

Upstream Bioprocessing in Food and Feed Applications

Ulrike Rasche

Eppendorf AG, Bioprocess Center, Juelich, Germany

Contact: rasche.u@eppendorf.com

Executive Summary

Bioprocessing has been used for decades for the industrial production of beverages and for the production and preservation of food and feed ingredients. While these applications are still crucial, new ones are being added, such as the production of new food and feed additives, functional foods and various types of novel foods. Many interventions are needed to improve product characteristics, ensure consistent quality, and reduce

production costs: strain engineering, optimization of process parameters, handling of difficult process conditions, and more. There are many interesting examples in the literature of how researchers tackle the challenges in food and feed applications, both old and new. In this white paper we introduce several of them that provide valuable insights and ideas.

Introduction

“Mix water and flour with a little yeast and incubate the dough in a warm place until its volume has approximately doubled” – such household baking instructions will (or may not) produce a decent result, but are not what is needed in industry. Bioprocess engineers in the food and feed industry carefully optimize producer strains and process conditions to manufacture products of high and consistent quality in an economical way. The ability to monitor and control critical process parameters is a prerequisite. Which strain is best? What temperature is optimal? And what process run time? Bioreactors and fermentors are the cultivation system of choice when process conditions need to be monitored, controlled, and reproduced, either to optimize a production process or to mimic environmental factors in applied research.

Find the right producer strain – Make meaningful comparisons

Microbial fermentation is a valid alternative to chemical synthesis for the production of many food and feed additives. It uses organisms, mostly bacteria and fungi, to naturally synthesize the product of interest. The demand for food and feed additives is high. The global lysine market for animal nutrition applications, for example, was estimated at over 1.9 million tons in 2014 [1]. It is not surprising that wild-type strains are not always suitable for developing a commercially viable production process. Bioprocess engineers often aim to optimize the producer strain to increase product yields, either by random mutagenesis and/or by genetic engineering of the metabolic pathways.

An example was given by Chen *et al.* who aimed to improve lysine production in *Corynebacterium glutamicum*

[2]. They engineered *C. glutamicum* strain LP917, which contains a mutation in the phosphoenolpyruvate carboxylase (PEPC) gene. The enzyme is at a relevant switch point for carbon flux distribution within the central metabolism. The mutation made the enzyme insensitive to feedback inhibition. To compare the performance of the mutant LP917 with the host strain LC298, the researchers performed parallel batch fermentations controlled at 30 °C, 30 % dissolved oxygen and pH 7.2. They found that the deregulation of the PEPC's feedback inhibition led to reduced cell growth but increased lysine yield by 37 %.

Gassel *et al.* chose a combinatorial approach to intensify the production of astaxanthin by the fungus *Xanthophyllomyces dendrorhous* [3]. This carotenoid is used as feed additive for salmon and trout farming. They first applied random mutagenesis and screened for astaxanthin-overproducing mutants. They then further optimized one of the mutants by genetically modifying the astaxanthin biosynthesis pathway. When comparing cell growth and astaxanthin production in controlled fermentations, differences in growth rate, total biomass production, and astaxanthin concentration were found, indicating that the mutational and engineering effects were efficient and stable.

Researchers from the Technical University of Denmark and Chalmers University of Technology in Sweden went one step further to improve production of the antioxidant resveratrol, which is used as a dietary supplement and cosmetic ingredient [4]. To avoid the need for expensive substrates, the researchers introduced a biosynthesis pathway for *de novo* resveratrol production directly from glucose or ethanol into *Saccharomyces cerevisiae*. In batch fermentations

controlled at 30 °C, pH 5.0, and DO > 30 % they compared cell growth, resveratrol production, yield, and productivity of strains expressing different combinations and copy numbers of relevant enzymes.

In all examples bioprocess engineers systematically compared the performance of different producer strains to improve production of the molecule of interest. Comparisons of cell growth, yield, and productivity are only meaningful, if data is acquired under comparable process conditions. Fermentors have proven their value, because they allow monitoring and control of critical process parameters like temperature, pH, and dissolved oxygen.

Produce more and better products – Optimize process parameters

Strain optimization is only one factor in improving bioprocesses. To optimize cell growth, product yield, and product quality, culture conditions must also be optimized.

