University of Natural Resources
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Master thesis

Experimental design to screen the impact of chemical chaperones on a recombinant CHO-K1 cell line producing the human IgG_1 antibody PG9

submitted by

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STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

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Eidesstaatliche Erklärung

Ich erkläre eidesstattlich, dass ich die Arbeit selbständig angefertigt habe. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Die aus fremden Quellen direkt oder indirekt übernommenen Formulierungen und Gedanken sind als solche kenntlich gemacht. Diese schriftliche Arbeit wurde noch an keiner Stelle vorgelegt.

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Abbreviations

BiP	Binding protein
BLI	Biolayer interferometry
CDR	Complementary determining region
CHO cell	Chinese hamster ovary cell
CIP	Cleaning in place
d8mAb	Day 8 antibody titer
DMSO	Dimethyl sulfoxide
DoE	Design of experiments
DSC	Differential scanning calorimetry
ER	Endoplasmatic reticulum
F_{ab}	Fragement antigen-binding
F_c	Fragment crystallizable
F_v	Variable domain
GA3P-DH	Glyceraldehyde-3-phosphate dehydrogenase
HQ-H_2O	High quality water
hrInsulin	human recombinant insulin
Hsp	Heat shock protein
IgG	Immunoglobulin G
LA/CD	Linoleic acid ß-cyclodextrin complex
mAb	Monoclonal antibody
mqP	Maximal specific productivity
mTiter	Maximal antibody titer
mTOR	Mechanistic target for rapamycin
mVCC	Maximal viable cell concentration
NC	Negative control
OFAT	One-factor-a-time
ØqP	Average specific productivity
4-PBA	4-Phenylbutyric acid
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
P_{lac}Con	Process lactate consumption rate
TMAO	Trimethylamine-N-oxide
VPA	Valproic acid

Abstract

Recombinant CHO cell lines are nowadays the commonly used expression system for antibody production. Cultivation is complicated due to the sensitivity of the cells, which can also affect the product expression level and quality. ‘Chemical chaperones’ are small molecules naturally accumulating in organisms during stress to prevent protein aggregation and stabilize the native form of the protein. Application as culture supplements during cultivation of CHO cells was reported to maximize product titers and ensure highest product quality attributes. In this study, 21 different chemical chaperones and derivatives have been tested by an experimental design approach to reduce the number of required experiments, but enhancing the statistical significance of the results. A recombinant CHO-K1 cell line producing the human anti-HIV-1 IgG_1 antibody PG9 was used as model cell line. After several round of screening dimethyl sulfoxide (DMSO) and 4-phenylbutyric acid (4-PBA) have been identified as the most promising candidates and tested in different cultivation modes. Purified protein samples from DMSO supplemented semi-perfusion cultures were analyzed for thermal stabilities. DMSO supplementation induced metabolic changes, indicated by an altered lactate and L-glutamic acid metabolism, but did not influence the final thermal stability of the protein.

Zusammenfassung

Rekombinante Zelllinien des aus Ovarien des chinesischen Zwerghamsters werden heutzutage standardmäßig für die Produktion von Antikörpern verwendet. Die Empfindlichkeit dieser Zellen macht die Kultivierung besonders aufwendig. Des Weiteren nehmen die Kultivierungsbedingungen auch Einfluss auf die Ausbeute sowie die Qualität des Produktes. Chemische Chaperone sind kleine Moleküle, welche natürlich in verschiedensten Organismen vorkommen, um dort die native Form der Zellulären Proteine während Stress zu gewährleisten. Des Weiteren verhindern sie die Aggregation von Proteinen, welche dem Organismus schaden könnten. Die Anwendung dieser Substanzen und derer Derivate in der Kultivierung von Ovarien Zellen des chinesischen Hamsters ist der Literatur zur Folge eine Strategie um die Ausbeute, aber auch die Qualität des Produktes zu steigern. In dieser Studie wurden insgesamt 21 verschiedene chemische Chaperone getestet unter Anwendung der statistischen Versuchsplanung. Durch die Anwendung von Teilfaktorplänen kann die Anzahl der notwendigen Experimente reduziert werden jedoch auch die statistische Signifikanz erhöht. Als Modell Zell Line wurde eine K1 Zell Line, welche den humanen IgG_1 PG9 Antikörper produziert herangezogen. Aus der Auswertung der statistischen Versuchspläne resultierte, dass Dimethylsulfoxid und 4-Phenylbuttersäure die vielversprechenden Substanzen sind. Um deren Wirksamkeit nochmals zu bestätigen wurden sie des Weiteren auch in anderen Kultivierungsmethoden getestet. Außerdem stellte sich heraus, dass die Supplementierung von Dimethylsulfoxid keinen Einfluss auf die Thermostabilität des Proteins hat. Außerdem wurde gezeigt, dass durch die Supplementierung von Dimethylsulfoxid auch der Laktat wie auch der L-Glutaminsäure Metabolismus beeinflusst wurden.

Aim of the study

The aim of this study was to screen the impact of various chemical chaperones on specific cell culture parameters related to productivity and process performance of a recombinant CHO-K1 model cell line producing the human anti-HIV-1 IgG_1 antibody PG9. Beside the classical chemical chaperones, already reported in literature, also new substances have been tested. Supplemented batch cultivation was chosen as screening assay, by supplementing the cultures on day three of cultivation to circumvent growth inhibition. Screening experiments were performed using an experimental design approach. Initial screening experiments were performed as fractional factorial screening designs with resolution III, where interactions between the factors are neglected. Evaluation of the effects has been used to design follow-up experiments to verify the obtained results in different cultivation modes including a glucose controlled batch and semi-perfusion. To investigate the effects in more detail, best-performing chemical chaperones were screened in a second screening design at resolution V+. In addition to the classical screening parameters including maximal antibody titer, maximal viable cell concentration and maximal specific productivity also metabolite concentrations have been determined in detail to monitor changes in the metabolism of CHO cells caused by the supplementation. Follow-up experiments included the monitoring of the thermal stability of the PG9 antibody by differential scanning calorimetry (DSC) of DMSO supplemented semi-perfusion cultivation experiments and the impact on the lactate metabolism in DMSO supplemented cultures.

Content

1 Introduction

1.1 Economic aspects of biopharmaceuticals

Chinese hamster ovary cells, commonly known as CHO cells, are predominantly used as mammalian expression system in biopharmaceutical protein production. In the period from January 2014 to July 2018, 155 new biopharmaceutical products have been approved in the EU and or the US, of which 68 have been monoclonal antibodies (Walsh 2018). In total 84% of the monoclonal antibodies are nowadays produced in CHO cell based systems. The annual total sales of monoclonal antibodies reached $123 billion in 2017, which corresponds to 65,5% of the annual total sales. These numbers indicate clearly the importance of monoclonal antibodies as biopharmaceuticals. From 2010 to 2014 monoclonal antibodies represented 27% of all first-time to market approvals. Already in the period from 2015 to 2018 the percentage of monoclonal antibodies all first time approvals increased to 53%. This trend is expected to steadily increase especially with the approval of biosimilars, which underlines the economic importance of monoclonal antibodies in the near future (Walsh 2018).

1.2 Characterization and historical aspects of Chinese hamster ovary cells

One major reason for their supremacy as mammalian expression system, is their ability to express highly complex proteins with human like glycosylation (O'Callaghan and James 2008). CHO cells are not susceptible to most of the human viruses, which makes cell line development and the production process safer. The ability of the cells to grow in suspension and in serum free chemically defined media make them even more interesting for large scale industrial applications (Lai, Yang, and Ng 2013). The origin of CHO cells dates back to the year 1956 in which Dr. Theodore Puck isolated spontaneously immortalized fibroblasts of cultured ovarian cells deriving from a Chinese hamster (Puck, Cieciura, and Robinson 1958). Subcloning and spontaneous mutagenesis of the ancestor resulted in a great genetic heterogeneity and led to various cell lineages who are nowadays available. The most widely used cell lines for the production of biopharmaceuticals are CHO-K1, CHO-S and CHO-DG44. Creation of CHO-K1 was obtained by subcloning of the originator. In 1980 the CHO DUKX-B11 was created by chemical mutagenesis and is characterized by its lack in dihydrofolate reductase activity. Furthermore CHO-DXB11 was used for the production of human tissue plasminogen activator, which was the first large scale application of a CHO cell line (Kaas et al. 2015). Deletion of both dihydrofolate reductase alleles in the originator

by mutagenesis led to the establishment of a selection marker, which is characteristic for the CHO-DG44 cell line, which was created in 1983 (Urlaub et al. 1983). The CHO-S cell line established in 1991, was obtained by another subclone of the originator cell line (Reinhart et al. 2018).

1.3 Differences of molecular, chemical and pharmaceutical chaperones

Prior the characterization of chemical and pharmaceutical chaperones, it is important to understand the purpose of molecular chaperones. First of all molecular chaperones are typically described as a diverse and large group of proteins found in the endoplasmatic reticulum (ER). Their main function is the assistance in disulfide bond rearrangement by protein disulfide isomerases (PDI) or non-covalent folding and unfolding of macromolecules. All cells require this chaperone function in order to prevent incorrect interactions by non-functional proteins, which will lead to a biological loss in function or toxic aggregation (Ellis 2006). Molecular chaperones can be further described as proteins that senor non-native structures and therefore act as a first line of a complex quality control system. For example immunoglobulin heavy-chain binding protein (BiP) is an organelle member of the heat shock protein (Hsp70) family that binds to the unfolded heavy chain constant domain (CH1) and prevents premature secretion unlike it is displaced by the antibody light chain (Feige and Buchner 2014). It is important to state that molecular chaperones are not part of the final structure, they just aid the re-folding process (Hoffmann, Bukau, and Kramer 2010).

The term chemical chaperone was established in order to describe the function of various substances. In general chemical chaperones comprise small molecules with low molecular weight that non-selectively enhance the protein stability (Bernier et al. 2004; Huang, Wang, and Tao 2017). Besides naturally occurring osmolytes also hydrophobic compounds can be considered as chemical chaperones due to their ability to stabilize the protein and inhibit aggregation. A description of the underlying principle of osmolytes is given hereinafter (Onitsuka et al. 2014). In comparison to this unspecific action of chemical chaperones, pharmaceutical chaperones bind selectively to protein domains and thereby favor the folded state, so that they can be used as medication and additive in cell culture media (Bernier et al. 2004).

1.3.1 Naturally occurring osmolytes

A majority of the cell dry weight is composed of protein that is estimated between 90 to 170 pg×cell^{-1} for CHO cells (Davies et al., 2012; Cheung et al., 2013). Proper functionality of these macromolecules is vital for the cell. Various mechanisms are known how the cell can stabilize the native form of endogenous proteins and thereby ensure their proper function even under cellular stress conditions. Not only the pH and the temperature influence or regulate the activity of proteins, but also varying concentrations of solutes in the cell plasm. These naturally occurring solutes also known as osmolytes can be categorized according to their chemical nature into amino acids and their derivatives, methylamines, polyols and sugars (Khan et al. 2010). As already mentioned accumulation of osmolytes occur especially during cellular stress to enhance the thermodynamic stability of the natively folded state of the proteins, which can be observed in microorganisms, plants and animals (R. Kumar 2009).

1.3.2 Natural occurrence of chemical chaperones and their application in recombinant protein production

In order to obtain properly folded and modified recombinant protein, different settings have to be met. Especially during bioprocessing, different factors can negatively impact the product quality. A strategy to enhance the performance of mammalian cells during bioprocessing is the supplementation of chemical chaperones to the cell culture media (Onitsuka et al. 2014). Furthermore it is already reported in literature that expression and secretion levels could be enhanced by this approach. The exact mechanism how chemical chaperones supplemented to the cell culture media influence the recombinant protein production in the cell remains elusive (Roth et al. 2012). In the following subsections the used chemical chaperones in this study, their effect and occurrence in nature will be described in brief. Beside the already established classification of osmolytes into amino acids and their derivatives, methylamines, polyols and sugars and biopolymers further categories were defined in this study. Pharmaceutical chaperones like valproic acid (VPA) and 4-Phenylbutyric acid (4-PBA), describe already approved pharmaceutical chaperones. The category ‘alternative chemical chaperones’ comprises substances cited in literature that cannot be categorized in any of the already established classification schemes.

Amino acids and derivatives

ß-alanine is naturally occurring in the central nervous system and acts as a neurotransmitter (Tiedje et al. 2010). This ß-amino acids showed a protective effect during hypoxia in cells and is also known to suppressor heat inactivation of proteins (Levy-Sakin et al. 2014). Another ß-amino acid with similar effects like ß-alanine is taurine, which can also be found in humans, mammals and marine invertebrates (Levy-Sakin et al. 2014; Tiedje et al. 2010). Both substances can be transported via a ß-amino acid transporter to regulate the osmolality in the cell plasm (Hammer and Baltz 2003). Cysteine is a non-essential sulfur containing amino acid and plays a major role in the formation of disulfide bridges in polypeptides, the correct concentration is critical for the process (Ritacco, Wu, and Khetan 2018).

Methylamines

Betaine is reported in literature to increase the secretion rate of recombinant produced FVIII in CHO cells (Roth et al. 2012). This substance naturally occurs in the human kidney beside several other chemical chaperones and osmolytes (R. Kumar 2009). It has already been reported that the supplementation of betaine also increased the integrated viable cell density, which can be interpreted as a function of the cell concentration (Johari et al. 2015a). Trimethylamine-N-oxide (TMAO) naturally occurs in bony fish, the accumulation of TMAO stabilizes the protein in deeper areas of the sea (Downing, Wallace, and Yancey 2018; Roth et al. 2012). Different studies show that the supplementation to cell culture cultivations lead to a decreased protein aggregation and an increased integrated viable cell density (Johari et al. 2015a).

Polyols

Glycerol is a well-known cryoprotectant in biological sciences and is also used as chemical chaperone to improve the production performance. The product titer and the specific productivity could be enhanced by the supplementation of glycerol to the cell culture media (Johari et al. 2015a). Another substance is myo-inositol, which is a cyclic polyol. This osmolyte can be found in deep sea fish, but is lacking in shallow species, indicating that the substance is necessary to cope with extreme conditions. Already very low concentrations of myo-inositol prevent aggregate formation (Downing, Wallace, and Yancey 2018).

Sugars and biopolymers

Accumulation of sugars in order to cope with osmotic stress is a strategy applied by various species (Arakawa et al. 2006). Trehalose, sucrose and mannitol accumulation is reported in various species in nature (R. Kumar 2009). Maltose is a disaccharide and known to enhance the thermal stability of proteins (Levy-Sakin et al. 2014). Supplementation of biopolymers like dextran is a known strategy as anti-aggregation agent. Furthermore it is also reported that dextran improve the cell growth and viability in the decline phase of the cultivation. Dextran also impact on glucose and lactate metabolism of the cells (Hyoung Park et al. 2016). Maltodextrin is structurally similar to dextran, similar effects are assumed by the supplementation to cell culture.

Pharmaceutical chaperones

Another substance already clinically approved and widely used as chemical chaperone in cell culture cultivation is 4-phenylbutyric acid (4-PBA). This low molecular weight fatty acid does reduce cellular stress by inducing the synthesis of endogenous molecular chaperones. Furthermore 4-PBA also binds to hydrophobic segments exposed by the protein and acts as histone deacetylase inhibitor. Studies already reported an enhanced specific productivity and reduced aggregate formation by the supplementation (Cortez and Sim 2014; Johari et al. 2015a; Perlmutter 2002). Another substance acting similar to 4-PBA is the valproic acid (VPA), which is also an already FDA approved histone deacetylase inhibitor. Again the unfolded protein response is up regulated by this substance (Segar, Chandrawanshi, and Mehra 2017).

Alternative chemical chaperones

Spermidine is a polyamine, which is essential for eukaryotic proliferation. Under physiological conditions spermidine is polycationic so that it is able to interact with negatively charged molecules like RNA and DNA. A critical function in terms of protein production is the activation of initiation factor 5 in eukaryotic cells (Mandal et al. 2013). The usage of water soluble polymers like ß-cyclodextrin only served as a carrier molecule for hydrophobic substances. The funnel like structure with a hydrophobic core provides a cavity suitable to incorporate hydrophobic substances (Inka, Francz, and Biesalski 2000). The incorporation of linoleic acid into this cavity leads to the formation of a ß-cyclodextrin-linoleic acid complex that is used as media supplement. Pure supplementation of linoleic acid is not suitable as oxygen oxidizes the fatty acid. Reports already showed that the cell incorporates a part of the fatty acid into cellular lipid fractions and thereby enhance the robustness of the cell, which led to the ability

to withstand higher shear forces during cultivation (Butler et al. 1999). Copper was supplemented in form of copper sulfate and is reported in literature to regulate the lactate metabolism in CHO cells and is essential for the formation of disulfide bridges in the proteins (Chaderjian et al. 2005; Pereira, Kildegaard, and Andersen 2018). Another substance used as chemical chaperone was dimethyl sulfoxide (DMSO), which is widely used as cryoprotectant in biological sciences (Noda et al. 2017). Application as chemical chaperone is reported in literature to enhance the specific productivity and product titer. Additionally protein aggregation is prevented by the supplementation. A major drawback of DMSO is its toxicity for the cell, which is manifested in a suppressed cell concentration during cultivation (Johari et al. 2015a).

1.4 Experimental design

Design of experiments (DoE), also known as experimental design, is a statistical method suitable to obtain mathematical relationships between process inputs and output. Furthermore, experimental design can be described as mass balancing of input and output variables. One big advantage of experimental design is that multiple factors can be tested simultaneously at varying factor settings or levels. The traditional one factor at the time (OFAT) approach only considers one factor with one setting during the experiment. Moreover design of experiment is able to determine interactions between different factors depending on the resolution of the design, which is almost impossible to characterize by OFAT approach (Mandenius and Brundin 2008).

1.4.1 Terminology of experimental design

A specific terminology is required to understand the concept of experimental design. At first there are factors, which can be set individually. The setting of the factor is also described as ‘level’ and can be a quantitative or qualitative measure. An interaction between two factors is described as interaction and is depended on the resolution of the chosen design. The resolution describes the ability of a design to separate different responses caused by different factors. A higher resolution design will yield more complex information about effects and interactions. Not all fractional factorial screening designs and resolution support the screening for interactions. Different quantitative responses also called ‘screening parameters’ in this study were measured during an experiment. Individual characterization of the used screening parameters for this study is done in section 2.9. Based on the obtained responses the main effects can be calculated, which are defined as the difference of the measured response at factor level +1 and level -1.The term ‘system’ describes the object under investigation, shown

as cultivation tube in Figure 1. Different factors and or factor levels show impact of various degree on the system and thereby change the response. It is important to distinguish between factors, that can be controlled and uncontrollable factors. Both factors define the finally obtained response, but only the controllable can be used for the mathematical model (Siebertz, Bebber, and Hochkirchen 2010).

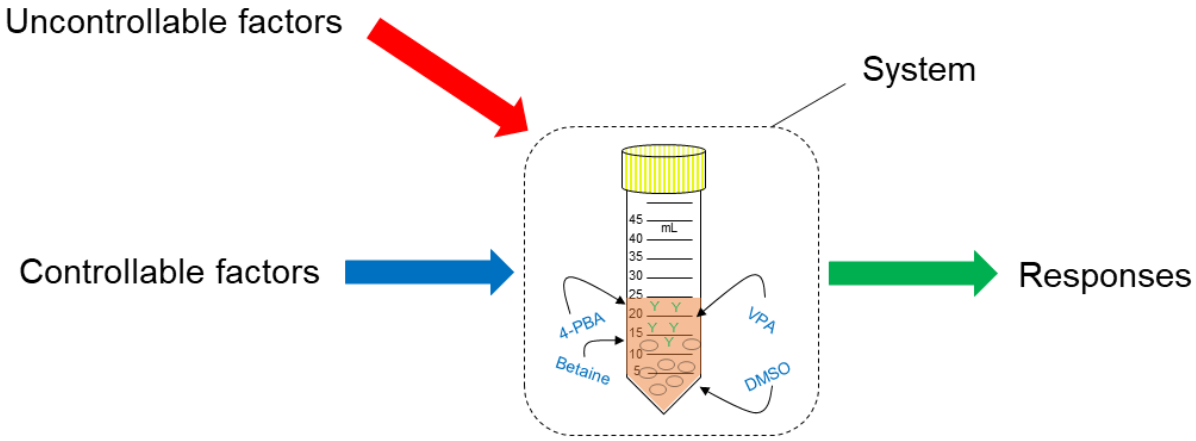


Figure 1: This schematic sketch summarizes all prior mentioned terms. Different factors can influence the system and define the response and thereby the obtained effect. Factors can be set and controlled (→) like the applied working concentration of "4-PBA", "VPA", "betaine" and "DMSO" or they cannot be controlled but do also influence the response (→), for example the sampling time. After all both types of factors contribute to the measurable response (→) like the cell concentration indicated by 'o' or the produced antibodies (Y). The system describes the object of investigation (Siebertz, Bebber, and Hochkirchen 2010). In this study mainly screening cultivation experiments have been performed in 50 mL bioreactor tubes

1.4.2 Identification of impacting factors

After definition of controllable 'factors', factor levels, quantitative responses and the system, a suitable design for screening must be selected. Depending on the purpose and the scope of the screening experiment, designs with different resolution can be chosen. The most commonly used design for screening is the fractional factorial design, in which only a subset of experiments of a full factorial design is used. In the fractional factorial design two different factor settings are screened in combination with additional center points to check the linear correlation between the two levels. These center points correspond to level 0 factor setting and should lie between the -1 level and 1 level factor setting in a good model (Mandenius and Brundin 2008). The design of the experiment and definition of different factor settings is nowadays performed by statistical software packages and analyzed by linear regression (Siebertz, Bebber, and Hochkirchen 2010). In this thesis mainly fractional factorial designs of resolution III were used. This resolution is characterized in that main factor effects are confounded with two factor interactions, but the aim of the screening experiments was to identify the most influential factors and not possible interactions (Gerlach et al. 2008).

1.5 Characteristics of the PG9 antibody

The model protein in this study was the PG9 antibody. This human derived immunoglobulin G subtype 1 (IgG_1) was isolated from an HIV infected patient (Davenport et al. 2011). Up to 80% of HIV-1 isolates can be neutralized by this broad antiviral neutralizing antibody. Critical aspects of virus neutralizing antibodies are viral evasion by specific glycosylation patterns and remarkable diversity in sequence. PG9 engages the envelope glycoprotein gp120 of the HIV-1. This glycoprotein is made up of variable regions 1 and 2 which folds as four anti-parallel ß-strands and consist of 50 to 90 residues. Especially the loops connecting the ß-strands are of special importance for antibody binding, but the biggest sequence variation and the highest glycosylation levels of the virus can exactly be found in these variable loop regions (Phogat et al. 2011).

