agitation was fixed at 1000 min^{-1} and antifoam agent was added regularly in intervals of 2 hours. The systems were monitored and controlled by ADI 1010 Biocontrollers and software BioXpert NT version 2.70.115 (Applikon, the Netherlands).

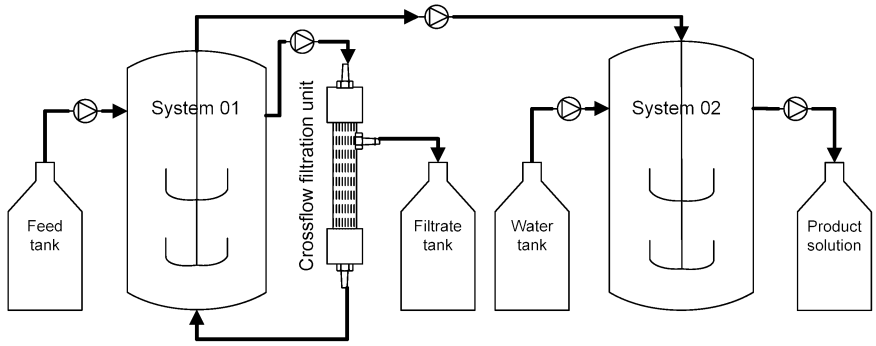

Figure 1: Setup of the bioreactor cascade and flows entering and leaving the systems.

System 01 also contained a crossflow filtration unit which was installed as a bypass. The filtration unit consisted of a hot-water-sterilizable MOLSEP FB02-CC/FUS-5081 hollow fibre module (DAICEN MembraneSystems, Japan) with a cutoff of $0.5 \cdot 10^6$ Dalton and a 55 series rotary lobe pump (JABSCO, Germany). Transmembrane pressure was set using an EPDM membrane valve type 612 (GEMÜ, Germany) and monitored by 3 pressure sensors (WIKA, Germany). Filtrate flow was measured by an oval gear flow meter (B.I.O.-TECH, Germany) and controlled using a EPDM membrane valve type 3233 (Bürkert, Germany).
The working volume of system 01 was 1.2 L (including the crossflow filtration module). The system was supplied by 1.0 L min^{-1} sterile air and 1.0 L min^{-1} sterile oxygen to guarantee dissolved oxygen concentrations greater 20 % saturation in the system. The feed stream was pumped into the system with pumps ISM 597 D and ISM 596 D (ISMATEC, Switzerland). System 02 was fed additionally with distilled water from a separate water tank. The working volume of system 02 was 2.0 L and the system was supplied by 2.0 L min^{-1} sterile air to guarantee dissolved oxygen concentrations greater 20 % saturation in the system.

2.3 Analytical Methods

Samples taken from the bioreactors every 30 min (batch cultivations) or in steady state (indicated by constant oxygen uptake rates in continuous mode) were centrifuged at 10.000 min^{-1} for 10 min. Supernatant and cell pellet were separated and stored at -20°C for further analysis.
Cell mass was analyzed by gravimetric measurement and cellular viability was determined (omitting centrifugation of the sample) by counting the colony forming units on Luria-Bertani agar plates (with 10 % w/v NaCl) after 24 hours of incubation at 36°C. Glucose concentration was measured using an enzymatic analyzer 2300 STAT Plus (YSI, Ohio, USA). Intracellular Ectoine and hydroxyectoine contents were quantified after Bligh & Dyer extraction (Bligh and Dyer, 1959) of the pelleted biomass. Extracellular concentrations were measured directly at wavelength 215 nm using a LaChrom^{TM} HPLC (Merck-Hitachi, Germany) with a NucleoSIL 120x5 C18 AQ-plus column (Knauer, Germany).

3. Results and Discussion

Temperature and salinity of the culture medium strongly influence growth characteristics and compatible solute production of *C. salexigens* (Calderon, Vargas et al., 2004). 26 batch cultivations were conducted to investigate the influence of temperature and salinity on specific growth rate μ_{max}, yield coefficient $Y_{x/s}$ (mass cells produced/mass glucose consumed) and the intracellular solute content a_{sol} within the range of temperatures (T) 32.0°C to 42.0°C and NaCl concentrations (c_{salt}) between 58 and 175 g L^{-1}. A Central Composite Design was chosen as basis for experimental setup following the guidelines of statistical Design of Experiments (Montgomery, 2005).
It was found that in constant hyperosmotic conditions the intracellular solute concentrations remained constant in growing cells (data not shown). In conclusion synthesis of compatible solutes could be treated as growth associated in the bioreactor which is explained by the simple fact that growing cells require compatible solutes for maintaining osmolytic balance inside the individual cells. Some authors reported increasing solute content for cells entering the stationary phase (Calderon et al., 2004), but this effect was not observed for *C. salexigens*, although the balance between ectoine and hydroxyectoine shifted towards the latter within the first hours after cessation of growth (data not shown).