A critical process parameter is the culture pH. A study from Subramanian *et al.* gives an example. The group studied lactic acid production by *Enterococcus faecalis* CBRD01 [5]. In the course of shake flask cultures, the pH of the culture decreased from initially 7.0 to 5.3 due to the production to lactic acid, and the decrease of the pH impaired lactate production. This problem was circumvented in cultures in fermentors, in which the pH was automatically controlled at pH 7.0. Cultivation in fermentors resulted in a 2.9-fold improvement in lactate production compared to the shake flask method.

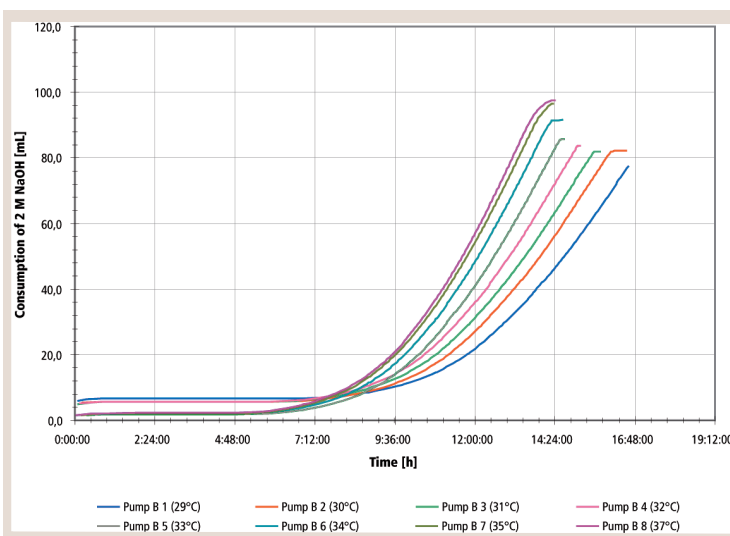


Figure 1: Cultivation of *Lactobacillus sp.* in an 8-fold DASGIP® Parallel Bioreactor System. Bacterial growth at various temperatures was compared. NaOH consumption was used as a proportional indicator for growth-dependent acidification.

Bioprocess engineers at the Austrian company Biomin® GmbH examined several process parameters to find optimal process conditions for the expansion of the silage inoculant *Lactobacillus sp.* [6]. Parameters included medium composition, agitation, pH, and temperature. The goal was to identify process conditions that lead to a maximum yield of viable cells. The need to test many different process conditions is typical for the development of bioprocesses. The researchers at Biomin tackled this challenge with a DASGIP Parallel Bioreactor Systems that allowed them to perform eight fermentations in parallel. Figure 1 shows the result of one such fermentation run, in which bacterial growth curves at eight different temperatures were compared.

A study by Martinez-Moreno *et al.* illustrates how the data from controlled fermentations can be analyzed [7]. In winery, ammonium is often added to the must to prevent nitrogen deficiency during fermentation. The researchers from the University of Rioja used parallel fermentation systems to systematically analyze how the nitrogen content of the fermentation broth influences the growth of the yeast and quality-related parameters, such as the production of volatile compounds, over time. They analyzed the data using principal component analysis. The results showed how the flavor profile of the wine was influenced by the fermentation time and must composition.

Scale-down models are often used to troubleshoot and implement process changes. The models recapitulate the parameters and performance of the original bioprocess, but require less media and supplements. An example was recently described by researchers at Evonik® [8]. They reduced the working volume of an amino acid fermentation process by a factor of ten. By transferring critical process parameters such as feeding profiles, tip speed, and pH, DO, and temperature set points from a 2-liter vessel to a DASbox® Mini Bioreactor System, they achieved comparable cell growth, product concentrations, and product yields at both scales. To recapitulate process parameters during scaling, it is important to choose fermentors with similar vessel geometries (Figure 2) and capabilities across scales, for example in terms of tip speed and oxygen transport capabilities.