2 Materials and Methods

This section describes the applied methods and used materials for conducting this study. Within the sections only the most important materials are listed, a detailed list summarizing the equipment, chemicals and disposables is given in section 2.1, 2.2 and 2.3.

2.1 Equipment

Table 1: Summary of the equipment

Device	**Brand**	**Serial number**
BioProfil 100 plus	nova biomedical	T34712020
DSC	Malvern	MAC1172720
forteBio Octet^{QK} system	PALL	FB-90057
Gilson^{TM} Pipeteman Classic P100	Fisher scientific	
Gilson^{TM} Pipeteman Classic P1000	Fisher scientific	
GilsonTM Pipeteman Classic P20	Fisher scientific	
Gilson^{TM} Pipeteman Classic P200	Fisher scientific	
Heraeus Megafuge 16 centrifuge	Thermoscientific	31006337
Heraeus oven	Thermo Scientific	41020862 50042312 31006316
MSC-Advantage (Laminarhood)	Thermoscientific	31006223
Osmomat® 030	gonotec	100404
pH7110	WTW	15371179
Routine culture shaker	Kuhner	30005976
Ultra clear UV Reinstwasser	SGwater	0919859-02
ViCell XR - Cell analyzer	Beckman Coulter	BA06052
Vortex	Scientific industries	2-151510

2.2 Chemicals

Table 2: Summary of the chemicals

Name	Brand	Product number
ß-Alanine	Sigma aldrich	A9920
Betaine	Sigma aldrich	B2629
Bioprofile 100 plus Reagent Pack	nova biomedical	38291
Bioprofile® Control Level 1	nova biomedical	22600
Bioprofile® Control Level 2	nova biomedical	22601
Bioprofile® Control Level 3	nova biomedical	22602
Bioprofile® Control Level 4	nova biomedical	24617
Bioprofile® Control Level 5	nova biomedical	24619
Calibration standard	gonotec	30.9.0020
Copper(II)sulfate pentahydrate	Sigma aldrich	C8027
ß-Cyclodextrin	Sigma aldrich	C4805
L-Cysteine	Sigma aldrich	C7352
Dextran from Leuconostoc spp.	Sigma aldrich	31387
DMSO	Sigma aldrich	D2650
Dulbecco's Phosphate Buffered Saline	Sigma aldrich	D8537 Sigma
Glacial acetic acid	Sigma aldrich	A6283
L-glutamine	Merck	1.00286.1000
Glycerol	VWR	24386298
Human recombinant insulin	SAFC Biosciences	91077C
HyClone ActiPro™	GE Lifesciences	SH31039.02
Linoleic acid	Sigma aldrich	L1012
Maltodextrin	Sigma aldrich	419672
D-(+)-Maltose monohydrate	Sigma aldrich	M9171
D-(+)-Mannose	Sigma aldrich	M6020
Myo-inositol	Sigma aldrich	I7508
$Na_2HPO_4 \times 2H_2O$	Carl Roth	4984.3
$NaH_2PO_4 \times H_2O$	E.Merck	6346
Phenol red	Sigma aldrich	P0290
4-Phenylbutyric acid	Sigma aldrich	P21005
Sodium hydroxide	VWR chemicals	28245.367
Spermidine	Sigma aldrich	S0266
Sucrose	Sigma aldrich	S1888
Taurine	Sigma aldrich	T8691
TMAO	Sigma aldrich	92277
D-Trehalose	Serva	36770
Trypan blue	Sigma aldrich	T8154
Tween20®	Sigma aldrich	9000-5-64-5
Valproic acid	Sigma aldrich	P4543

2.3 Disposables

Table 3: Summary of the disposables

Name	Brand	Product number
Cellstar Tubes 15 mL	greiner bio-one	188271
Cellstar Tubes 50 mL	greiner bio-one	227261
Cellstar Tubes 50 mL	greiner bio-one	210261
Measuring vessels	gonotec GmbH	30.9.0010
Micro centrifuge tubes	VWR	211-0015
Nunclon™ Delta Surface	Thermo Scientific	163371
Nunclon™ Delta Surface	Thermo Scientific	153732
PD-10 Desalting column	GE Healthcare	17-0851-01
Protein A (ProA) Dip and Read™ Biosensors	PALL	1806191
Protein A HP SpinTrap	GE Healthcare	28903132
Rotilabo®-Syringefilter	Roth	P668.1
4 mL sample cup	Beckmann coulter	08229NT
Serological pipettes 10 mL	Corning	4488
Serological pipettes 2 mL	Corning	4486
Serological pipettes 25 mL	Corning	4489
Serological pipettes 5 mL	Corning	4487
Serological pipettes 50 mL	Corning	4490
125 mL shaking flask	Corning	431143
Tubespin Bioreaktor 50	TPP®	87050
ultratip 1000 µL	greiner bio-one	740290
ultratip 200 µL	greiner bio-one	685261

2.4 Thawing of the cells

Developed cell lines may not be used permanently so that storage and conservation of the cells is of special interest. Not only maintenance of routine culture but also risk of contamination by microorganism, genetic drift by sub culturing, senescence and other genetic or phenotypic issues can be prevented by cryopreservation. For this to happen cells suspended in the media should have a high viability and authenticity. Perfect storage conditions are provided by antifreeze agents like glycerol or DMSO. These substances are supplemented to the cell suspension to prevent crystal formation intra or extracellular by partial dehydrate the cell plasm by forming a mixture with water. Afterwards the cell suspension is stored at -196°C in liquid nitrogen, at which no biological reactions take place (Gstraunthaler and Lindl 2013).

2.4.1 Materials

70% Ethanol

HyClone ActiPro™ (GE Lifescienes, SH31039.02)

125 mL shaking flask (Corning, 431143)

2.4.2 Procedure

The vial containing the desired cell line, stored at -196°C containing was taken out of the liquid nitrogen storage for the thawing procedure. After that, the whole vial was put in to a 50 mL tube containing 10 mL ethanol for disinfection. In the meanwhile 8 mL of cultivation media were aliquoted under sterile conditions into a 10 mL tube and cooled to 4°C. After the disinfection procedure of the vial, the closed vial still suspended in ethanol was poured out on to a piece of paper towel in a laminar workbench for thawing. Afterwards the vial was opened and 1000 µL of the cell suspension were transferred to the pre-cooled cultivation media. The cool cell suspension was centrifuged for 10 minutes at 188×g to remove the cryoprotectant DMSO from the cell suspension. In the meantime, 10 mL of cultivation medium were tempered at 37°C. After the centrifugation procedure the supernatant was discarded and the cell pellet was re-suspended in 10 mL of the pre-warmed cultivation medium and then subsequently transferred in to the 125 mL shaking flask. The established routine culture was passaged every 3rd and 4th day and expanded to 30 mL after the 4th passage. The procedure of cell passaging is described in *“2.8 Routine culture maintenance”*.

2.5 Determination of cell and cultivation parameters

Information about the overall state of the cultivated cells is crucial to correctly interpret the results obtained by the experiment. Fully automatic electronic devices for cellular analysis are nowadays common for such purposes. The optical based system injects the cells into a flow cell and determines the total cell concentration, viability and morphologic characteristics like cell size and cell circularity by optical evaluation. The principle of viability evaluation is based on trypan blue exclusion. Only dead cells with a damaged cell membrane are able to incorporate the blue dye and appear blue on the screen. The viability is then obtained by the number of viable, uncolored cells divided by the total cell number (Gstraunthaler and Lindl 2013).

2.5.1 Materials

Dulbecco's Phosphate Buffered Saline (Sigma Aldrich, D8537)

4 mL sample cup (Beckmann coulter, 08229NT)

2.5.2 Equipment

ViCell XR - Cell analyser (Beckman Coulter, Serial number BA06052)

2.5.3 Procedure

Evaluation of default cell cultivation parameters was performed daily for cultivation experiments, this comprised the cell concentration, viability and morphologic characteristics like the cell size and circularity. For this to happen at least 1.5 mL of the cell suspension were sampled in a standard 15 mL tube under sterile conditions. The analysis required only 700 µL of the cell suspension so that the remaining cell suspension was spun down at 317×g, stored at -20° and used for further analysis, described in later sections. If required, the cell suspension was diluted to an expected cell number below 10×10^6 $c\times mL^{-1}$ with phosphate buffered saline (PBS). The analysis was initiated by pipetting 700 µL of the prepared diluted or undiluted cell suspension into a sampling beaker (Beckmann coulter). Especially semi-perfusion cultures required higher dilutions due to higher obtained cell concentrations. Parameters like the cell type, in our case 'CHO-K1' and the dilution factor were set into the acquisition software for each sample individually before the analysis. Up to 12 samples can be placed into the auto sampler and are analyzed fully automatically.

2.6 Batch Cultivation

A batch process is characterized in that it operates as a closed system. During the process no material is transported beyond the system boundaries (Doran 2013). That means that cultivation conditions are changing steadily over the process time. The cell concentration increases until a maximum is reached and essential nutrients and other growth providing factors deplete. Furthermore, toxic metabolites, like lactate and ammonia accumulate in the cultivation media, which has an influence on the pH and thereby on the cell growth and productivity of the cells. In addition the redox status of each individual cell changes over time (Gstraunthaler and Lindl 2013). In this study batch cultivations in addition do not upregulate the oxygen supply with increasing cell concentration, which is another limiting factor.

2.6.1 Equations

Specific growth rate μ [day^{-1}]

CC…Total cell concentration in 10^6 cells×mL^{-1}

Δt…..Time difference in days

$$\mu = \frac{\ln(CC_i) - \ln(CC_{i-1})}{\Delta t}$$

Viable specific growth rate [day^{-1}]

VCC…Viable cell concentration in 10^6 cells×mL^{-1}

Δt……..Time difference in days

$$\mu_v = \frac{\ln(VCC_i) - \ln(VCC_{i-1})}{\Delta t}$$

Viable cell days [10^6 cells×day×mL^{-1}]

VCC…Viable cell concentration in 10^6 cells×mL^{-1}

μ_v…….Viable specific growth rate in day^{-1}

$$VCD_i = \frac{VCC_i - VCC_{i-1}}{\mu_v}$$

Viable cumulative cell days [10^6 cells×day×mL^{-1}]

VCCD…Viable cumulative cell days in 10^6 cells×day×mL^{-1}

VCD……Viable cell days in 10^6 cells×day×mL^{-1}

$$VCCD_i = VCCD_{i-1} + VCD_i$$

Specific productivity q_p [pg×cell^{-1}×day^{-1}]

titer…Product titer in μg×mL^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$q_p = \frac{titer_i - titer_{i-1}}{VCD_i} \times 10^6$$

Metabolite consumption rate [pg×cell^{-1}×day^{-1}]

MC…Metabolite concentration in g×L^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$MCR_i = \frac{(MC_{i-1} - MC_i) \times 10^9}{VCD_i}$$

Metabolite production rate [pg×cell^{-1}×day^{-1}]

MC…Metabolite concentration in g×L^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$MPR_i = \frac{(MC_i - MC_{i-1}) \times 10^9}{VCD_i}$$

2.6.2 Materials

Cultivation media

HyClone ActiPro™ (GE Lifesciences, SH31039.02)

6 mM L-glutamine (Merck, 1.00286.1000)

15 mg×L^{-1} Phenol red (Sigma aldrich, P0290)

Cultivation tubes

TubeSpin® Bioreaktor 50 (TPP®, 87050)

2.6.3 Procedure

Batch cultivations were initiated by inoculating 0.3×10^6 cells×mL^{-1} in 30 mL cultivation media. Then the cells were incubated at default cultivation conditions, summarized in Table 4. For determination of characteristic measures, samples ranging from 1.5 mL to 2 mL were drawn in a 24 hours interval and analyzed according to the procedure described in section 2.5. After sampling the cells were further cultivated until the termination criteria of a viability below 60% were reached. Furthermore supplements of the supplemented batches were added on day 3 of the cultivation to circumvent growth inhibition. The working concentrations of the supplements are summarized in Table 6.

Table 4: Default cultivation settings for batch and semi-perfusion cultivation.

Parameter	Setting
Temperature	37°C
Humidity	80%
CO_2	7%
Shaking speed	220 rpm
Orbital shaking diameter	50 mm
Shaking angle	90°

Table 5: Summary of the supplements with applied working concentrations and prepared stock concentrations. If not otherwise stated, the shown concentration is in mM. The concentration shown for the Linoleic ß-cyclodextrin complex (LA/CD) refers to the concentration of linoleic acid. Human recombinant insulin (Insulin) was prepared according to manufacturer's suggestions. The solubility of 4-phenylbutyric acid was enhanced by supplementation of 5 M NaOH.

Chemical	Stock concentration [mM]	Working concentration [mM]
ß-Alanine	700.02	0.1
Betaine	298.85	3
$CuSO_4 \times 5\ H_2O$	597.90	0.075
ß-Cyclodextrin	35.27	1.25
L-Cysteine	1066.84	0.5
Dextran	41.28	0.5
DMSO	100%	2%
Glycerol	50%	1%
Insulin	5000 $mg \times L^{-1}$	20 $mg \times L^{-1}$
LA/CD	8.91	0.0125
Maltodextrin	65.97% (w/v)	0.10% (w/v)
Maltose Monohydrate	913.17	0.5
Mannitol	552.07	7
Myo-inositol	222.11	0.1
4-Phenylbutyric acid	200	1
Spermidine	76.42	0.075
Sucrose	612.77	0.1
Taurine	182.12	0.5
$Trehalose \times 2\ H_2O$	92.39	0.5
Trimethylamine N-oxide	1854.97	50
Valproic acid	500	0.5

2.7 Semi-perfusion cultivation

High cell densities are often limited due to nutrient depletion or accumulation of toxic metabolites. The semi-perfusion cultivation approach is characterized in that the cultivation media is exchanged daily in a 24 hour interval, so that cultivation conditions are kept constant over the whole process time. The aim of this cultivation approach is to mimic perfusion cultivation in small scale, which normally requires extensive amounts of media and a complex control unit. By the daily media exchange toxic metabolites are removed and fresh nutrients were supplied. All the prior mentioned characteristics provide cultivation conditions suitable for very high cell densities and product titers (Gstraunthaler and Lindl 2013).

2.7.1 Equations

The herein described equations neglect the cell bleed caused by the sampling procedure. Only 100 µL of a 10 mL or 30 mL cultivation have been sampled for the cellular analysis so that the loss of biomass is neglect able. Cell specific antibody production and metabolite consumption or production rates are determined differently in semi-perfusion compared to batch cultivation cultivations as the media is daily exchanged. Thus, antibody product and toxic by-products are completely removed and nutrient concentrations set to the initial levels every 24 hours. In order to obtain correct results determination of the glucose, lactate, L-glutamine, L-glutamate and ammonia levels of the culture media was performed before the inoculation.

Specific growth rate µ [day^{-1}]

CC…Total cell concentration in 10^6 cells×mL^{-1}

∆t…..Time difference in days

$$\mu = \frac{\ln(CC_i) - \ln(CC_{i-1})}{\Delta t}$$

Viable specific growth rate [day^{-1}]

VCC…Viable cell concentration in 10^6 cells×mL^{-1}

∆t…..Time difference in days

$$\mu_v = \frac{\ln(VCC_i) - \ln(VCC_{i-1})}{\Delta t}$$

Viable cell days [10^6 cells×day×mL^{-1}]

VCC…Viable cell concentration in 10^6 cells×mL^{-1}

μ_v…Viable specific growth rate in day^{-1}

$$VCD = \frac{VCC_i - VCC_{i-1}}{\mu_v}$$

Viable cumulative cell days [10^6 cells×day×mL^{-1}]

VCCD…Viable cumulative cell days in 10^6 cells×day×mL^{-1}

VCD…Viable cell days in 10^6 cells×day×mL^{-1}

$$VCCD_i = VCCD_{i-1} + VCD_i$$

Specific productivity [pg×cell^{-1}×day^{-1}]

titer…Product titer in µg×mL^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$q_p = \frac{titer}{VCD} \times 10^6$$

Metabolite consumption rate [pg×cell^{-1}×day^{-1}]

MC..Metabolite concentration in g×L^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$MCR = \frac{(MC_{i-1} - MC_i) \times 10^9}{VCD}$$

Metabolite production rate [pg×cell^{-1}×day^{-1}]

MC..Metabolite concentration in g×L^{-1}

VCD..Viable cell days in 10^6 cells×day×mL^{-1}

$$MPR = \frac{(MC_i - MC_{i-1}) \times 10^9}{VCD}$$

2.7.2 Procedure

The semi-perfusion cultivation was initiated by seeding 5×10^6 cells×mL^{-1} at 10 mL or 30 mL working volume, depending on the purpose of the experiment. For screening of metabolite formation, growth behavior and product formation 10 mL were sufficient. A working volume of 30 mL was chosen to obtain protein for purification and further characterization. After seeding, the cells were incubated under default cultivation conditions (Table 4). Sampling was performed daily with 100 µL cell suspension diluted with PBS for determination of cell concentrations and viabilities. Then the 50 mL cultivation tubes were centrifuged for 7 minutes at 317×g and 2 mL of the cell free supernatant were sampled and stored at -20°C for further analysis. After that the remaining supernatant in the cultivation tube was pooled in a sterile containment in the laminar work bench. The collected supernatant can be used for protein purification via protein A chromatography. Afterwards the remaining cell pellet was suspended in 10 to 30 mL fresh and tempered cultivation media and re-cultured under default cultivation conditions, this procedure was performed in an exact 24 h interval.

2.8 Routine culture maintenance

Batch cultivations are characterized in that the cultivation conditions permanently change over the process time. Different factors impact on the cells due to changes in the environment, as already characterized in the batch cultivation description (see section 2.6). Furthermore the increasing cell concentration lead to a faster nutrient depletion and a decrease in proliferation rate. To circumvent different limitations cells must be passaged, every 3rd to 4th day (Gstraunthaler and Lindl 2013).

2.8.1 Equations

Optimal split ratio CC…Total cell concentration in 10^6 cells×mL^{-1}	$Optimal\ Split\ ratio = \frac{CC_i}{CC_{target}}$
Seeding volume of the cell suspension V_{target}…Desired culture volume in mL	$V_{Seeding} = \frac{V_{target}}{Split\ ratio}$
Media volume V_{Media}…Volume of fresh media in mL	$V_{Media} = V_{target} - V_{Seeding}$

2.8.2 Procedure

The routine cultures were treated similar to batch cultivations only that sampling took place every 3rd and 4th day, at which the viability as well as the cell concentration were determined. Split ratios were calculated based on the obtained total cell concentration.

Furthermore, the obtained cell free supernatant was used for the determination of the antibody titer. Routine culture performance was monitored by the cell specific productivity and viable specific growth rate, which should stay constant over all passages.

2.9 Screening experiments based on experimental design

Screening of different parameters impacting on the growth and productivity of cells can be a time and cost intensive task. The traditional way of a screening experiment would be the one-factor-at-a-time, in which only one factor is varied in a single experiment. Experimental design is an approach trying to circumvent time and cost issues and leveraging statistical confidence of the results. Fractional factorial designs of different solution were applied within this study to screen different factors. Furthermore, the number of required experiments was reduced in comparison to a full factorial design. Another advantage of experimental design is that different levels can be tested at the same time and interactions can also be identified depending on the resolution of the design, which is not possible by the traditional one-factor-at-the-time approach (Ritacco, Wu, and Khetan 2018).

2.9.1 Software

MODDE 12.1, Umetrics

2.9.2 Procedure

Prior every experimental screening design different settings must be defined. Factors were set according to the maximal working concentration which are summarized in Table 5. Level 1 setting corresponded to the maximal working concentration, level 0 or the center points represented the half maximal working concentrations and the -1 level represented a HQ-H_2O adjusted cultivation experiment. In addition, all fractional factorial screenings were conducted with an additional negative control (NC), that was not suggested by the software and therefore not considered for the evaluation. The whole concept of the level preparation and its application in this study is exemplarily explained in Figure 1. An example working matrix is shown in Table 6. The three factors in this case are A, B and C and are screened in a fractional factorial design of resolution III which was used as default screening design in this study. In total there are seven experiments, with three different levels. Experiment number one contained factor A at level -1, factor B at level -1 and factor C at level 1. In other words, the concentration of factor A and B was zero, but factor C was set to the maximal working concentration.

The center points with the experimental-IDs #5 to #7 contained the half maximal working concentration. As responses quantitative screening parameters like the maximal viable cell concentration are defined which are necessary to obtain a valid linear regression model, which balances the factors and responses. Used screening parameters are described later in this section (Siebertz, Bebber, and Hochkirchen 2010).

Table 6: Example design of experiment (DoE) matrix of a fractional factorial with resolution III. A brief description is given in the text before.