3.1 System 01: production of compatible solutes

An important index to compare the performance of different bioreactor systems is the biomass productivity P, or in this case – when the desired product is stored inside the biomass and a_{sol} denotes the fraction of compatible solutes in the biomass - the productivity of compatible solutes P_{sol}, which is given for continuous cultivation by

$$P_{sol} = a_{sol} \cdot D \cdot X = a_{sol} \cdot D \cdot Y_{x/s} \left(S_0 - \frac{k_s \cdot D}{\mu_{max} - D} \right) \quad (1)$$

assuming Monod growth kinetics and sterile feed streams (D = dilution rate, X = biomass concentration, S_0 = substrate concentration entering system 01, k_s = Monod constant). The solute productivity P_{sol} reaches a maximum value $P_{sol,max}$ when $d(a_{sol} \cdot D \cdot X)/dD = 0$:

$$P_{sol,max} = a_{sol} \cdot \mu_{max} \cdot Y_{x/s} \left(S_0 + 2 \cdot k_s - 2 \cdot \sqrt{k_s \cdot (S_0 + k_s)} \right) \quad (2)$$

The Monod constant k_s exerts only a small influence on equation (2) and was assumed to be constant under all conditions. Inserting the respective values μ_{max}, $Y_{x/s}$, a_{sol} = f (T, c_{salt}) obtained by batch cultivation experiments into equation (2), the maximum productivity in continuous cultivation was estimated at the investigated temperatures and salinities. These experimental data (not shown) were fitted to a quadratic optimization model (Montgomery, 2005) and yielded the estimated maximum productivity of compatible solutes (combined ectoine and hydroxyenctoine content) $P_{sol,max}$ = 1.51 mmol L^{-1} h^{-1} at T = 37.5°C and 108 g L^{-1} NaCl.
Continuous cultivations were conducted to verify the predicted productivities at various dilution rates D and under optimal cultivation conditions (T= 37.5°C and 108 g L^{-1}

NaCl) without integrated crossflow filtration. Biomass as well as the intracellular content of ectoine, hydroxyectoines and compatible solutes (sum of ectoine and hydroxyectoine) was quantified and the respective productivities calculated according to equation (2) (Figure 2a).

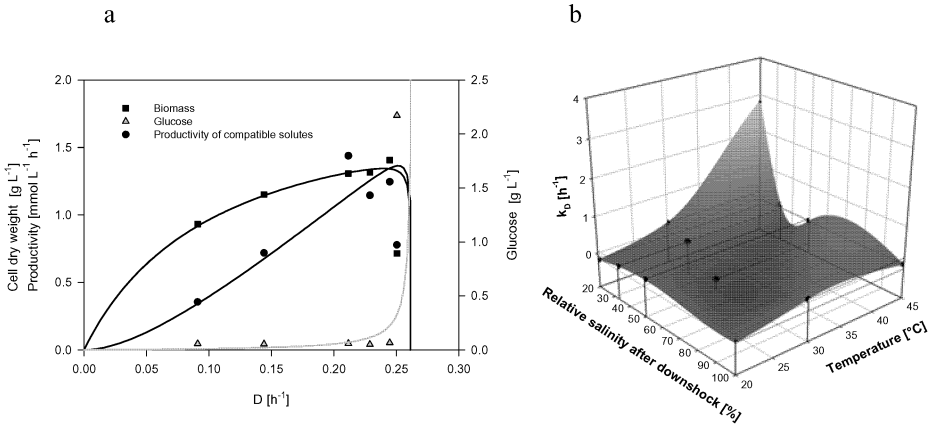

Figure 2: Multi-objective optimization. a) Biomass, substrate and max. productivity of total combined compatible solutes in system 01 for S_0 = 5 g L^{-1} glucose, k_s = 0.014 g L^{-1}. b) Specific death rate k_d under various hypoosmotic downshock conditions

To fit the data with a suitable model substrate consumption for maintenance was considered (Pirt, 1975). This provided a reasonable fit for the experimental data (maintenance coefficient m = 0.27 g L^{-1}, true yield $Y_{x/s\ (true)}$ = 0.4). The maximum productivity of compatible solutes was experimentally found to be 1.36 mmol L^{-1} h^{-1} which comes close to the predicted value of 1.51 mmol L^{-1} h^{-1}.
After establishing and validating continuous compatible solute production, high cell densities of up to 61 g_{cdw} L^{-1} were achieved at a dilution rate of 2.45 h^{-1} and S_0 = 10 g L^{-1} glucose by cell retention applying crossflow filtration. This resulted in an intracellular productivity of $P_{sol,max}$ = 14.9 mmol L^{-1} h^{-1} which sums up to approximately 2.2 g L^{-1} h^{-1} compatible solutes (relating to the operating volume of 3.2 L).

3.2 System 02: secretion of compatible solutes

Preliminary experiments conducted with cells transferred to various hypoosmotic conditions in shake flasks yielded optimal secretion conditions of the accumulated compatible solutes when the cells where shocked from 108 g L^{-1} to 54 g L^{-1} NaCl and 30°C (data not shown). At these conditions, no cell death (Figure 2b) or unspecific release of proteins into the medium occurred, secretion was almost complete and dilution was minimal as compared to hypoosmotic shocks to < 20 g L^{-1} NaCl (data not shown). When connected to system 01, the secretion unit served the purpose of forcing *C. salexigens* to release its compatible solutes into the medium. In continuous operation, up to 95 % (2.1 g L^{-1} h^{-1}) of the intracellular solutes were secreted by the cells into the medium of system 02 and were recovered as product solution.

4. Conclusions

Only negligible cell death was observed in the crossflow filtration or the secretion unit at optimal operating conditions. No citrate, formate, acetate, propionate or butyrate was detected in noteworthy amounts. No ectoine or hydroxyectoine was detected in the cultivation broth of system 01. Together these findings indicate that high cell density cultivation, pumping the cells through a filtration unit and exposure to a hypoosmotic shock did not alter the cell metabolism in undesirable ways or kill the cells.

The introduced cascade outperforms hitherto reported processes for production of compatible solutes (Sauer and Galinski, 1998; Schiraldi et al., 2006; Schubert et al., 2007) in terms of productivity and exemplifies the potential of process optimization by continuous cultivation with integrated downstream processing modules.

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