These examples highlight some of the challenges in process development in food and feed applications: Critical process parameters need to be monitored and controlled, often many process conditions need to be compared, and the results need to be statistically analyzed. Parallel bioprocess systems have the advantage that multiple experimental

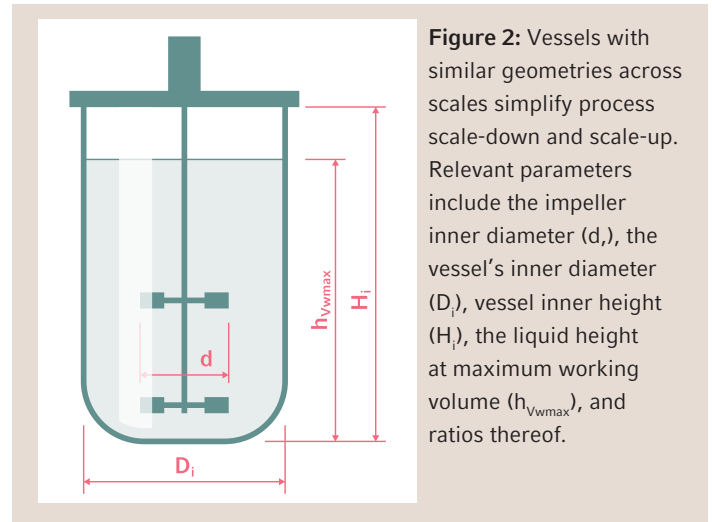


Figure 2: Vessels with similar geometries across scales simplify process scale-down and scale-up. Relevant parameters include the impeller inner diameter (d), the vessel's inner diameter (D_i), vessel inner height (H_i), the liquid height at maximum working volume ($h_{V_{wmax}}$), and ratios thereof.

parameters can be tested simultaneously in one run, which ensures maximal comparability between runs and saves precious time. Process scalability should always be considered during process development, in order to be able to develop scale-down models and to scale-up the process from development to production.

Analyse complex systems – Mimic the environment

We don't always need to optimize process parameters alone. In some application in the food and feed sector it is more important to mimic *environmental* parameters.

An example is the improvement of silage inoculants. During ensiling, process conditions change from aerobic (when the green fodder is prepared), to anaerobic (during the ensiling process itself), and back to aerobic (when the silage is opened). The occurrence of aerobic conditions after opening is critical for silage quality, because it might



Figure 3: DASGIP Parallel Bioreactor System.

promote the growth of spoiling microorganisms. Inoculating the fresh biomass with certain *Lactobacillus* strains can enhance silage stability after opening. Researchers from Bielefeld University and the University of Natural Resources and Life Sciences in Vienna mimicked the changing aeration conditions during ensiling in a bioreactor, to analyze changes in gene expression of the silage inoculant *Lactobacillus buchneri* CD034 [9]. They cultivated *L. buchneri* in stirred tank bioreactors, first under aerobic conditions (aerating with 21 % oxygen), the switching to anaerobic conditions (aerating with 2 % carbon dioxide and 98 % nitrogen), and again under aerobic conditions (aerating with 21 % oxygen). Experimental control of the gas phase composition facilitated identification of oxygen-responsive transcripts. The results suggest that oxygen exposure could lead to an increase in the production of acetate, which has antimicrobial properties and therefore could contribute to the aerobic stability of silage inoculated with *L. buchneri*. The results of the study may help to identify new, beneficial silage inoculants.

extended the gastric digestion model to also simulate digestion in the small intestine (Figure 4). They did so by pumping in a pancreatin/bile mixture. This model was used to analyze the coagulation and digestion of a protein supplement intended to stimulate muscle protein synthesis [12].

Researchers from the University of Wageningen used a similar bioreactor system to analyze survival of therapeutic microbes in the gastric system [13]. They encapsulated *Akkermansia muciniphila* –a promising therapeutic microbe– in a water-in-oil-in-water double emulsion, to protect it from harmful gastric conditions and facilitate its delivery to the small intestine. Using the gastric digestion model they showed that encapsulated bacteria were more resistant to the gastric phase than non-encapsulated ones. In a simulated intestinal system the emulsion was digested and the bacteria released.

In these studies, researchers simulated environmental conditions in a bioreactor. The bioprocess systems made it possible to add liquids, control process parameters, and change them over time, which made complex systems experimentally accessible.