Experimental-ID	Factor A	Factor B	Factor C	Response 1	Response 2
1	-1	-1	1	y_1	z_1
2	1	-1	-1	y_2	z_2
3	-1	1	-1	y_3	z_3
4	1	1	1	y_4	z_4
5	0	0	0	y_5	z_5
6	0	0	0	y_6	z_6
7	0	0	0	y_7	z_7

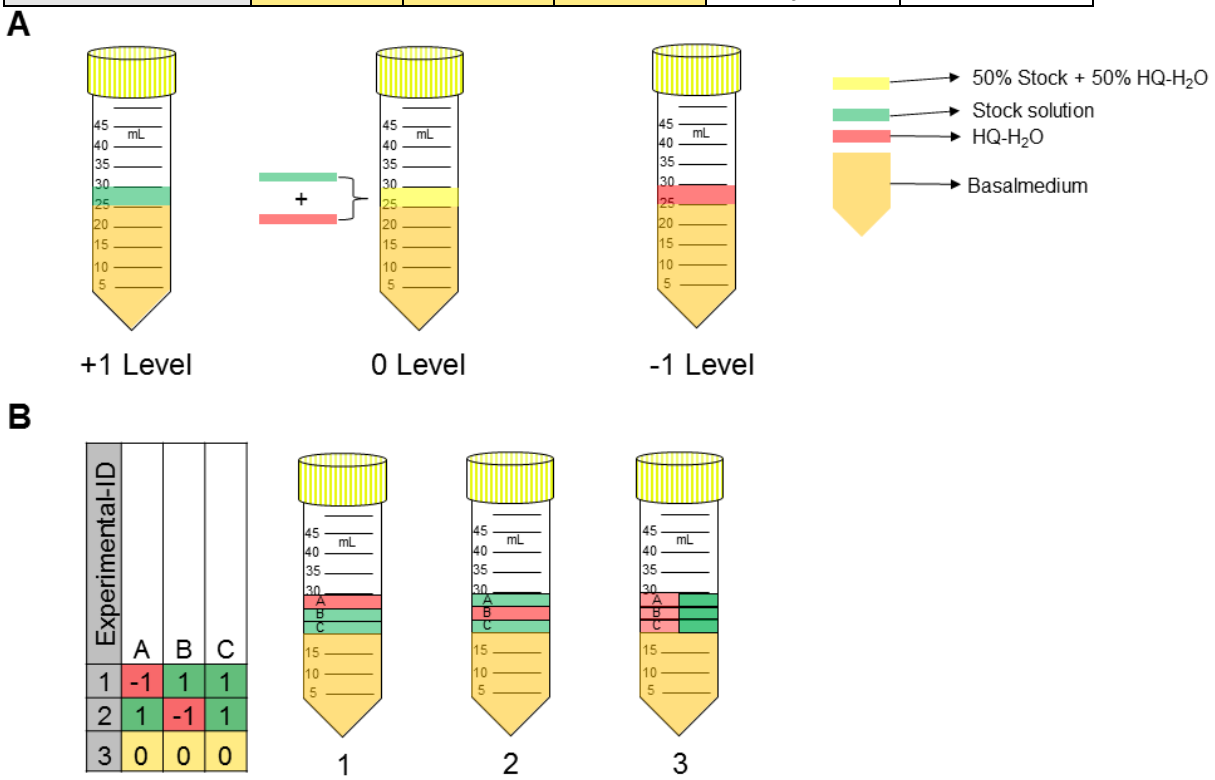


Figure 2: An overview of the level preparation (Part A) and an example design of experiment working matrix (Part B). Part A of the figure describes the procedure to obtain different levels. Green indicates a stock solution. The desired volume to obtain +1 level was calculated based on the required dilution to obtain the needed working concentration. Center points were obtained by mixing 50% of the stock solution with 50% of the HQ-H_2O, yielding in 50% of the maximal working concentration of level 1. The -1 levels were obtained by adding the same volume of HQ-H_2O as for the 1 level setting to obtain an adjusted cultivation. Part B considers an example working matrix. Experiment 1 does contain factor A in -1 level setting, factor B and C in 1 level setting. The second experiment is made up of 1 level of factor A and C and -1 level of factor C. Center points are obtained by mixing equal amounts of level 1 and -1, as indicated in experiment 3.

2.9.3 Evaluation of the results

Depending on the purpose of the screening experiment different responses or screening parameters must be defined and monitored. Responses used in this study are summarized and characterized in Table 7. The effect of certain factors is then summarized in a linear model, which can then be used for predictive models. The center points are not considered for the effect analysis, but are important as they proof the linearity of the linear model (Siebertz, Bebber, and Hochkirchen 2010).

Overall mean of the experiment $\bar{y}$	$\bar{y} = \frac{1}{n}\sum_{i}^{n} x_i$
Effect of factor E_i	$E_i = \frac{1}{n}\sum_{i}^{n} x_{i(1\ level)} - \frac{1}{n}\sum_{i}^{n} x_{i(-1\ level)}$
Regression model	$\hat{y} = \bar{y} + \frac{E_i}{2}x_1 + \frac{E_j}{2}x_2 + \frac{E_k}{2}x_3$ $\hat{y}$ Predictor x_i Level (1/0/-1)

The equations below describe exemplarily the effect analysis of the working matrix shown in Table 6. After all a linear regression model is obtained to quantify the impact of different factors on the response. Statistical software is also able to compute a model error (Ɛ), which is not indicated in the example.

$$\bar{y} = \frac{y_1 + y_2 + y_3 + y_4}{4}$$

$$E_A = \frac{y_2 + y_4}{2} - \frac{y_1 + y_3}{2}$$

$$E_B = \frac{y_3 + y_4}{2} - \frac{y_1 + y_2}{2}$$

$$E_C = \frac{y_1 + y_4}{2} - \frac{y_2 + y_3}{2}$$

$$\hat{y} = \bar{y} + \frac{E_A}{2}x_1 + \frac{E_B}{2}x_2 + \frac{E_C}{2}x_3$$

The evaluation if an effect can be considered as significant was determined by so-called effect plots, who directly compare the different levels described by 'E_i' above. In a fractional factorial design the obtained effect is double the coefficient of the linear

model. In general the effect described the difference between 1 level and -1 level. Figure 3 characterizes an example effect analysis. The y-axis quantifies the effect caused by the factors. Factor A in this example had a significant positive effect on the response indicated by the error bar, which do not cross the y-axis at zero. In general the error bars indicate a 95% confidence interval. Factor B is an example for a non-significant effect. There might be an enhanced response caused by the effect, which is statistically not significant. The last response characterized is caused by factor C, which show in this example a significant negative effect on the response.

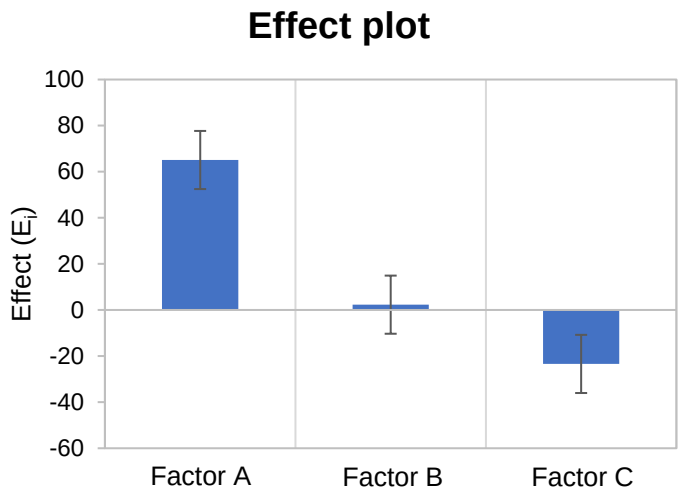


Figure 3: An example effect plot, with factors A, B and C. Error bars represent a 95% confidence interval. A detail description is given in the text.

The overall quality of the model was assessed by the R^2 and Q^2 value. R^2 describes the goodness of the fit and should be higher than 0.8. In general this parameter described the percentage described by the model. An R^2 of 0.8 describes that at least 80% of the data can be described by the obtained model. The Q^2 describes the goodness of prediction. A high Q^2 indicates that a high percentage of the data can be predicted by the model. In literature a Q^2 above 0.6 is recommend, indicating that 60% of the responses can be predicted by the model (Mandenius and Brundin 2008).

Table 7: Summary and overview of the screening parameters and responses respectively taken for the effect analysis and also for evaluation of the cultivation process.

Screening parameter	Description
Maximal viable cell concentration [$\times 10^6$ cells$\times$mL^{-1}]	The maximal viable cell concentration is an important measure to characterize the biomass formation. Only viable cells are able to produce recombinant product of proper quality. Despite all, a high biomass concentration is not always favorable in terms of specific productivity.
Maximal antibody titer [µg×mL^{-1}]	Product formation is essential for process evaluation. Maximization of product formation is always a driving force in process development.
Maximal specific productivity [pg×cell^{-1}×day^{-1}]	Cell specific productivity balances the product and biomass formation within a certain time period. Improvement of the specific productivity is also driving force in process development. Processes with a low specific productivity are economically not feasible. At this point is has to be kept in mind that especially the specific productivity is biased by error propagation of several methods.
Process specific productivity [pg×cell^{-1}×day^{-1}]	The process specific productivity describes the specific productivity of a cell during process. It is calculated by equation below and compares the total product output to the total biomass formation. $Pq_P = \frac{titer_2 - titer_1}{VCD_{last\ day}}$
Viable specific productivity [pg×cell^{-1}×day^{-1}]	The maximal specific productivity is error prone due to error propagation as already mentioned. To circumvent biased results, viable specific productivities have been screened. This measure only concerns specific productivities obtained during positive viable specific growth rates. Specific productivities obtained by negative viable specific growth rates are not considered.
Metabolite concentrations [g×L^{-1}]	Specific metabolite concentrations may yield information about the status of the cell. Depletion or accumulation of specific metabolites may alter the behavior of the cell.
Maximal average diameter [µm]	The diameter of a cell can be influenced by various parameters and do change in course of the process. Monitoring of the cell size during the process time may yield information about certain supplementations.
Process metabolite consumption/production rates [pg×cell^{-1}×day^{-1}]	The rate of consumption or production of metabolites over the process time is essential to describe the metabolic state of the cell.

2.10 Determination of the antibody titer via biolayer interferometry

Determination of the antibody titer is important to evaluate the production performance of a cell line under the chosen conditions. Biolayer interferometry (BLI) is an optical method which can be used for the purpose of antibody quantification via protein A coated fiber biosensors. The *Staphylococcal* protein A is characterized in that it provides five binding domains for the F_c region of various Ig subclasses. Only two to three sites are accessible for IgG binding, due to steric hindrance (Carta and Jungbauer 2010). The binding event of IgG to the protein A changes the thickness of the layer on the biosensor, which can be used for quantification of the antibody titer. In general a light signal is sent down the biosensor and only a minor amount is reflected due to changes in layer thickness by antibody binding. This causes a wavelength shift which is directly proportional to the amount of antibodies bound to the biosensor (Do et al. 2008).

2.10.1 Materials

Protein A (ProA) Dip and Read™ Biosensors (Pall, 1806191)

Nunc-Immuno™ MicroWell™ 96 well polystyrene plates (Nunclon®, P8616)

2.10.2 Equipment

Octet RED96e (ForteBio – A division PALL life sciences, FB-90057)

2.10.3 Procedure

The quantification of antibodies was performed in cell free crude culture supernatants. Dilutions were assumed based on estimated antibody titers. Furthermore, dilutions were prepared with phosphate buffered saline supplemented with 0.1% Tween20. A target concentration of 50 $\mu g \times ml^{-1}$ was set as maximum, due to non-linearity in the standard above this concentration. Dilutions were prepared and 200 µL of the obtained suspension were transferred into the 96 well plate according to the scheme shown in Figure 4. A summary of the assay is given in Table 8. The whole assay was conducted with one set of protein A tips so that a regeneration and neutralization step are necessary. For the regeneration 200 µL glycine-HCl buffer with a pH of 2.5 were used. Neutralization was obtained in 200 µL PBS supplemented with 0.1% Tween20. The prepared 96 well plate was then put into the BLI device for the measurement. Octet Data Acquisition software conducted the measurement fully automatically. The measurement procedure was initiated by dipping the protein A tips 10 minutes into 200 µL PBS supplemented with 0.1% Tween20 at 25°C. In the meantime, the plate,

harboring the samples were shook at 1000 rpm. After the so-called preconditioning of the protein A tips, the measurement was started by dipping the biosensors into the first column. Shaking was set at 1000 rpm and quantification was obtained for 120 seconds. After the quantification step a recycling cycle was performed to remove antibodies bound to the biosensors and to ensure proper measurement conditions for the next sample. For this to happen the protein A tips were dipped into column 11 for 5 seconds at 1000 rpm to ensure proper regeneration. Wells in column 11 contained 200 µL glycine-HCl with pH 2.5. After this step the protein A tips were subsequently dipped into 200 µL PBS supplemented with 0.1% Tween20 as neutralization step for 5 seconds at 1000 rpm. This so-called recycling cycle was performed three times, before the next measurement was started in column 2. The obtained binding rates were then evaluated by the octet data analysis software version 11. For quantification an in-house standard was established, which was based on a pharmacy Humira IgG antibody.

Table 8: Assay conditions for human IgG1 quantification at 25°C

Step	Duration	Shaking
Quantification	120 s	1000
Regeneration	5 s	1000
Neutralization	5 s	1000

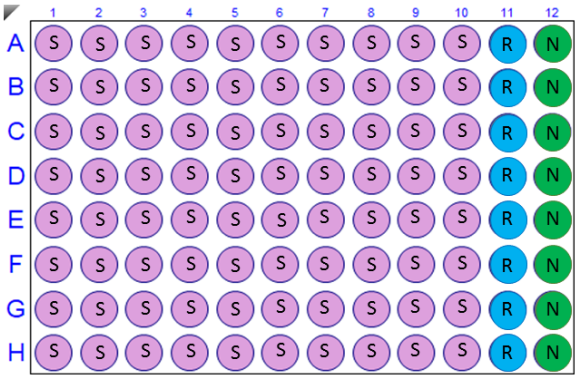


Figure 4: 96 well plate scheme. Magenta wells labelled with *"S"* indicate wells for the diluted protein sample. Blue wells labelled with *"R"* indicate regeneration wells filled with Glycine-HCl with pH=2.5 and the green wells labelled with *"N"* indicate the neutralization wells filled with Phosphate buffer saline supplemented with 0.1% Tween20. The direction of the measurement was from column number one to column number 10.

2.11 Determination of osmolality

The osmotic pressure can be defined as the concentration of all osmotically active substances dissolved in a solution and is a critical measure in cell culture processes. The osmolality with the unit osmole per kilogram solvent describes this measure. An osmole is defined as the amount of one mole molecules which impact on the osmotic pressure in the solution. Change in the osmotic pressure can derive from various sources, also proteins contribute to the change in osmotic pressure, which is then termed colloid osmotic pressure. Quantification of this measure is performed by application of the principle of the freezing point depression. In general the freezing point is the temperature at which solid and liquid state are in equilibrium at atmospheric pressure. Addition of osmotic active molecules depresses this temperature. The amount of osmotically active substances added to the solution is directly proportional to the freezing point depression, thereby the osmolality can be determined (Ge and Wang 2009; Gstraunthaler and Lindl 2013; Koumantakis and Wyndham 1989).

2.11.1 Materials

Measuring vessel (Gonotec GmbH, 30.9.0010)

300 mOsmol×kg^{-1} aqueous calibration standard (Gonotec GmbH, 30.9.0020)

2.11.2 Equipment

Osmomat® 030 (Gonotec GmbH, Serial number: 100404)

2.11.3 Procedure

Determination of osmolality over cultivation time was obtained by cell free crude culture supernatants. For the measurement 50 µL of the culture supernatant were pipetted in specific tubes for the Osmomat®. All following steps were performed to manufacturer's suggestions.

2.12 Enzymatic quantification of cell culture metabolites

Knowledge about nutrient levels during cultivation is crucial for process understanding. Nutrient depletions may explain certain events during the cultivation. The BioProfile 100 plus (nova biomedical) is a device, which uses enzymatic reactions to determine levels of glucose, L-glutamine, L-glutamate, lactate and ammonia.

2.12.1 Materials

Bioprofile® reagent pack (nova biomedical, 38291)

2.12.2 Equipment

Bioprofile 100 plus (nova biomedical, Serial number: T34712020)

2.12.3 Procedure

For quantification of specific metabolites 700 µL cell culture supernatant or cell suspension are required. Prior the measurement, it should be checked whether all sensors of the Bioprofile 100 plus are calibrated, if that is not the case a calibration should be performed. After the calibration the samples can be placed into the auto sampler and the whole procedure is carrying out fully automatic.

2.13 Quantification of purified protein by spectrophotometry

Protein quantification in solution is crucial to conduct further experiments. Various methods are available for this purpose. Amino acids, which are the building blocks of proteins have a plethora of properties. In this context the aromatic amino acid tryptophan is of special interest as it shows intrinsic fluorescence due to the indole group. Tyrosine and phenylalanine show intrinsic fluorescence as well, but are not as abundant as tryptophan in proteins and show a lower absorptivity. Tryptophan shows a dominant UV absorbance at 280 nm and can therefore be used for label free quantification of proteins in solution (Ghisaidoobe and Chung 2014). At this point it should be mentioned that this method is only suitable to quantify purified protein as the method is not selective like BLI (Do et al. 2008). Quantification of the protein is then performed by measuring the absorbance (A) of the solution at 280 nm, which is then further used to calculate the concentration via the path length (l) and the molar extinction coefficient (ε_m) at 280 nm. The prior mentioned coefficient must be calculated individually for each protein depending on the sequence and possible modifications like glycosylation (Carta and Jungbauer 2010).

$$A = -log\frac{I}{I_0}$$

$$c = \frac{A}{\varepsilon_m \times l}$$

2.13.1 Procedure

Prior the measurement specific information about the protein was required. For example the molar extinction coefficient (ε_m) needed to be calculated. ProtParam[i] is an online tool designed to estimate parameters like the molar extinction coefficient. Only the amino acid sequence of the protein is necessary to obtain information about the molar mass and the extinction coefficient. Furthermore the precision of the calculation can be optimized by indicating the glycosylation sites. Protein quantification was then initiated by setting a blank with the buffer in which the protein is suspended. The spectrophotometer, 1 µL of the buffer solution were applied on the measuring lens and the lid were closed. Then the blank procedure was carried out. After the blank procedure, a measurement of the buffer was performed to check if the success of the procedure, variations within ±0.005 $mg \times mL^{-1}$ were accepted. Afterwards the lens was rinsed with HQ-H_2O and cleaned with lens paper. Then 1 µL of the sample, containing the protein of interest was applied on the measurement lens for quantification. The lid was again closed and the quantification was performed. Two measurements were performed to avoid errors or outliers. If a neglectable deviation of 10% was not exceeded, no further measurement was performed. After the last measurement, a cleaning procedure was performed again and the device was switched off.

2.14 Protein A affinity chromatography for Immunoglobulin G purification

Protein characterization is sensitive and often requires highly purified protein. Protein A is a *Staphylococcal* protein, which provides five binding sites for the F_c region of an IgG and can therefore be used to selectively purify IgG. Crude cell culture supernatant can therefore be taken for purification of the IgG. Highly purified protein is then obtained by affinity chromatography. Different buffers and solutions provide conditions mediating binding or elution by changing the chemical environment (Carta and Jungbauer 2010).

[i] https://web.expasy.org/protparam/ - 4th of January 2019

2.14.1 Materials

HiTrap Protein A HP (GE Healthcare, 17040203)

Equilibration buffer, PBS, pH 7

Wash buffer, 50 mM Sodium acetate, pH 5.6

Elution buffer, 50 mM Sodium acetate, pH 3.5

Strip solution, 100 mM Acetic acid

CIP solution, 100 mM NaOH

Storage solution, 20% Ethanol

2.14.2 Equipment

Äkta Start (GE Healthcare)

2.14.3 Procedure

Prior the chromatographic purification all liquids needed to be filtrated with at least 0.45 µm pore size filter to remove interfering particles. All purification steps were performed on an ÄKTA Start (GE Healthcare). Initiation of the purification was obtained by checking and fitting outlets and inlets to the corresponding containments and buffers and liquids. Then the system was purged with HQ-H_2O to remove residual 20% ethanol. All outlets were washed separately by a predefined purification program at a flow rate of 1 mL×min^{-1}. Afterwards the column was equilibrated by equilibration buffer, PBS. The conductivity signal indicated the progress of the flow as the solutions show a different conductivity. As soon as a constant UV signal was obtained a set to zero was performed. Then the valve was changed to load the sample on to the column, the flow rate kept with 1 mL×min^{-1}. The replacement of PBS can be seen by a decrease in conductivity. As first fraction the so-called flow through eluates and was collected in a suitable containment. After loading the desired volume of supernatant, depending on the concentration, onto the column, a washing step was performed. For this to happen five column volumes of washing buffer and PBS were purged through the column. Again the flow through of the wash fraction was collected into a new containment. The flow progress of the washing buffer could be monitored by an increase in conductivity. Washing was then followed by the elution step. For this to happen the containment was changed again and elution buffer was filled into the column. Elution of the protein of interest could be monitored by the change in UV signal. Subsequently after the elution a neutralization step of the eluate was performed by the addition of HEPES.

After elution, the column was washed with the strip solution to remove unwanted protein attached to the column. Again the containment was changed and the so-called clean fraction was collected. At the end of the purification all outlets and the column were flushed with five column volumes HQ-H_2O. After all the whole chromatography system was purged with 20% ethanol to prevent contamination. The protein A column was then stored in 20% ethanol and at 4°C in the freezer.

2.15 Determination of thermal stability by differential scanning calorimetry

Thermal protein stability is a crucial quality attribute in manufacturing of therapeutic proteins. The discipline of calorimetry in general deals with heat transfer during reactions and processes. During the event of protein unfolding a change in heat transfer can be monitored. Differential scanning calorimetry commonly known as DSC is a method for determination of heat flow during different processes. The measuring principle relies on the temperature difference of the sample cell and reference cell. Unfolded protein characteristically absorbs more heat due to the higher heat capacity than natively folded protein. Thermodynamically natively folded protein has a lower free energy state than the unfolded state. A characteristic value for the thermal stability of the protein is the melting temperature (T_m), at which 50% of the proteins are in the unfolded state and 50% are folded state. This value is characterized by a peak in the thermogram (Gill, Moghadam, and Ranjbar 2010).

2.15.1 Materials

Sample buffer, 30 mM Phosphate, 150 mM NaCl, pH 6

Elution buffer, 50 mM Sodium acetate + HEPES, pH 7

PD-10 Desalting column (GE Healthcare, 17-0851-01)

2.15.2 Equipment

MicroCal PEAQ-Differential scanning calorimeter (Malvern Panalytical, MAC1172720)

2.15.3 Procedure

In order to get information about thermal stability of the recombinant produced protein, differential scanning calorimetry was performed. Protein A purified human IgG1 samples were used for the measurement. An aliquot of the purified protein was used for buffer exchange from sodium acetate to the phosphate buffer system, which was carried out in PD-10 columns (GE Healthcare) according to manufactures suggestions. Afterwards the protein concentration was determined by a spectrophotometric method

described in section 2.13 and the concentration of the protein containing solution was set to 0.7 mg×mL^{-1} by the buffer in which the protein is suspended. At the end 325 µL of the sample and of the buffer were pipetted into the sample cell and reference cell. A default scheme is given in Figure 5 and a short description is given in the figure legend. Then the plate was spun down to ensure bubble free solutions and subsequently placed into the device. Default parameters were set which are summarized in Table 9.