Caution: Hot! – Challenging process conditions

Process development in food and feed applications often means coping with challenging process conditions. Bioprocessing for the manufacture of dairy products may require the stirring of viscous material. Pasteurization processes need reliable control at high temperatures. Some bioprocesses use side products of the food production chain as substrate, like wheat bran or potato peels, and therefore contain solid components.

If dealing with challenging process conditions, bioprocess engineers need to look carefully at the capabilities of their equipment. When processing solid suspensions it is crucial to optimize the agitation conditions to keep the solid in suspension. To be able to pump solid suspensions, such as in continuous processes, testing the capabilities of the pumps is required. For example, our application engineers tested pumping of suspensions of 100 g/L and 200 g/L cellulose with the DASGIP MP4 and MP8 pump module. They compared pump head tubing of different inner tube diameters to identify the best tubing for pumping an accurate amount of solid suspension in a reproducible manner.

Solid suspensions and viscous fermentation broths may

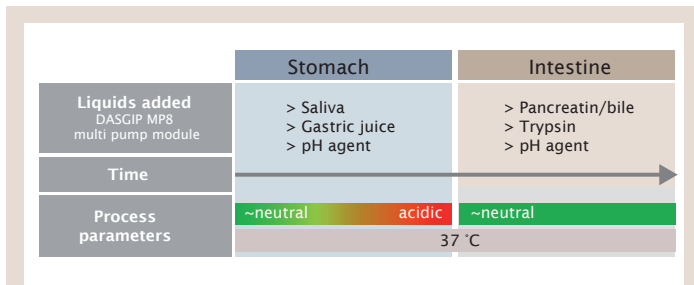


Figure 4: Schematic representation of experimental concept to mimic the gastrointestinal tract in a bioreactor (adapted from [12]).

To analyze enteral nutrition products, researchers at Danone® Research established a bioreactor system that mimics gastric digestion [10]. The system consisted of eight parallel bioreactors, each equipped with four feed lines connected to DASGIP MP8 pump modules (Figure 3). Pumps and feed lines were used to add HCl and NaOH for pH control, artificial gastric juice, and saliva to the bioreactors. Gastric digestion was simulated by incubating the system at 37 °C, continuously lowering the pH down to 2.0, and adding artificial saliva and gastric juice (Figure 4). With this system, researchers analyzed the coagulation of individual proteins and protein mixtures [10] and the effect of fibers on the coagulation of casein-based enteral nutrition [11]. The findings are relevant to the development of new enteral nutrition products for the nutrition of critically ill patients.

In another study researchers from Nutricia Research

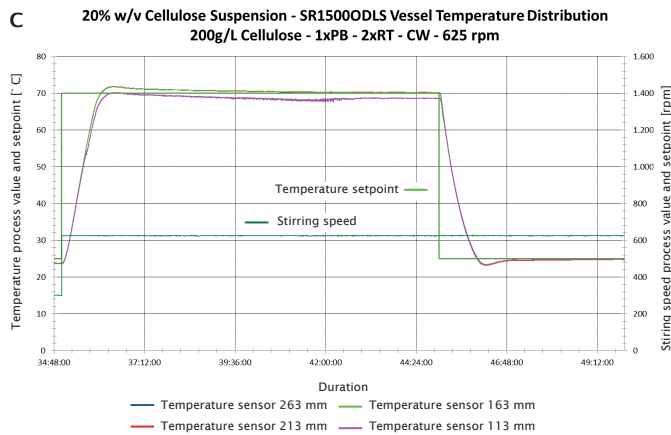
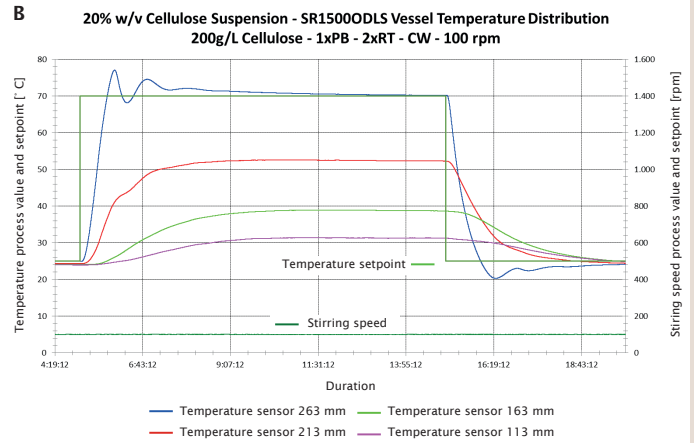
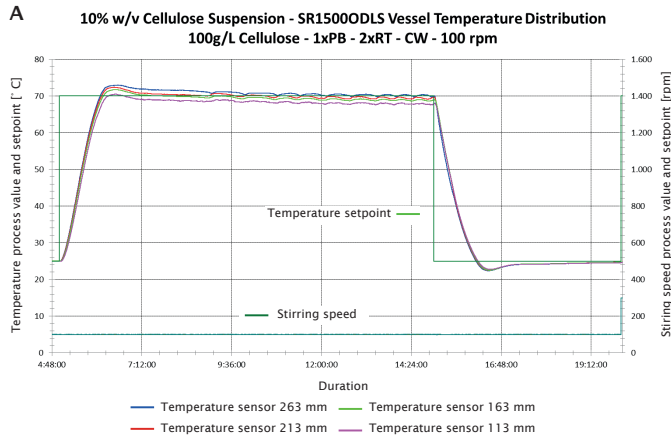


Figure 5: Analysis of temperature distribution.

In all experiments a modified DASGIP Bioblock Stirrer Vessel (maximum working volume 1.8 L) with one pitched-blade (PB), two Rushton-type impellers (RT), and four temperature sensors was used. The temperature sensors were installed at different distances from the vessel head plate liquid heights. The temperature was set to 70 °C and controlled with a DASGIP Bioblock (heating at the vessel bottom). The temperature was measured at different locations in the vessel (blue, red, green, and magenta curves).

A: A 10 % (w/v) cellulose suspension was agitated at 100 rpm (clockwise (CW)). The temperature distribution within the vessel was uniform.

B: A 20 % (w/v) cellulose suspension was agitated at 100 rpm (CW). The temperature distribution within the vessel was not uniform.

C: A 20 % (w/v) cellulose suspension was agitated at 625 rpm (CW). The temperature distribution within the vessel was uniform.

influence mixing times. It might be necessary to optimize the agitation speed to ensure proper mixing and therefore a uniform distribution of metabolites and temperature within the vessel. An experiment performed by the Eppendorf applications team gives an example (Figure 5). Agitating 1.5 L of a 10 % (w/v) cellulose suspension at 100 rpm was sufficient to achieve a uniform temperature distribution within the vessel (Figure 5A). When agitating a 20 % (w/v) cellulose suspension at the same conditions, a temperature gradient within the vessel was observed (Figure 5B) .

Agitating at 625 rpm resulted in a uniform temperature distribution within the 20 % cellulose suspension (Figure 5C).

The more extreme the conditions, the more carefully the bioprocess engineer needs to verify that set points are met in the course of the bioprocess. By carefully choosing the right equipment and conducting some optimization work it is often possible, however, to deal with challenging process conditions.

Conclusion

Bioprocess engineers in the food and feed sector compare the performance of strains, optimize bioprocesses, and simulate complex systems. To do so they need to be able to control and reproduce bioprocess conditions. Stirred-tank bioreactor systems have proven their value, because they offer online control of critical process parameters,

including temperature, pH, and dissolved oxygen and facilitate automated acquisition of bioprocess data tracks. Parallel bioprocess systems help accelerate process development and maximize the reproducibility between runs.