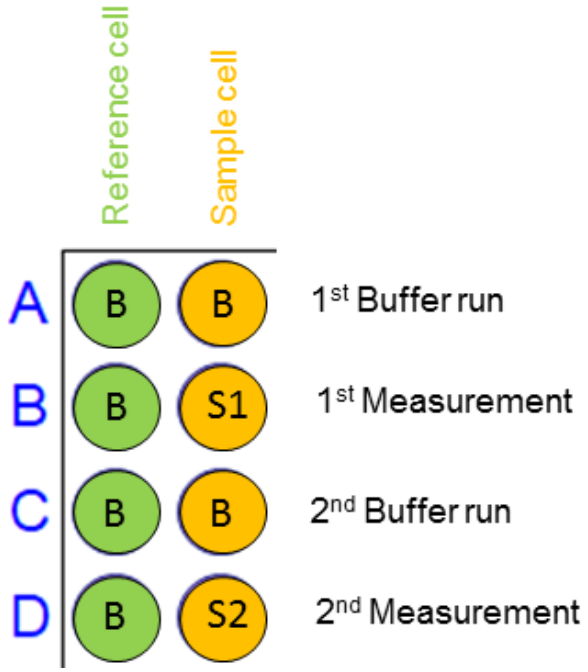


Figure 5: Scheme for a DSC measurement. As indicated in the picture there are two different cells. Green indicates the reference cell in which the buffer (B) of the protein is placed and the sample cell indicated in yellow contains either a buffer (B) for a buffer run or a sample (S). It is recommended to run at first a buffer (see 1st buffer run) to set a baseline or to monitor impurities or other undesired effects (row A). Then the 1st measurement can be performed (see row B), in which the buffer is placed into the reference cell and the 1st sample (S1) placed into the sample cell. In order to run a second measurement for the 2nd sample (S2) an additional buffer run must be performed to remove accumulated impurities.

Table 9: Summary of the default settings for a DSC measurement.

Parameter	**Setting**
Scan rate	60°C×h^{-1}
Scanning range	20-100°C
Cleaning mode	Wash

3 Results

Two fractional factorial designs of resolution III at two levels were defined to screen 21 different substances. It was assumed that there is no interaction between the substances so that the resolution III is sufficient for the purpose of this study. After the initial screening phase, one additional screening experiment was performed to verify the obtained results from the prior screenings by applying a fractional factorial design of resolution V+. The candidate substances for this final screening have been chosen based on their beneficial impact on defined responses or screening parameters. A positive impact on the antibody titer was set as first criterion, followed by a positive effect on the maximal viable cell concentration and as last criterion the maximal cell specific productivity. The order of the criteria was chosen according to estimated error robustness. Betaine, DMSO, maltodextrin and valproic acid performed best according to the ranking scheme. In addition, 4-PBA was selected as extraordinary candidate due to its ability to improve the cell specific productivity and to limit the maximal viable cell concentration without affecting the viability over the process time. Furthermore, follow-up experiments have been designed and performed based on the results obtained during the first two fractional factorial screening designs of resolution III. This comprised a DMSO supplemented semi-perfusion cultivation and a glucose controlled and DMSO supplemented batch. The produced amount of antibodies obtained in the semi-perfusion cultivation was purified via protein A chromatography and characterized by DSC, to evaluate whether DMSO supplementation had any effect on the thermal stability of the protein. Additionally, different buffer systems have also been tested to identify possible bottlenecks of the protein purification and storage. As an outcome of this study, promising chemical chaperones could be identified and characterized for a recombinant, IgG_1 producing, CHO-K1 clone. Especially DMSO and 4-PBA showed promising potential for further evaluation as they impact on the specific productivity of the CHO cell.

3.1 Characterization of chemical chaperones by experimental design

The following sections will characterize the obtained results in detail. Especially significant effects are highlighted.

3.2 First fractional factorial screening design for effect analysis of 14 individual substances

The conducted fractional factorial screening experiment of resolution III comprised 20 experiments indicated by the “Experimental-ID” in Table 10. These 20 experiments consisted of a center point conducted as biological triplicates with factor setting 0, 16 experiments with different factor setting combinations and one negative control (NC) obtained by setting all levels to -1, which was not suggested by the software. In general the screening comprised 14 different chemical substances defined as factors, which have been supplemented on the third day of cultivation. Furthermore Table 10 summarizes the obtained responses and performance parameters. For the statistical evaluation multiple responses were taken into account. Maximal viable cell concentration (mVCC) of 5.5×10^6 c×mL^{-1} to 10.3×10^6 c×mL^{-1} were reached with a cumulating antibody titer on day 8 (d8mAb) between 267 µg×mL^{-1} and 374 µg×mL^{-1}. This translates into maximal specific productivities (mqP) of 8 pg×cell^{-1}×day^{-1} to 17 pg×cell^{-1}×day^{-1}. Additional values describing the performance of each individual experiment are summarized in Table 10. The maximal antibody titer (mTiter), which was not taken for the evaluation due to a high variability in the center points ranged from 227 µg×mL^{-1} to 374 µg×mL^{-1}. Final process times of 8 to 10 days were reached. The composition and the obtained responses for experiment #1 is exemplarily explained. This experiment was not supplemented with ß-alanine, betaine, copper sulfate, cysteine, dextran, maltodextrin and maltose, but received +1 level of mannitol, spermidine, sucrose, taurine, trehalose and TMAO. For this experiment a maximal viable cell concentration of 8.8×10^6 cells×mL^{-1}, an antibody titer of 227 µg×mL^{-1} on day 8 and a maximal specific productivity of 8 pg×cell^{-1}×day^{-1} was reached as responses. Furthermore, a maximal antibody titer of 227 µg×mL^{-1} and a process duration of 9 days were reached as performance parameters.

Table 10: Summary of the experimental design matrix used for the first screening experiment. 14 individual substances were combined at two different factor levels and a triplicate culture at half-maximum working concentration representing the center points. The measured responses, including maximal viable cell concentration (mVCC), antibody titer on day 8 (d8mAb) and the maximal specific productivity (mqP) were used as input data for subsequent regression analysis. One negative control (NC) with basal media only was included, which was not required for the evaluation. Experiment #16 is described in all following graphs as “all supplements”.

	Factors														**Responses**			**Performance**	
Experimental-ID	ß-Alanine	Betaine	$CuSO_4$×5 H_2O	Cysteine	Dextran	Dimethylsulfoxid	Maltodextrin	Maltose×H_2O	Mannitol	Spermidine	Sucrose	Taurine	Trehalose×2 H_2O	Trimethylamine N-oxide	mVCC [×10^6 cells×mL^{-1}]	(d8mAb) [µg×mL^{-1}]	mqP [pg×$cell^{-1}$×day^{-1}]	mTiter [µg×mL^{-1}]	Process duration [days]
1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	8.76	226.85	8.02	226.85	9
2	1	-1	-1	-1	1	-1	1	1	-1	-1	-1	-1	1	1	8.35	256.7	8.60	256.70	9
3	-1	1	-1	-1	1	1	-1	1	-1	-1	1	1	-1	-1	5.54	333.6	17.01	333.60	8
4	1	1	-1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	6.05	373.5	17.22	373.50	8
5	-1	-1	1	-1	1	1	1	-1	-1	1	-1	1	-1	1	6.40	326.85	14.29	326.85	8
6	1	-1	1	-1	-1	1	-1	1	1	-1	1	-1	-1	1	6.27	317.75	13.89	317.75	8
7	-1	1	1	-1	-1	-1	1	1	1	-1	-1	1	1	-1	9.30	281.6	9.87	281.60	9
8	1	1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	9.33	257.6	9.92	257.60	9
9	-1	-1	-1	1	-1	1	1	1	-1	1	1	-1	1	-1	6.29	342.25	16.57	342.25	8
10	1	-1	-1	1	1	1	-1	-1	1	-1	-1	1	1	-1	6.13	329.4	15.52	329.40	8
11	-1	1	-1	1	1	-1	1	-1	1	-1	1	-1	-1	1	9.62	243.35	6.98	243.35	10
12	1	1	-1	1	-1	-1	-1	1	-1	1	-1	1	-1	1	9.87	254.05	7.82	254.05	9
13	-1	-1	1	1	1	-1	-1	1	1	1	-1	-1	-1	-1	8.97	342.25	9.36	342.25	9
14	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	-1	-1	8.62	256.6	10.19	256.60	9
15	-1	1	1	1	-1	1	-1	-1	-1	-1	-1	-1	1	1	6.67	308.6	14.49	308.60	8
16	1	1	1	1	1	1	1	1	1	1	1	1	1	1	7.22	307.65	14.62	307.65	8
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.36	292.95	12.53	292.95	9
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8.82	299.8	12.05	299.80	9
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9.46	290.2	11.95	290.20	8
NC	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.32	234.70	9.34	234.70	9

Figure 6 summarizes the obtained experimental results graphically. Already the graphs show that the supplementation of different chemical chaperones impacted on different cultivation parameters. Each chemical chaperone which impacted significantly on a defined screening parameter is characterized separately in the course of this section. The obtained maximal values are summarized in Table 10. In general the maximal cell concentration was reached between day 6 and 8 and the viability did start to decrease at day 5 in most of the cultures. The negative control indicated by the red dashed line did show the highest cell concentration and additionally showed a higher viability over the course of the process time. Furthermore, most of the cultures reached their maximal antibody titer on day 7. The viable specific growth rates showed a typical batch profile, characterized by continuously decreasing values. Some cultures seem to stabilize the viable specific growth rate between day 5 and 7. Specific productivities show the biggest difference after the supplementation on day 3 of the cultivation. Maximal values for the specific productivity were obtained between day 4 and 6 of the cultivation in all cultures. Furthermore, the slope of the graph showing the viable cumulative cell days against the antibody titer indicates the specific productivity and already shows different slopes of the curves.

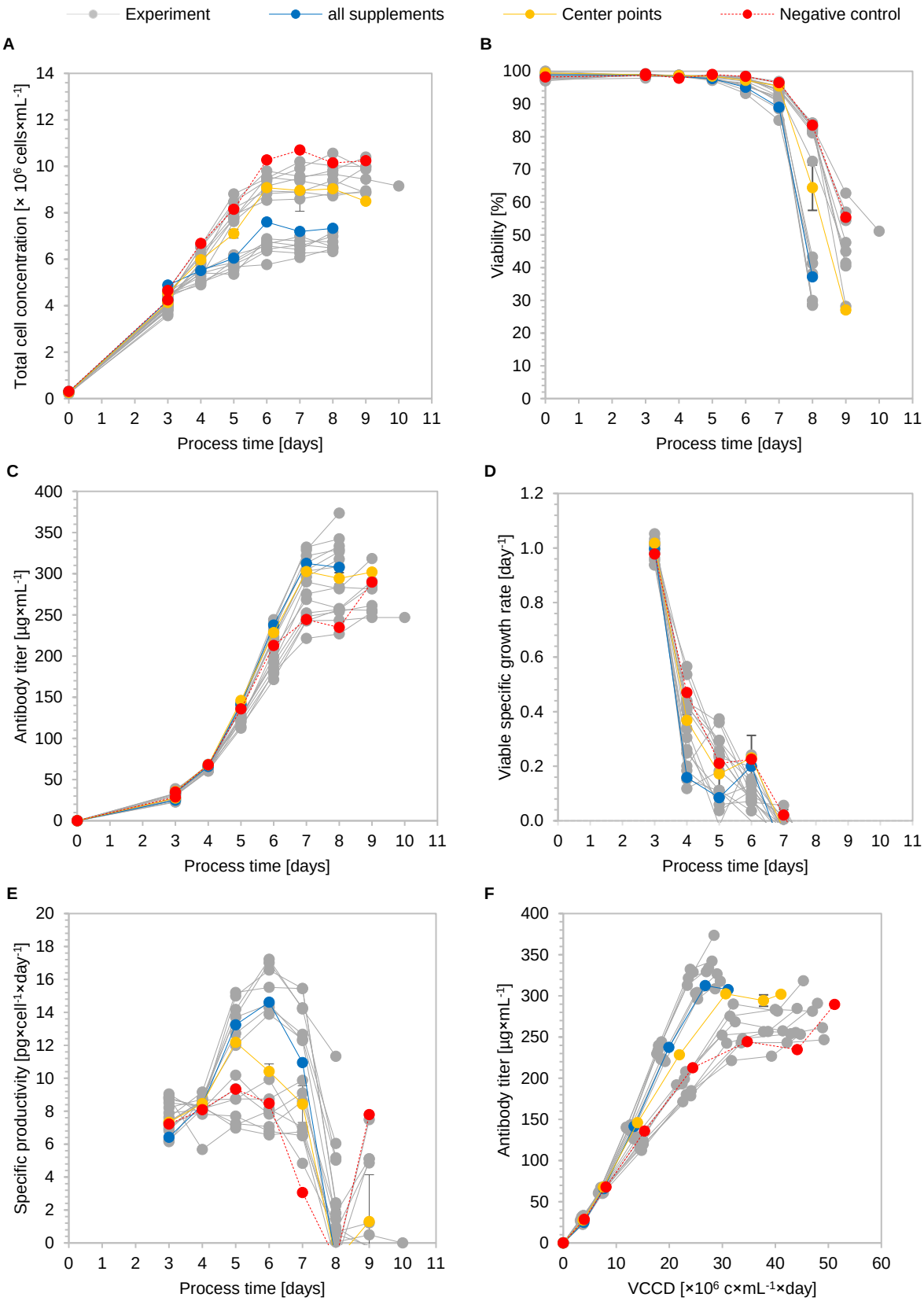


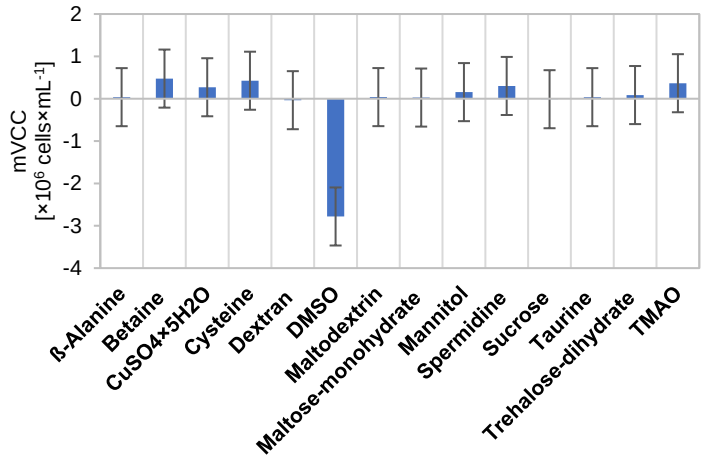
Figure 6: Change of cell culture parameters over process time in the first fractional factorial screening design of 14 substances. Experiment #16 included all 14 tested substances at level 1. Center points represent experiments #17 to #19, with a half-maximum factor setting of level 0 and are summarized by the mean and the corresponding standard deviation displayed in the error bars. The negative control (NC) was obtained by a volume adjusted culture, which corresponds to a factor setting of -1 level for all factors. (A) describes the total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate, (E) specific productivity over the process time and (F) the antibody titer in the course of the viable cumulative cell days, in which the slope describes the specific productivity.

3.2.1 Statistical evaluation of the main effects

The resolution of the fractional factorial screening design was III, so that only main effects were considered for the characterization of the substances, neglecting two factor interactions. Impact of substances on the maximal viable cell concentration (mVCC), antibody titer on day 8 (d8mAb) and maximal specific productivity (mqP) will be characterized in detail by effect plots (Figure 7). In this evaluation the antibody titer on day 8 was chosen due to a smaller variance of the center points compared to the maximal antibody titer values. Furthermore, a detailed characterization was only done if a factor impacted significantly on a response.

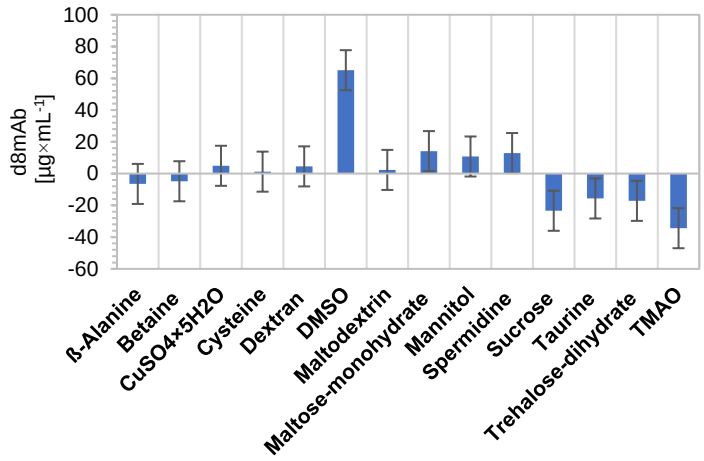
The effect plot shows that DMSO influenced various screening parameters. In all evaluations a good model quality could be obtained, represented by a high R^2 and Q^2. All obtained R^2 exceed a threshold of 0.8 and Q^2 also reach a threshold of at least 0.75. Only the antibody titer had a lower model quality in terms of Q^2. The maximal viable cell concentration was mainly negatively affected by the DMSO supplementation. Furthermore, DMSO enhanced the maximal specific productivity and thereby the antibody titer on day 8. Enhancement of antibody titers was also induced by mannitol and spermidine supplementation, whereas all other substances did not show any improvement of the antibody titer. Similar observations can be seen for the maximal specific productivity. DMSO had a significantly positive effect, whereas TMAO reduced the maximal specific productivity.

A



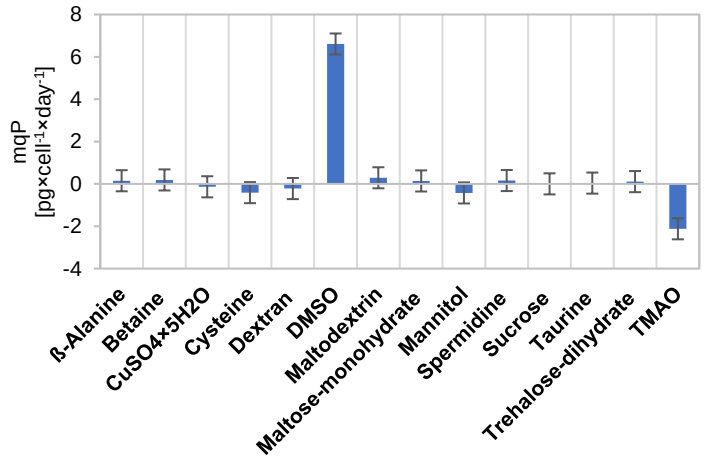
Parameter	Value
R^2	0.83
Q^2	0.79

B



Parameter	Value
R^2	0.98
Q^2	0.63

C



Parameter	Value
R^2	0.98
Q^2	0.97

Figure 7: Effect plot obtained by regression analysis of the experimental responses and the quality of the analysis expressed by R^2 and Q^2 for the maximal viable cell concentration (A, mVCC), antibody titer on day 8 (B, d8mAb) and maximal specific productivity (C, mqP). Error bars represent the calculated 95% confidence interval.

3.2.2 Dimethyl sulfoxide

Supplementation of DMSO showed significant impact on several screening parameters. The maximal viable cell concentration was significantly reduced as shown in the effect plot and the dot plot (Figure 7A and Figure 8B). All cultures supplemented with 2% DMSO showed a lower cell concentration after the supplementation on day 3 (green in Figure 8A). Furthermore, the viability decreased faster in DMSO supplemented cultures and the 60% viability threshold was reached on day 8 (Figure 8B).

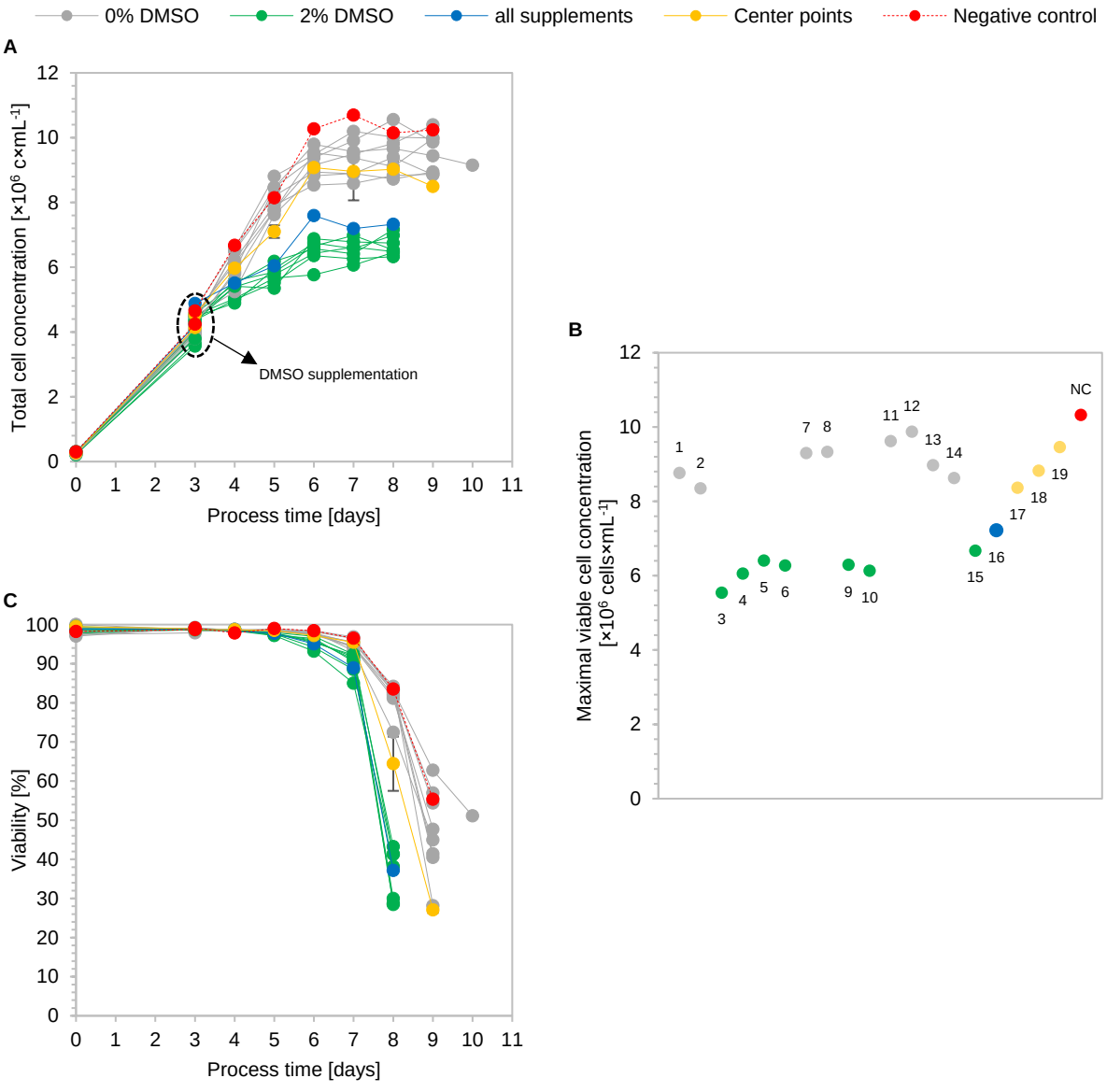


Figure 8: Overview of DMSO supplemented cultures and the impact on the total cell concentration over the process time (A), obtained maximal viable cell concentrations (B) and the course of the viability (C). The data plot (B) describes each experiment individually, exact compositions are shown in Table 10. The number adjacent to the dots correspond to the experimental-ID. The time point of the supplementation was day 3 and is indicated by a dotted circle in (A). Two measurements have been performed on day 3, one before and one after the supplementation for all obtained data.

The antibody titer was positively affected by the supplementation of DMSO as evaluated by the effect analysis (Figure 7B). Already on day 5 of the screening experiment, antibody titers were enhanced in DMSO supplemented cultures, despite lower cell concentrations (Figure 9A). The obtained antibody titer values for DMSO supplemented cultures on day 8 showed the highest values among all experiments as shown in Figure 9B.

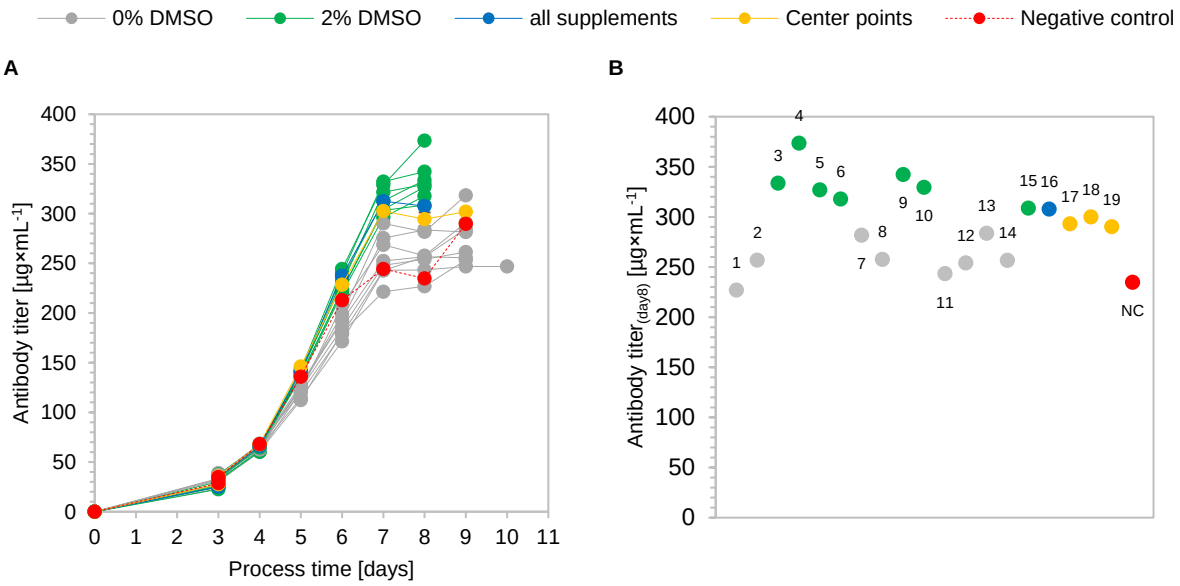


Figure 9: The course of the antibody titer over the process time (A) and the obtained antibody titers on day 8 (B) of DMSO supplemented cultures. Numbers adjacent to the dots in the obtained screening values (B) correspond to the experimental-ID as described in Table 6.

The negative impact on the maximal viable cell concentration and the positive effect on the antibody titer was due to a maintained specific productivity leading to a high maximal specific productivity (Figure 10A, B). DMSO supplemented cultures showed a higher specific productivity already one day after the supplementation on day 3. All DMSO supplemented cultures reached their maximal specific productivity on day 6 with a valid viable specific growth rate (Figure 10C). Furthermore, a stabilization of the specific productivity can be seen between day 5 and 7 for DMSO supplemented cultures. The specific productivity was enhanced by at least 1.5-fold in comparison to the negative control on day 6.

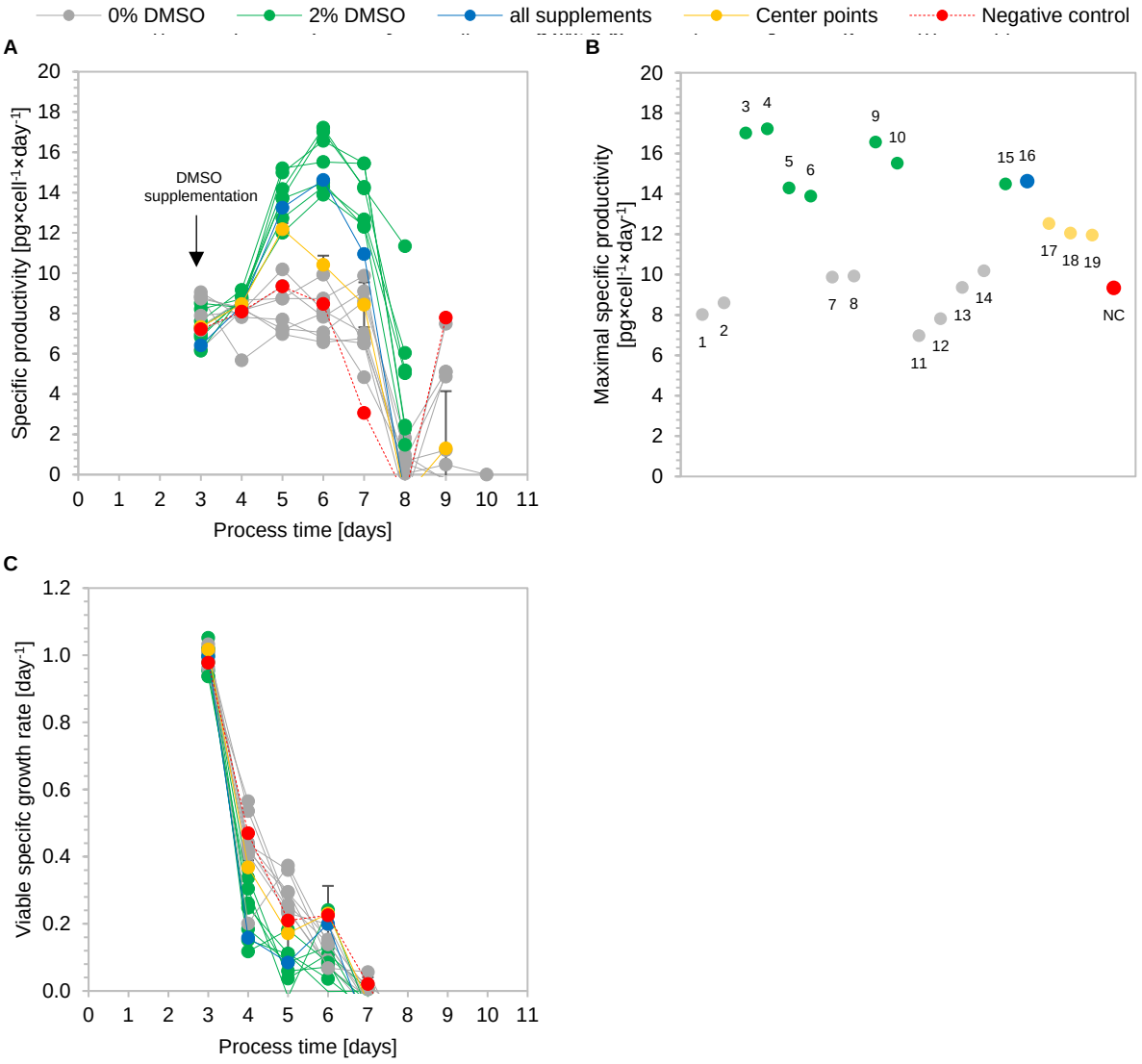


Figure 10: The specific productivity (A) and viable specific growth rate (C) of DMSO supplemented cultures in the course of the process time and the obtained maximal values (B) used for the effect evaluation.

Osmolality and cell size have also been recorded in the course of process time (Figure 11A). Information obtained about cell morphology may explain or lead to hypotheses explaining the effect of DMSO supplementation. Graphs show that the osmolality in DMSO supplemented cultures have been enhanced as well as the average cell diameter. This led to a decreased surface to volume ratio as shown in Figure 11C.

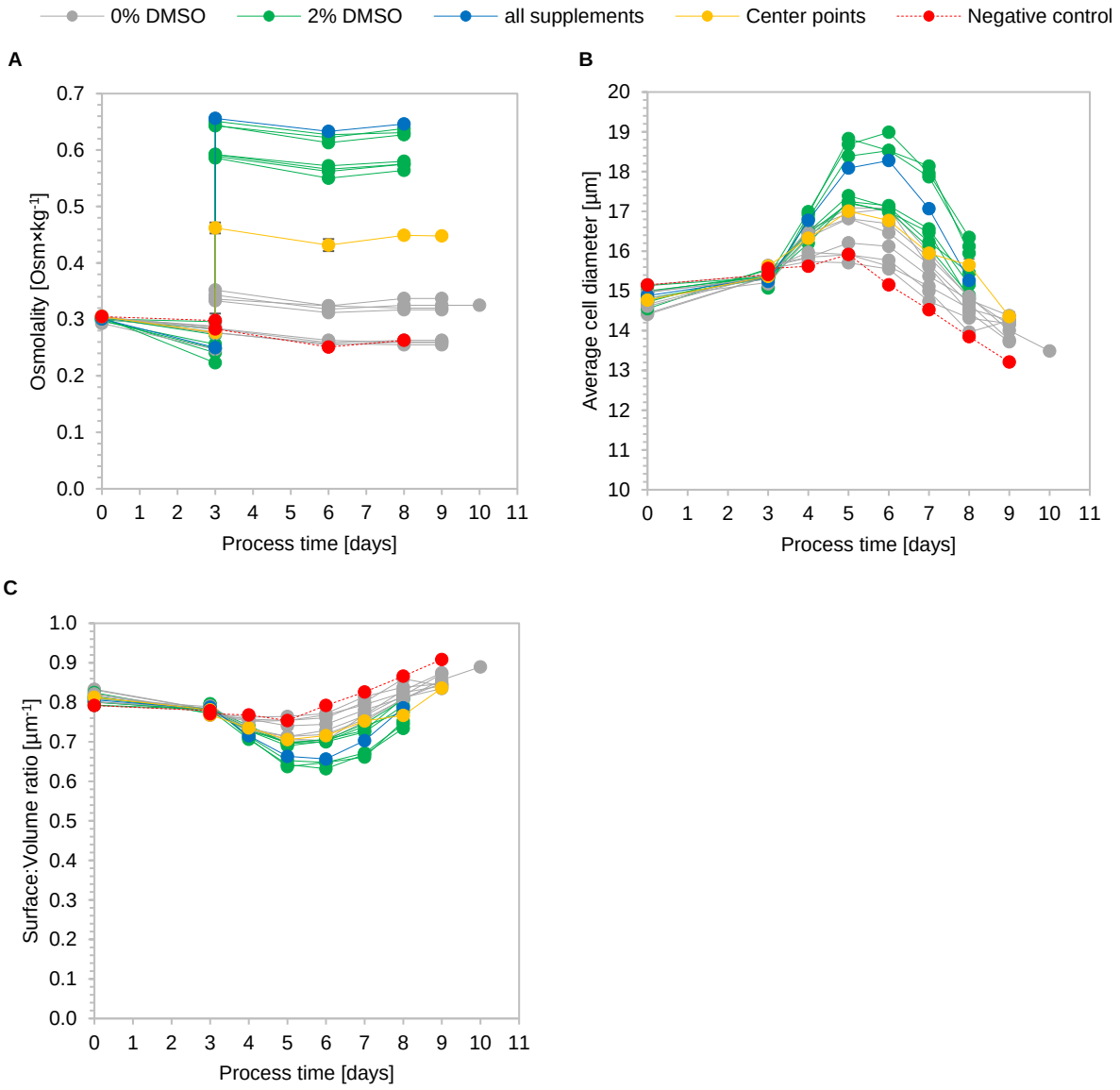


Figure 11: The impact of the DMSO supplementation on the osmolality (A), average cell diameter (B) and the surface to volume ratio over the process time (C).

3.2.3 Taurine

The evaluation did show that the supplementation of taurine led to a decreased antibody titer. This significant negative effect can already be seen in Figure 12A describing the course of the antibody titer and the obtained maximal values taken for the evaluation in section B. The green lines and dots (Figure 12), who have been supplemented with taurine and without DMSO did show a low antibody titer comparable to the negative control, which was not considered for the statistical evaluation of the effects. In contrast the purple lines and dots do show a higher antibody titer, which is mainly caused by the DMSO supplementation and therefore indicated in the graphs.

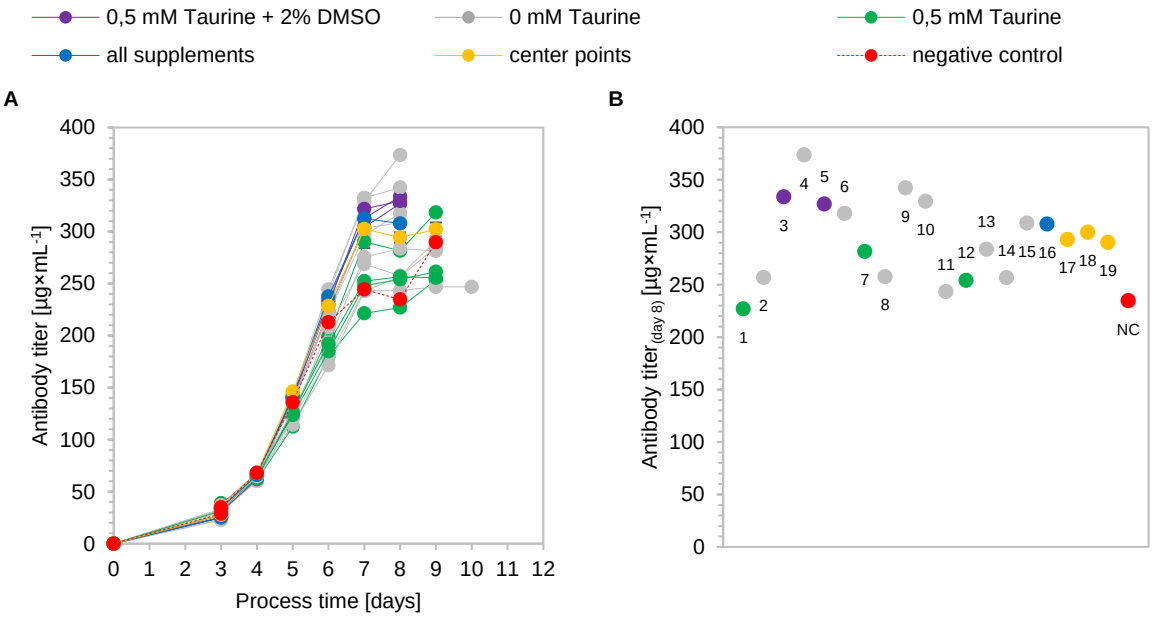


Figure 12: The impact of taurine supplementation on the antibody titer over process time (A) and the obtained antibody titers of day 8 taken for the evaluation (B).

3.2.4 Trehalose×2 H_2O

As the data plot already indicates, supplementation of trehalose×2 H_2O had a significant negative impact on the antibody titer. The graph and the dot plot (Figure 13A and B) already show a lower antibody titer for supplemented cultures in comparison to other experiments. More precisely the dot plot show that the supplementation led to a clear separation of supplemented and non-supplemented cultures but again the effect of the DMSO supplementation is present and indicated in purple.

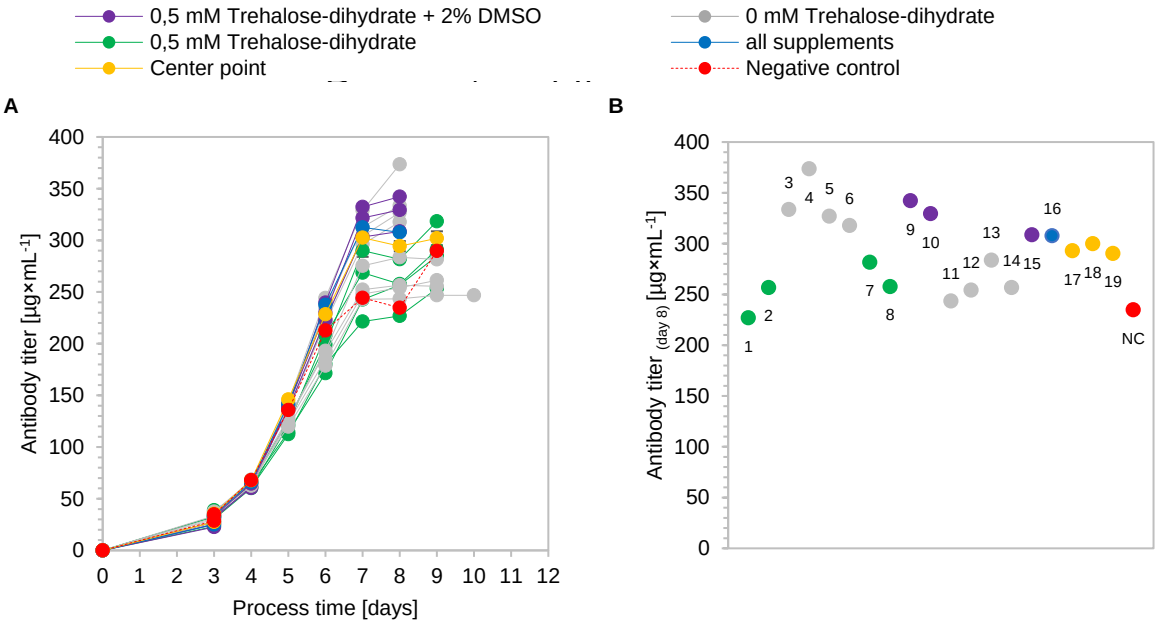


Figure 13: The figures characterize the course of the antibody titer of trehalose×2 H_2O supplemented cultures (A) and the obtained antibody titer values used for effect evaluation (B).

3.2.5 Trimethylaminoxid

The effect evaluation did show that the TMAO supplementation resulted in a reduced antibody titer. This effect was considered to be significant in the effect analysis. Already the graph describing the antibody titer in the course of the process time indicates, that the antibody titer was always lower for plain TMAO supplemented cultures after day 3 (Figure 14A) of the cultivation in comparison to the other cultures. Furthermore, day 8 antibody titers shown in the dot plot were lower for the supplemented cultures in comparison to the non-supplemented cultures. The light green dots and lines indicate the combination of TMAO and DMSO, which showed an enhanced antibody titer mainly caused by the DMSO.

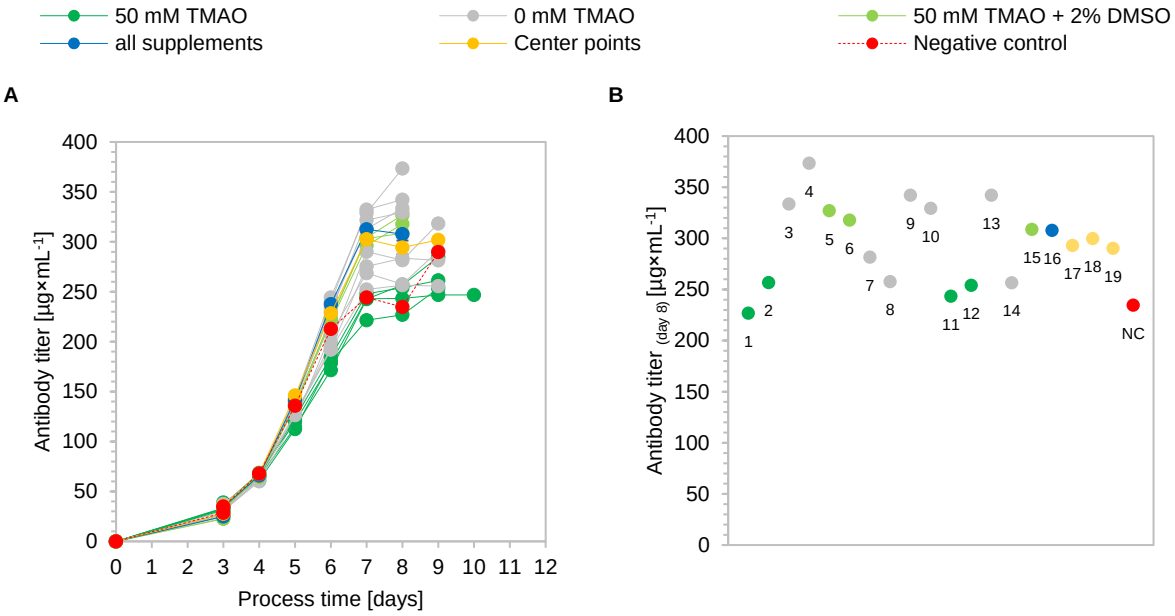


Figure 14: Supplementation of TMAO and the observed impact on the antibody titer in the course of the process time (A) and the obtained antibody titers used for the effect analysis (B).

A typical decrease in the specific productivity over the process time can be seen in Figure 15A for plain TMAO supplemented cultures. Furthermore, the cultures supplemented with TMAO and without DMSO show a lower specific productivity than the negative control. The supplemented cultures show an at least 1.2-fold reduced maximal specific productivity. Cultures supplemented with the combination of DMSO and TMAO show an enhanced maximal specific productivity mainly caused by the DMSO supplementation.