References

- [1] <https://www.gminsights.com/industry-analysis/lysine-methionine-market>. Accessed on January 18, 2019.
- [2] Chen Z, Bommareddy RR, Frank D, Rappert S, Zeng AP. Dereglulation of feedback inhibition of phosphoenolpyruvate carboxylase for improved lysine production in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 80(4): 1388-1393. 2014.
- [3] Gassel S, Schewe H, Schmidt I, Schrader J, Sandmann G. Multiple improvement of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* by a combination of conventional mutagenesis and metabolic pathway engineering. *Biotechnol. Lett.* 35:565-569. 2013.
- [4] Li M, Kildegaard KR, Chen Y, Rodriguez A, Borodina I, Nielsen J. *De novo* production of resveratrol from glucose or ethanol by engineered *Saccharomyces cerevisiae*. *Metab. Eng.* 32:1-11. 2015.
- [5] Subramanian MR, Talluri S, Christopher LP. Production of lactic acid using a new homofermentative *Enterococcus faecalis* isolate. *Microb. Biotechnol.* 8(2): 221-229. 2015.
- [6] Kreici A, Strohmayer F, Huether-Franken CM, Schlottbom C. Process development for silage inoculants – optimization of *Lactobacillus sp.* fermentations with parallel bioreactor systems. *Eppendorf Application Note 299*. 2009.
- [7] Martínez-Moreno R, Quirós M, Morales P, Gonzales R. new insights into the advantages of ammonium as a winemaking nutrient. *Int. J. Food Microbiol.* 177: 128-135. 2017.
- [8] Schlottbom C, Kleebank S, Hauser M, Hans S. Amino acid fermentation: Evaluation of scale-down capabilities using DASbox Mini Bioreactors. *Eppendorf Application Note 290*. 2013.
- [9] Eikmeyer FG, Heintz S, Marx H, Pühler A, Grabherr R, Schlüter A. Identification of oxygen-responsive transcripts in the silage inoculant *Lactobacillus buchneri* CD034 by RNA sequencing. *PLOS ONE*. Doi: 10.1371/journal.pone.0134149. 2015.
- [10] van den Braak CCM, Kleebank S, Abrahamse E, Minor M, Hofman Z, Knol J, Ludwig T. A novel protein mixture containing vegetable proteins renders enteral nutrition products non-coagulating after *in vitro* gastric digestion. *Clin. Nutr.* 32: 765-771. 2013.
- [11] Luttikhof J, van Norren K, Minor M, Buijs N, van den Braak CCM, Ludwig T, Abrahamse E, Rijna H, van Leeuwen PAM. The effect of fibers on coagulation of casein-based enteral nutrition in an artificial gastric digestion model. *Food Funct.* 5: 1866-1871. 2014.
- [12] Luiking YC, Abrahamse E, Ludwig T, Boirie Y, Verlaan S. Protein type and caloric density of protein supplements modulate postprandial amino acid profile through changes in gastrointestinal behaviour: A randomized trial. *Clin. Nutr.* 35(1): 48-58. 2016.
- [13] van der Ark KCH, Wahyu Nugroho AD, Berton-Carabin C, Wang C, Belzer C, de Vos WM, Schroen K. Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. *Food Res. Int.* 102: 372-379. 2017.

Ordering information

Description	Order no.
DASbox® Mini Bioreactor System for Microbial Applications , max. 25 sL/h gassing, 4-fold system	76DX04MB
DASGIP® Parallel Bioreactor System for Microbial Applications , max. 250 sL/h gassing, 4-fold system, benchtop	76DG04MB
DASGIP® Parallel Bioreactor System for Microbial Applications , max. 250 sL/h gassing, 4-fold system with Bioblock	76DG04MBBB
DASware® control , incl. PC, OS, and licenses, for 4-fold DASGIP® system	76DGCS+4

About Eppendorf

Eppendorf is a leading life science company that develops and sells instruments, consumables, and services for liquid-, sample-, and cell handling in laboratories worldwide. Its product range includes pipettes and automated pipetting systems, dispensers, centrifuges, mixers, spectrometers, and DNA amplification equipment as well as ultra-low temperature freezers, fermentors, bioreactors, CO₂ incubators, shakers, and cell manipulation systems. Associated consumables like pipette tips, test tubes, microtiter plates, and disposable bioreactors complement the instruments for highest quality workflow solutions.

Eppendorf was founded in Hamburg, Germany in 1945 and has more than 3,100 employees worldwide. The company has subsidiaries in 26 countries and is represented in all other markets by distributors.

Your local distributor: www.eppendorf.com/contact
Eppendorf AG · Barkhausenweg 1 · 22339 Hamburg · Germany
eppendorf@eppendorf.com · www.eppendorf.com

www.eppendorf.com