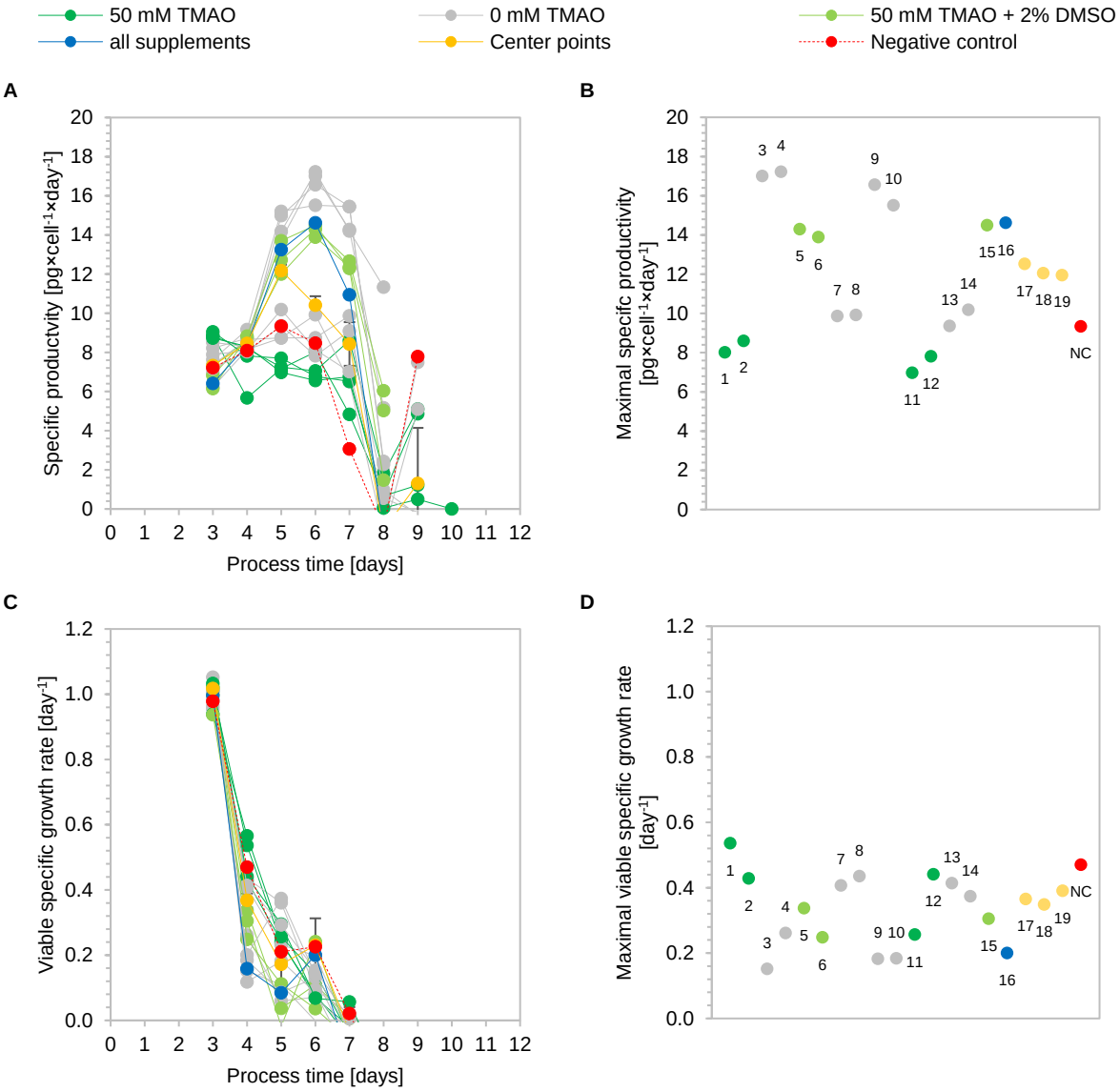


Figure 15: TMAO supplementation and the effect on the course of the specific productivity (A) and viable specific growth rate (C) over the process time and the obtained maximal values taken for the effect analysis (B and D).

3.2.6 Sucrose

In general the evaluation showed that the supplementation of sucrose led to a significant decrease in the antibody titer. The graphs shown in Figure 16A, elucidates the impact of the sucrose supplementation. Experiment #1, #8, #11 and #14 show the lowest antibody titers on day 8 among all other supplemented cultures, only the negative control indicated in red shows an even lower antibody titer on day 8, but a higher maximal antibody titer at the end of the cultivation. Again, the effect of DMSO is still visible as indicated by the light green dots and lines.

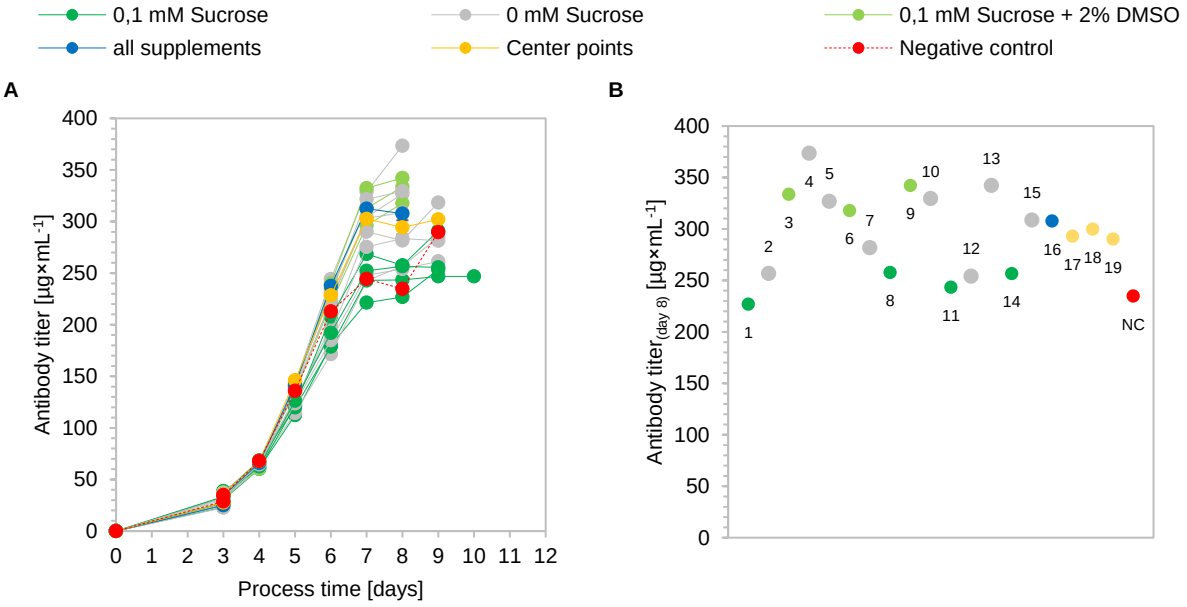


Figure 16: The impact of sucrose supplementation on the course of the antibody titer (A) and the obtained values (B) used for the evaluation.

3.3 Second fractional factorial screening design for effect analysis of 12 individual substances

Table 11 gives an overview of the different factor combinations used for this fractional factorial screening experiment of resolution III. In total 12 different chemical chaperones have been screened in 19 different experiments indicated by "Experimental-ID". In addition, a negative control was included to compare the obtained results to a non-supplemented culture. The obtained responses of the maximal viable cell concentration (mVCC) ranged from 4.8×10^6 c×mL^{-1} to 12.1×10^6 c×mL^{-1}, maximal antibody titer (mTiter) from 196 µg×mL^{-1} to 375 µg×mL^{-1} and maximal specific productivities (mqP) from 9 pg×cell^{-1}×day^{-1} to 12 pg×cell^{-1}×day^{-1}. In general the process times reached 9 to 11 days.

Table 11: A summary matrix of the experimental design and the obtained responses used for the effect analysis. Responses were defined as maximal viable cell concentration (mVCC), maximal antibody titer (mTiter) and the maximal specific productivity (mqP). Additionally the process duration is also summarized as performance parameter. Linoleic acid ß-cyclodextrin complex (LA/CD). Experiment #16 will be indicated by "all supplements" in the following graphs.

	Factors												Responses			Performance
Experimental-ID	Insulin	Valproic acid	4-Phenylbutyric acid	Glycerol	LA/CD complex	Myo-inositol	Spermidine	Maltodextrin	Trehalose×2 H_2O	Betaine	Beta-Alanine	Maltose×H_2O	mVCC [×10^6 c×mL^{-1}]	mTiter [µg×mL^{-1}]	mqP [pg×cell^{-1}×day^{-1}]	Process duration [days]
1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	10.59	334.40	9.54	9
2	1	-1	-1	-1	1	-1	1	1	-1	-1	-1	-1	10.09	307.60	9.20	10
3	-1	1	-1	-1	1	1	-1	1	-1	-1	1	1	12.08	331.20	10.22	9
4	1	1	-1	-1	-1	1	1	-1	1	1	-1	-1	8.55	287.20	10.68	10
5	-1	-1	1	-1	1	1	1	-1	-1	1	-1	1	5.58	324.80	10.85	11
6	1	-1	1	-1	-1	1	-1	1	1	-1	1	-1	5.39	204.00	9.22	10
7	-1	1	1	-1	-1	-1	1	1	1	-1	-1	1	6.02	297.20	11.25	11
8	1	1	1	-1	1	1	-1	-1	-1	1	1	-1	4.84	205.00	11.15	9
9	-1	-1	-1	1	-1	1	1	1	-1	1	1	-1	10.80	356.00	9.17	10
10	1	-1	-1	1	1	1	-1	-1	1	-1	-1	1	7.76	375.20	11.48	10
11	-1	1	-1	1	1	-1	1	-1	1	-1	1	-1	10.86	317.20	11.71	10
12	1	1	-1	1	-1	-1	-1	1	-1	1	-1	1	8.05	339.20	9.88	10
13	-1	-1	1	1	1	-1	-1	1	1	1	-1	-1	5.78	299.00	9.67	11
14	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	4.76	196.20	10.76	9
15	-1	1	1	1	-1	1	-1	-1	-1	-1	-1	-1	5.59	280.00	12.16	10
16	1	1	1	1	1	1	1	1	1	1	1	1	5.55	197.80	9.47	9
17	0	0	0	0	0	0	0	0	0	0	0	0	6.07	276.40	10.51	10
18	0	0	0	0	0	0	0	0	0	0	0	0	5.92	255.80	9.54	10
19	0	0	0	0	0	0	0	0	0	0	0	0	5.79	274.60	11.01	10
NC	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	10.87	286.40	11.13	10

Figure 17 show again different impacts caused by the addition of different chemical supplements on day 3 of the cultivation. Already the viability indicates a nick, which was caused by the maltodextrin supplementation on day 3. A major difference can be seen in the total cell concentration over the process time. After supplementation on day 3, the 4-PBA supplemented cultures stopped growing and reached low maximal cell concentrations. Other cultures reached maximal total cell concentration on day 7 of the cultivation. Furthermore, the decrease in viability over the process time was also different. Up to day 6 the negative control accumulated equal antibody titers comparable to the other cultures, but deviates afterwards. The growth rate decreased steadily, as expected for a typical batch culture as shown in the negative control. Some supplements seem to stabilize the cell specific productivity or even enhance it during the process time, but with a critical viability at day 8. Similar observations can be seen in the plot showing viable cumulative cell days against the antibody titer. The slope describes the cell specific productivity, which is decreasing in the course of the process for the negative control but is maintained for other experiments from day 4 to 6 due to chemical supplementation.

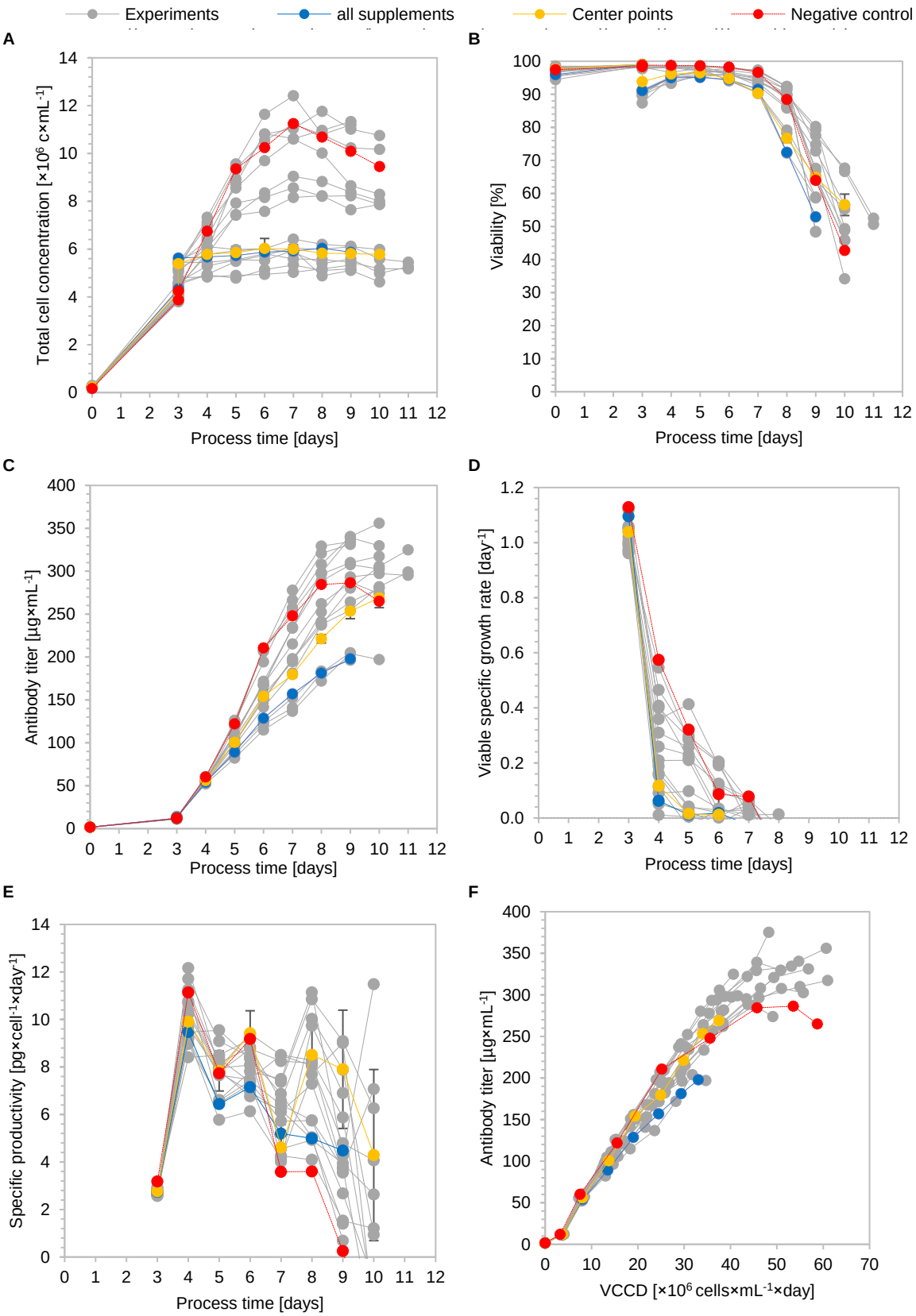
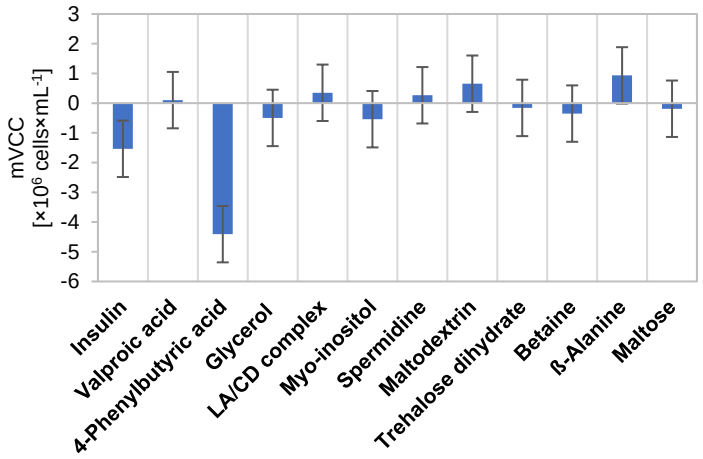


Figure 17: Changes in the course of the process time. 'All supplements' represents experiment #16 in which all factors were set to level 1. Center points represent experiments #17 to #19, with a factor setting of level 0, error bars represent the standard deviation of the triplicates. The negative control (NC) was obtained by a volume adjusted culture, which corresponds to a factor setting of -1 level for all factors. Section (A) describes the total cell concentration, (B) viability, (C) antibody titer, (D) viable specific growth rate and (E) specific productivity over the process time. Section (F) describes the antibody titer in the course of the viable cumulative cell days, in which the slope describes the specific productivity.

3.3.1 Statistical evaluation of the main effects

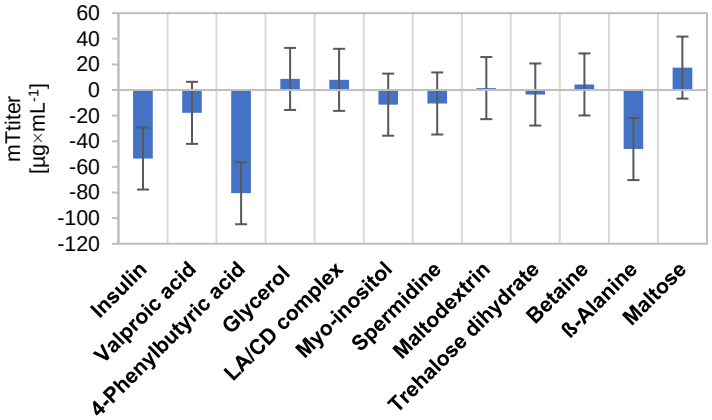
Analysis of the obtained responses by regression analysis yielded a quantitative description of the effect of different chemical chaperones. The maximal viable cell concentration (mVCC) was significantly negatively affected by the supplementation of human recombinant insulin (Insulin) and 4-PBA. Maltodextrin supplementation resulted in an enhanced maximal cell concentration, which was not significant, in contrast to the ß-alanine supplementation which showed a significantly increased maximal viable cell concentration. The obtained results are reliable due to a high model quality indicated by R^2 and Q^2. No significant positive effects could be obtained for the maximal antibody titer (mTiter). Insulin, 4-PBA and ß-alanine had a negative effect on the maximal antibody titer. Again the model quality is high indicating a reliable regression model. The last screening parameter was the maximal specific productivity (mqP), which was significantly positively influenced by the supplementation of valproic acid. 4-PBA also showed an elevated effect on the maximal specific productivity, which was not significant. Only betaine and maltodextrin negatively affected the maximal specific productivity at significant confidence intervals. Again a good model quality ensures reliable interpretation of the results.

A



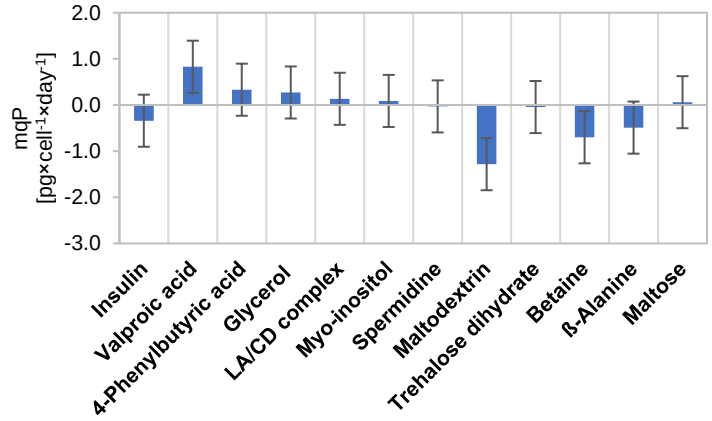
Parameter	Value
R^2	0.89
Q^2	0.86

B



Parameter	Value
R^2	0.86
Q^2	0.79

C



Parameter	Value
R^2	0.75
Q^2	0.68

Figure 18: Effect plot obtained by regression analysis of the experimental responses for the maximal viable cell concentration (A,mVCC), maximal antibody titer (B,mTiter) and maximum specific productivity (C, mqP). Error bars correspond to the 95% confidence interval. (Insulin = human recombinant insulin)

3.3.2 4-Phenylbutyric acid

The graphs hereinafter characterize the impact of 4-PBA supplementation on different screening parameters. Supplementation of 4-PBA had a significantly negative impact on the maximal viable cell concentration, which is shown by a limitation of the cell concentration to about 6×10^6 cells×mL^{-1} after the supplementation on day 3. This cell concentration was kept constant over the process time with acceptable viabilities above 80% until day 8 or 9 (Figure 19A). This negative effect on the maximal viable cell concentration can clearly be seen in the dot plot (Figure 19B), in which all 4-PBA supplemented cultures showed a lower maximal viable cell concentration.

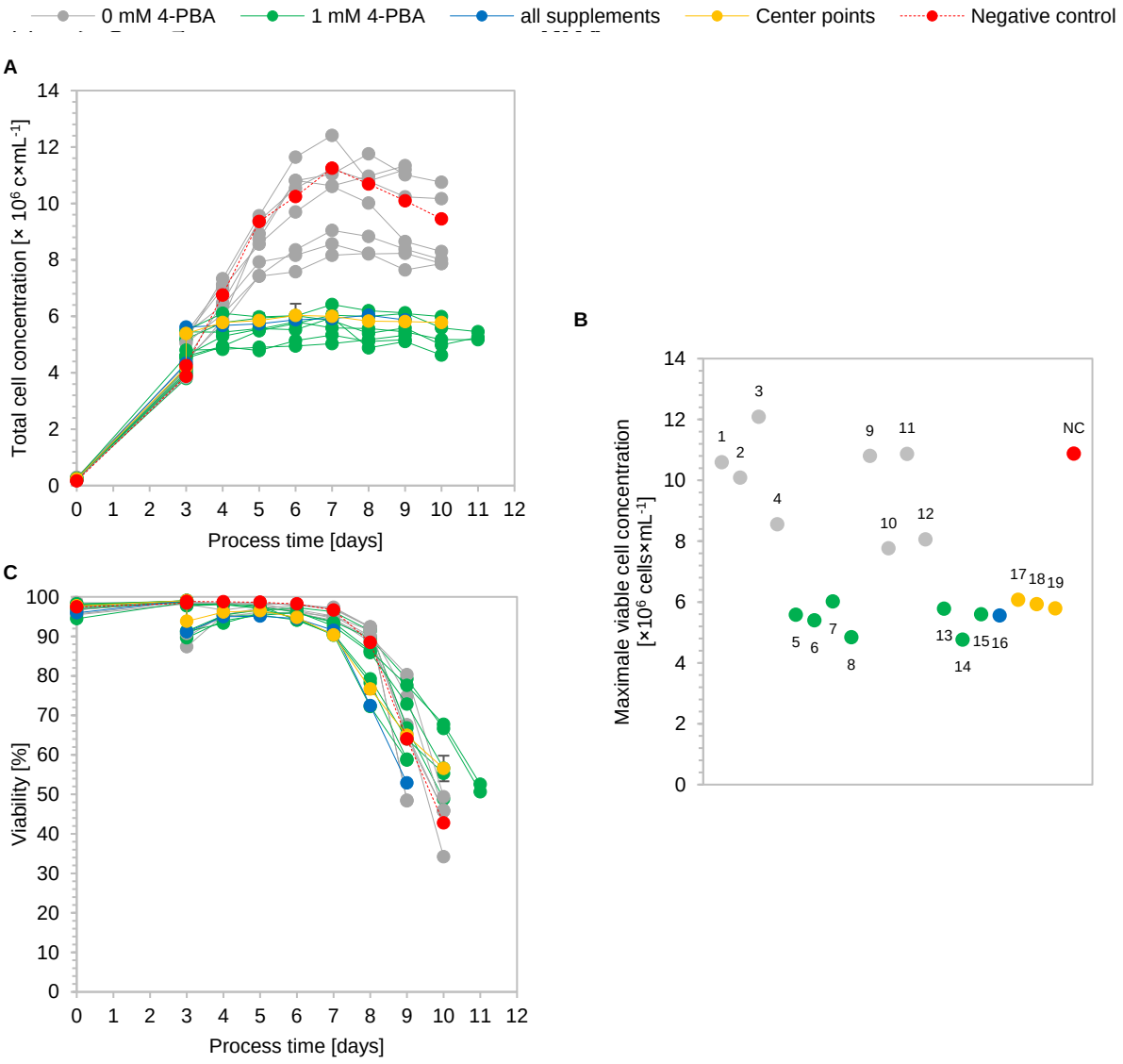


Figure 19: The effect of 4-PBA supplementation on the total cell concentration (A) and viability (C) over the process time and the obtained maximal viable cell concentration (B) used for the effect analysis.

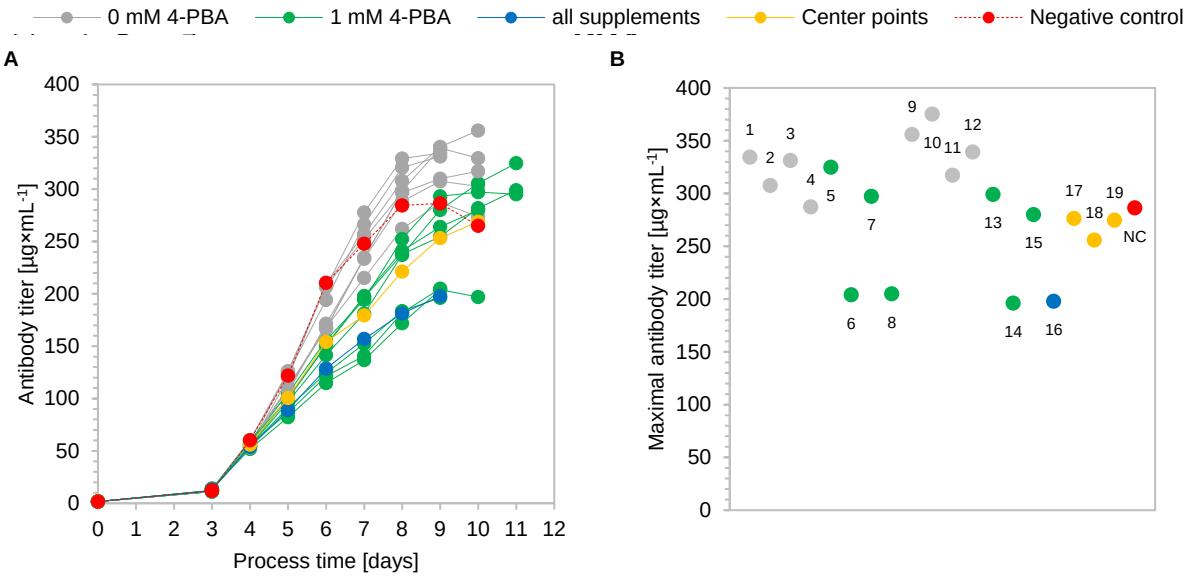


Figure 20: The course of the antibody titer over the process time (A) and the obtained maximal values used for the evaluation of the effect (B) for 4-PBA supplemented cultures.

Lower maximal antibody titers were produced because of lower cell concentrations and not because of lower specific productivities (Figure 20A and Figure 21A). A stabilization of specific productivity was observed by 4-PBA supplementation in the course of the process (Figure 21A). The limitation of the cell concentration mentioned earlier led to extremely low viable specific growth rates. Already on day 4, one day after the supplementation, viable specific growth rates decreased to 0.2 day^{-1} (Figure 21C).

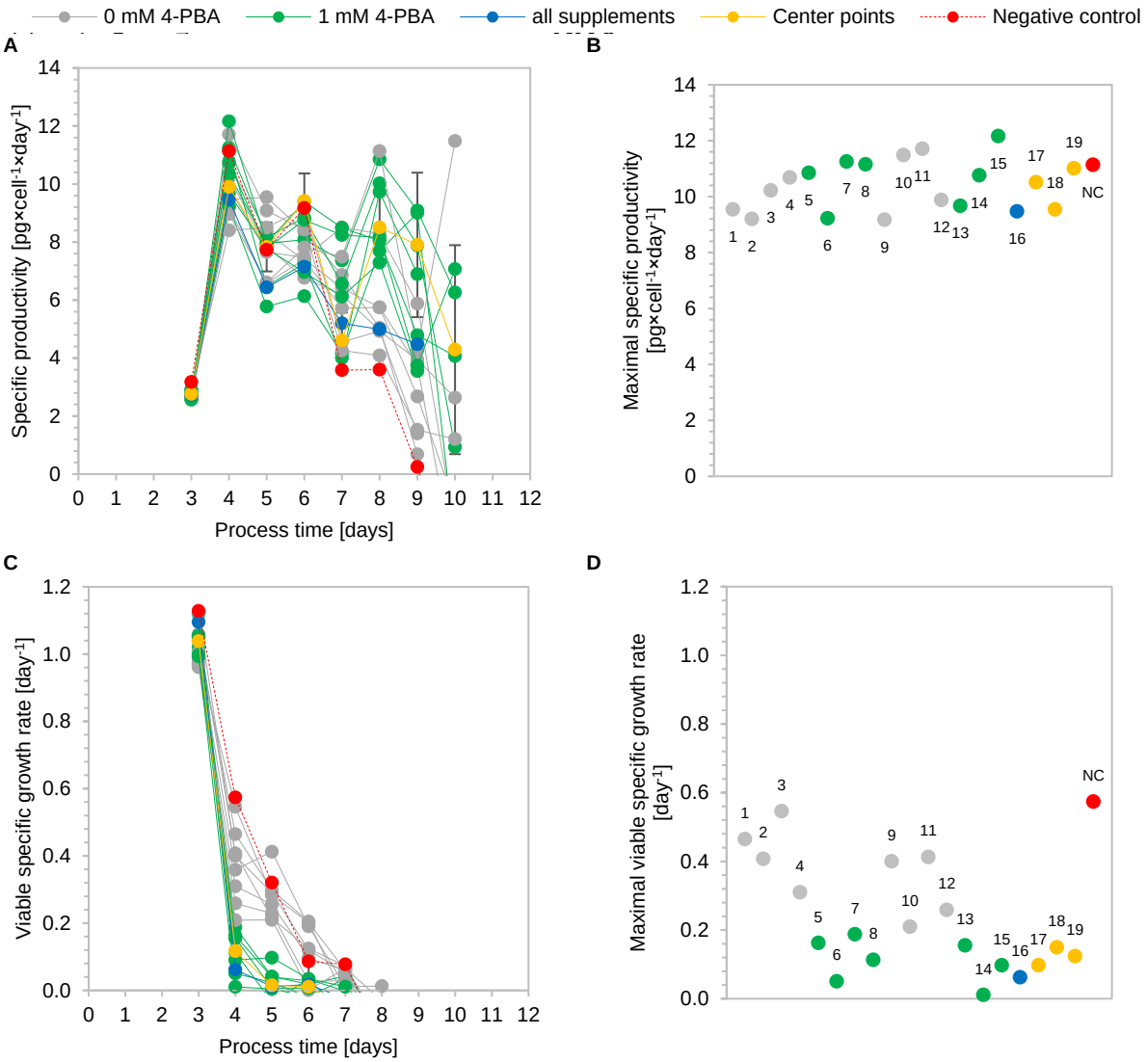


Figure 21: The course of the viable specific growth rate (A) and specific productivity (B) over the process time and the obtained maximal values used for the evaluation of 4-PBA supplemented cultures.

3.3.4 Valproic acid

The evaluation provided information about the positive and significant impact of valproic acid on the maximal specific productivity (Figure 22B). Supplemented experiments showed a higher maximal specific productivity. On average the response was higher for supplemented cultures than for non-supplemented ones. Valid interpretation of the maximal specific productivity is possible until day six due to a positive viable specific growth rate. After all a poor model quality may bias the evaluation of the specific productivity (Figure 18C).

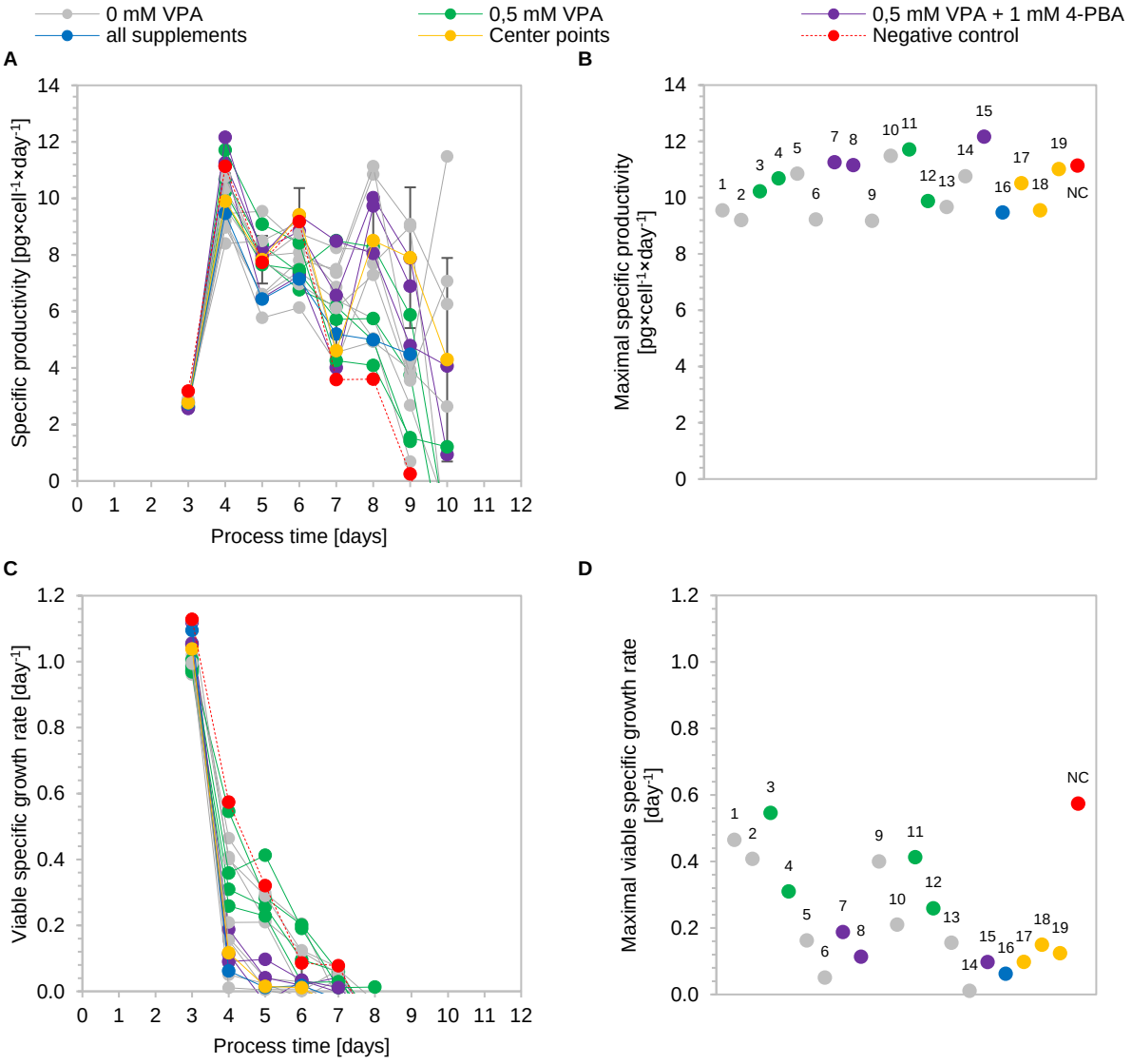


Figure 22: The effect of valproic acid supplementation on the specific productivity (A) and the viable specific growth rate (C) in course of the process time. Dashed dots in the maximal specific productivity plot (B) indicate the specific productivity on the 4th day of cultivation. Panel D summarizes the obtained maximal viable specific growth rates.

3.3.5 Human recombinant insulin

The maximal viable cell concentration was significantly reduced by the supplementation of human recombinant insulin (Figure 24A), viability dropped earlier and reached the 60% threshold one day prior the other cultures. As a consequence of reduced cell concentration and viabilities, maximal antibody titers were reduced in the first week of culture but, interestingly, similar maximal antibody titers were reached on day 9, but only in cultures without 4-PBA supplementation (Figure 24A green lines).

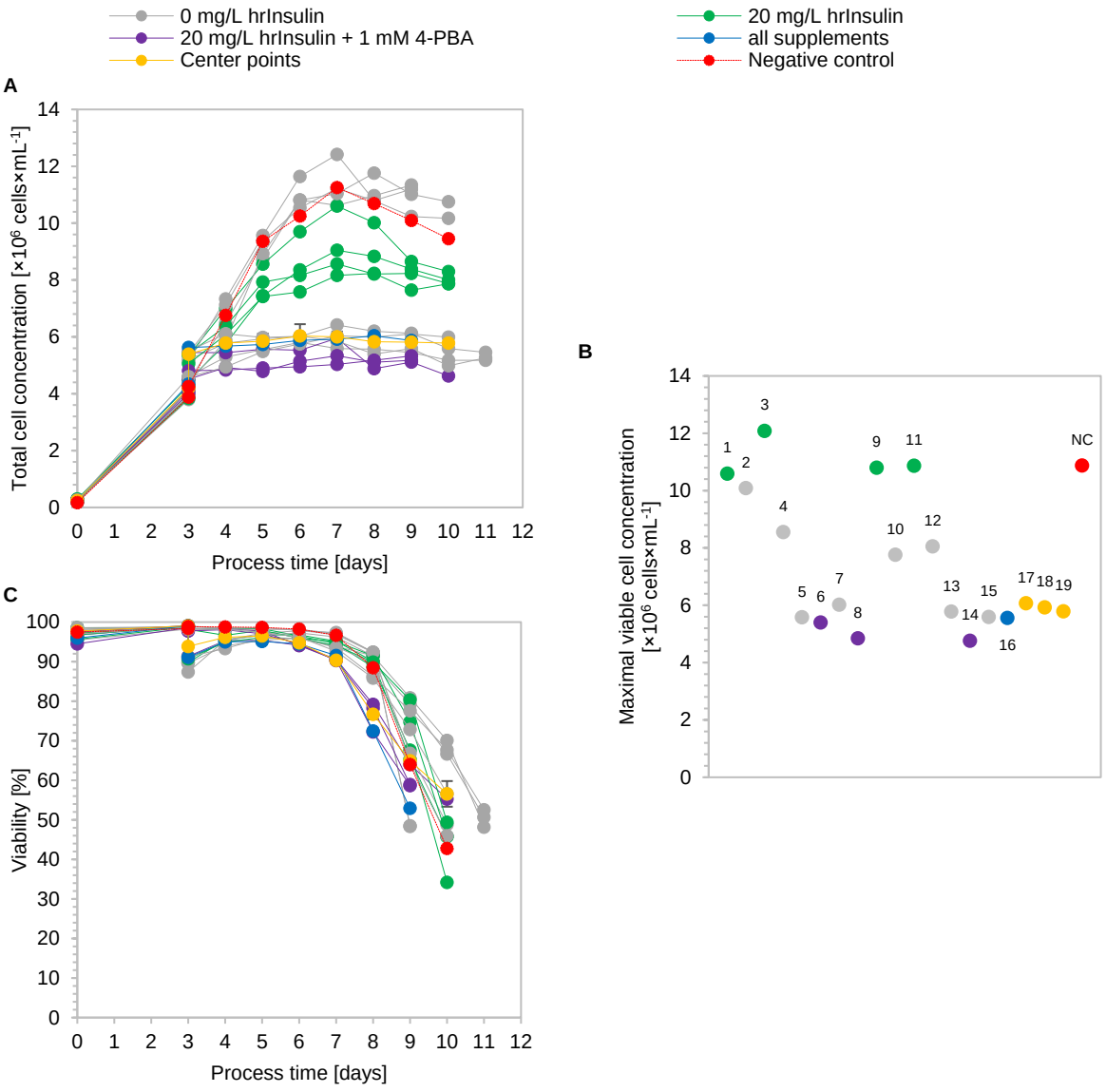


Figure 23: The effect of human recombinant insulin supplementation on the total cell concentration over the process time (A), and the corresponding maximal viable cell concentration (B) and the course of the viability (C).

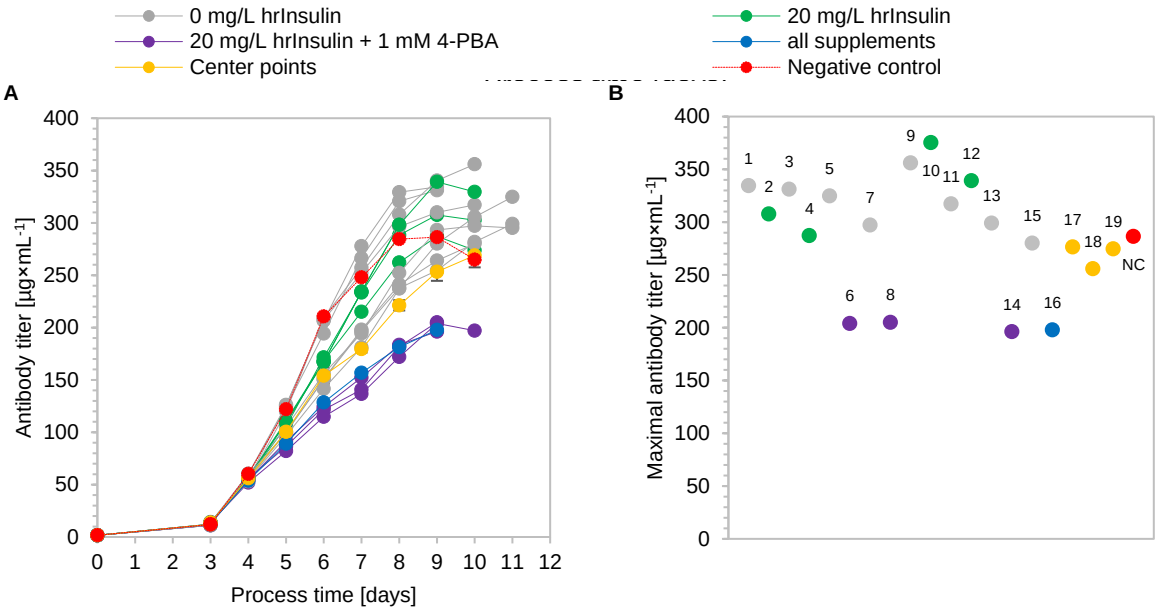


Figure 24: The impact of recombinant human insulin supplementation on the antibody titer over the process time (A) and the obtained maximal values (B).

3.3.6 ß-Alanine

ß-alanine supplementation caused a positive impact on the maximal viable cell concentration leading up to 12×10^6 c×mL^{-1} (Figure 25A). This accumulation of high cell concentration was accompanied with a higher viable specific growth rate on day 6 comparable to the negative control (Figure 26C, D). Adversely, ß-alanine supplementation also led to a significantly decreased maximal antibody titer, probably caused by the combination with 4-PBA supplementation shown in Figure 26A and B.

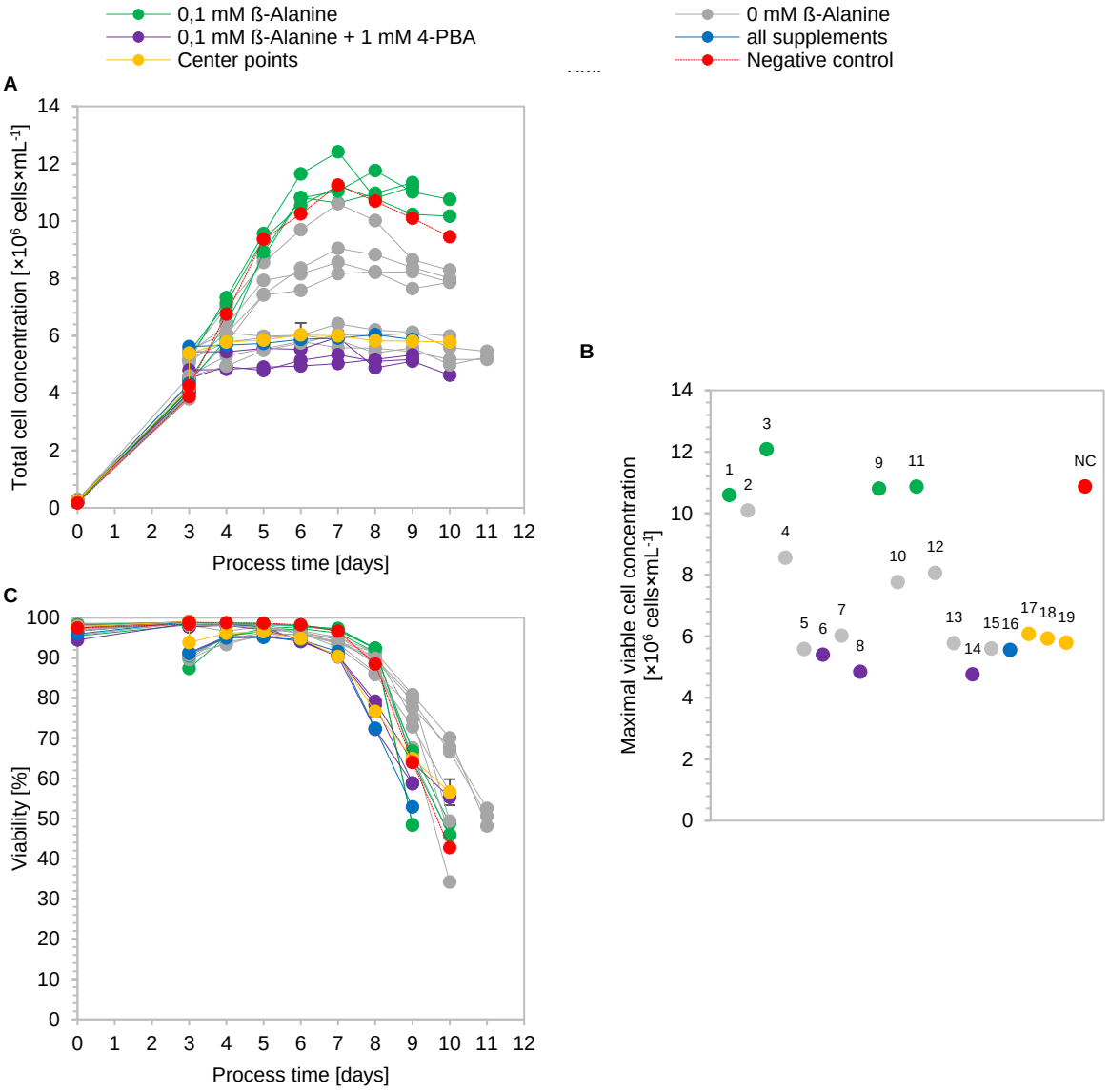


Figure 25: Overview of the total cell concentration (A) and the corresponding viability (C) and the thereby obtained maximal viable cell concentration (B) used for the evaluation of ß-alanine.

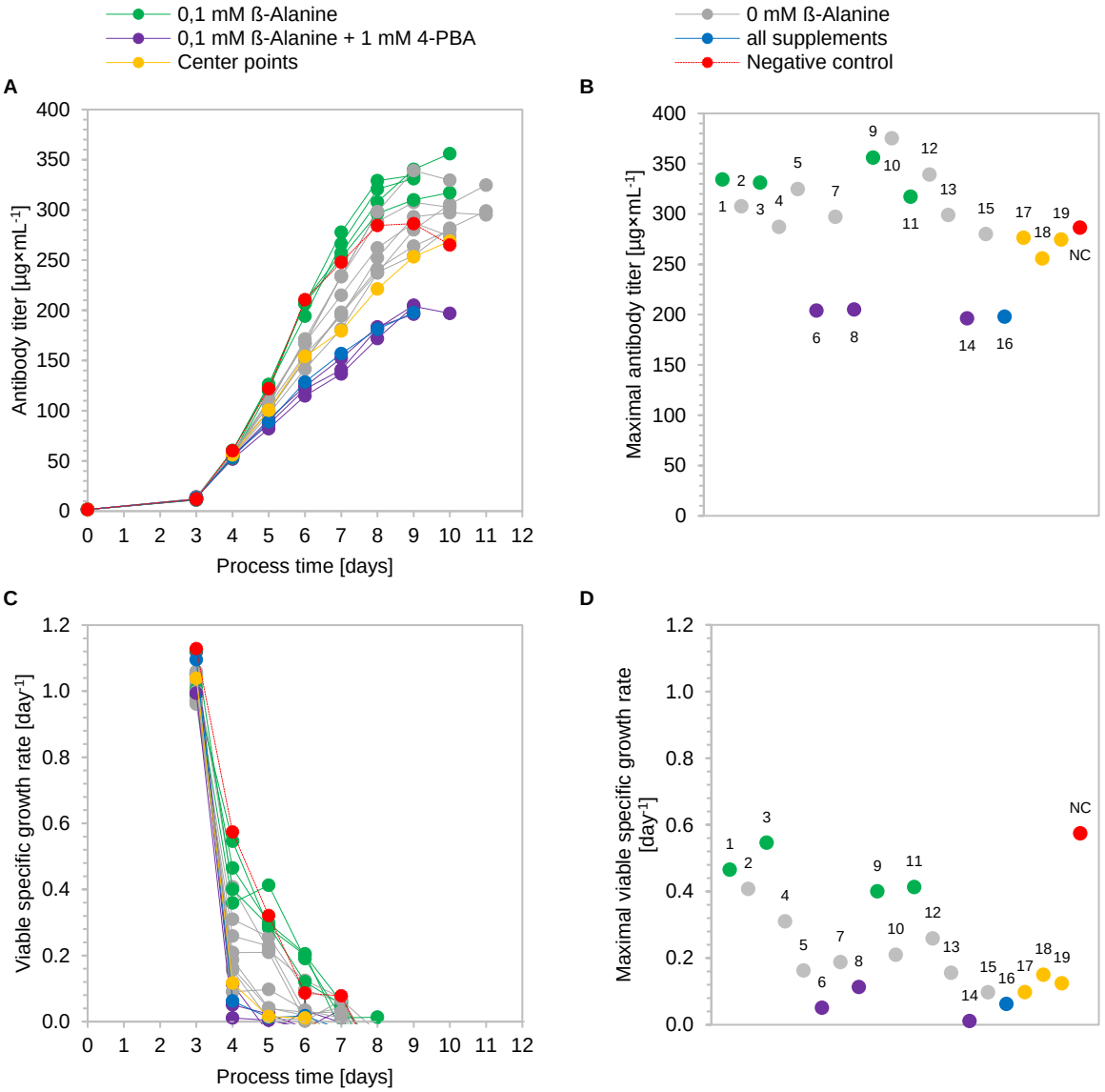


Figure 26: Overview of the impact of ß-alanine supplementation on the antibody titer over the process time (A) and the obtained maximal values (B), the viable specific growth rate (C) and the obtained maximal viable specific growth rates (D).

3.3.7 Maltodextrin

The effect obtained for maltodextrin for the maximal specific productivity was significantly negative. Dot plot as well as the graph (Figure 26A and B) showing the specific productivity in the course of the process time indicate that the supplemented cultures always had a lower specific productivity compared to the negative control. Only culture #7 supplemented with 4-PBA and maltodextrin showed an enhanced specific productivity as shown in purple. Furthermore the viable specific growth rate seem to be stabilized by the supplementation of maltodextrin as indicated on day 5.

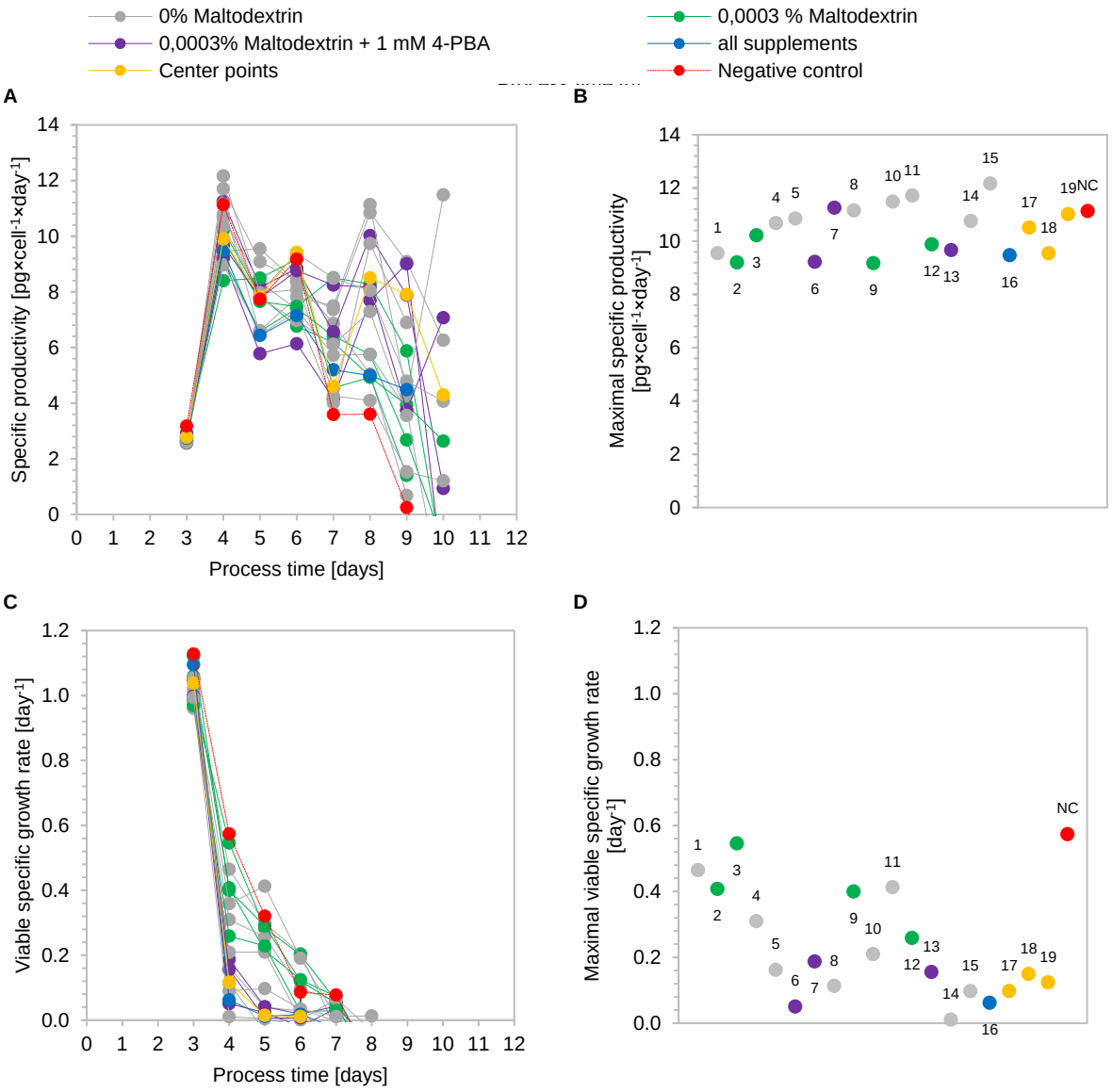


Figure 27: The effect of maltodextrin supplementation on the specific productivity (A) and the viable specific growth rate (C) in the course of the process time. Dot plots represent the obtained maximal values taken for the evaluation of the effects of the maximal specific productivity (B) and the maximal viable specific growth rate (D).

3.3.8 Betaine

The effect evaluation showed that supplementation of betaine led to a significant decrease of the maximal specific productivity. Already the dot plot (Figure 28B) shows that betaine supplemented cultures did show comparable or lower maximal specific productivities compared to the negative control, only the combinatorial supplementation of 4-PBA and betaine led to an enhanced maximal specific productivity as indicated in purple.

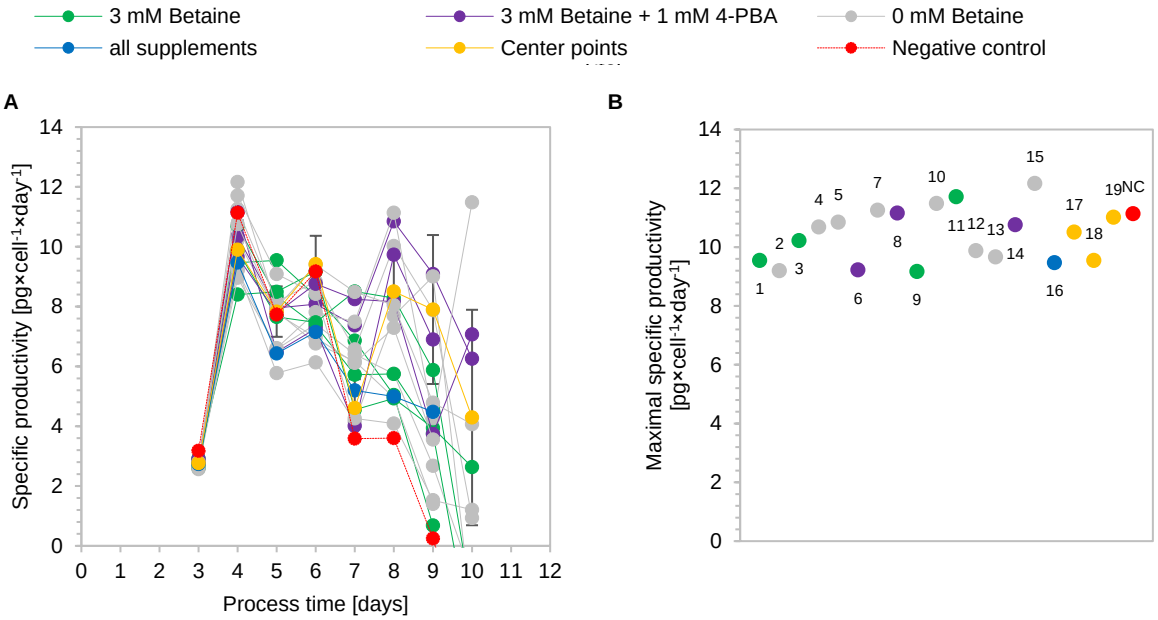


Figure 28: The effect of betaine supplementation on the specific productivity (A) in the course of the process time and the obtained maximal specific productivity (B) values taken for the evaluation.

3.4 Third screening experiment for detailed investigation of 4-PBA, betaine, DMSO, maltodextrin and valproic acid

The aim of the previous fractional factorial screening experiments of resolution III was to identify suitable chemical substances that positively impact on selected screening parameters. A second follow-up experiment should then further characterize the 4-PBA, betaine, DMSO, maltodextrin and valproic acid by an experimental design at a higher resolution to obtain more precise results by estimating factor interactions in addition to the linear (main) effects. Furthermore, metabolite concentrations have also been determined in the course of the process time to identify possible metabolic changes during the cultivation.

3.4.1 Criteria for selection of promising substances

All screened supplements were ranked and chosen according to their effect on defined screening parameters, which comprised the maximal antibody titer, maximal viable cell concentration and maximal specific productivity. A high maximal antibody titer was set as first criterion, due to the assumption that a high antibody titer may yield in a higher chance of properly folded and modified recombinant protein. The maximal viable cell concentration was set as second criterion, as only viable cells are relevant for the production process and are capable to express the recombinant protein. Maximization of the specific productivity was set as last criterion. This sequence order of the three criteria was based on error robustness from high to low. So it is assumed that the determination of the viable cell concentration and the antibody titer is a robust method and not prone to errors, whereas the maximal specific productivity is set as last parameter due to error propagation of several methods. Furthermore, a correct estimation of the maximal specific productivity is critical due to various parameters including viability, antibody titer and cell growth that contribute to the calculation of specific productivity. Table 12 summarizes the data obtained during the previous screenings. DMSO, maltodextrin, betaine and valproic acid performed best according to the ranking and 4-PBA was selected as additional chemical due to its ability to impact on cell growth and maintain the cell specific productivity as shown in section 3.3.2.

Table 12: Overview of the effect of all screened chemical substances. The obtained effects can be discriminated by the color. Green indicates a positive effect, red a negative and yellow indicates that there was no response. The selected supplements are highlighted by a bold frame in the 'Supplements' column.

	Criterion 1	Criterion 2	Criterion 3
Supplements	**Maximal antibody titer [µg×mL^{-1}]**	**Maximal viable cell concentration [×10^{6} c×mL^{-1}]**	**Maximal specific productivity [pg×cell^{-1}×day^{-1}]**
DMSO	56.09	-2.78	6.61
Maltodextrin	0.00	0.65	-1.28
Betaine	0.00	0.48	-0.70
Valproic acid	0.00	0.00	0.83
Spermidine	0.00	0.00	0.00
Trehalose×2 H_2O	0.00	0.00	0.00
Maltose monohydrate	0.00	0.00	0.00
Myo-inositol	0.00	0.00	0.00
beta-Cyclodextrin	0.00	0.00	0.00
Copper sulfate	0.00	0.00	0.00
Dextran	0.00	0.00	0.00
Glycerol	0.00	0.00	0.00
Linoleic acid	0.00	0.00	0.00
Taurine	0.00	0.00	0.00
Mannitol	0.00	0.00	-0.43
Sucrose	-16.57	0.00	0.00
Cysteine	-19.76	0.00	0.00
Trimethylamine N-oxide	-27.03	0.00	-2.12
ß-Alanine	-46.05	0.93	0.00
Insulin	-53.45	-1.54	0.00
4-Phenylbutyric acid	-80.50	-4.41	0.33

3.4.2 Graphical representation of the obtained results

The graphs below summarize the obtained data in the course of the process. Already the graph showing the total cell concentration over time indicates that the negative control reached the highest total cell concentration among all experiments (Figure 29A). Some cultivation experiments reached similar cell concentrations, but no significant improvement could be achieved by the supplementation of the selected chemicals. Again the effect of substances limiting the total cell concentration is clearly visible. The viability shown in Figure 29B seems to be stabilized by the supplementation of some chemicals. Already on day 4 some cultivations show a decrease in viability due to the supplementation on day 3 shown in Figure 29B. Highest viability over time was reached by the center point. Also the antibody titers were affected by supplementation (Figure 29C). Until day 7, antibody titers of the negative

control and other well performing cultivations reached similar values of 155 µg×mL^{-1}, then some supplements tended to enhance the maximal antibody titer leading up to approximately 20% enhanced antibody titers in comparison to the negative control. The viable specific growth rate of the negative control is steadily decreasing as expected in a batch cultivation (Figure 29D). Especially experiment #3 and #15 show an enhanced viable specific growth rate in the course of the process.

Experimental-ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Center points			NC
DMSO	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	0	0	0	-1
Betaine	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	0	0	0	-1
Maltodextrin	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	0	0	0	-1
Valproic acid	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	0	0	0	-1
4-PBA	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1	0	0	0	-1

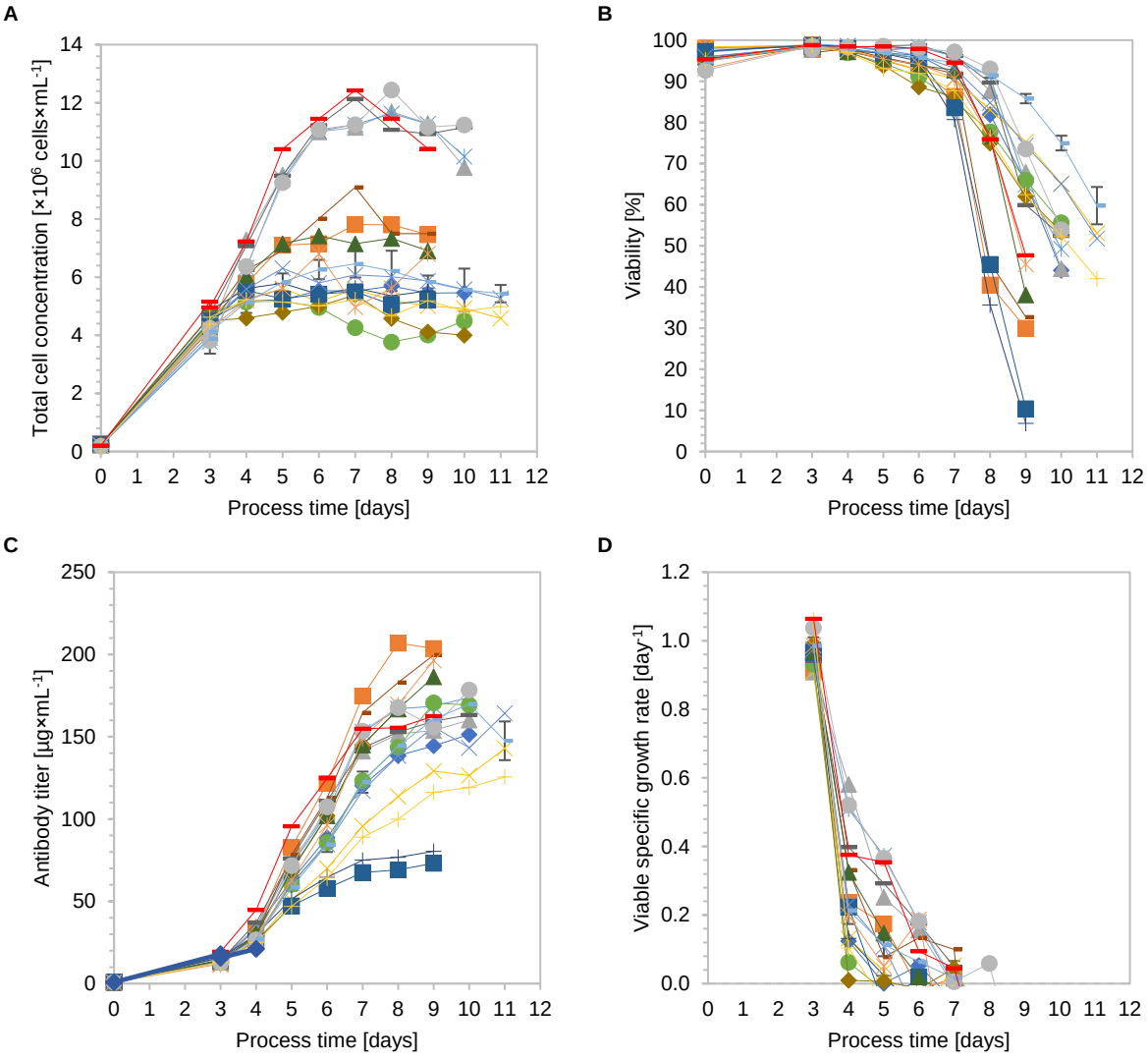


Figure 29: The cell concentration (A), viability (B), antibody titer (C) and the viable specific growth rate (D) over process time of the fractional factorial screening design of resolution V+.

Different observations could be obtained for the cell specific productivity. The negative control shows a steadily decreasing cell specific productivity and a maximum on day 5 at 6 pg×cell^{-1}×day^{-1}. Other experiments show a higher and lower specific productivity than the negative control at day 5, depending on the supplementation. Especially DMSO supplemented cultures show an enhanced specific productivity and reached their maximal specific productivity of 9 pg×cell^{-1}×day^{-1} with still acceptable viabilities on day 5 (Figure 30A Experiment #2, 6, 8, 10, 12). Of special interest is experiment #2, which is supplemented only with DMSO and no other supplements. Furthermore, some supplements seem to improve or stabilize the specific productivity in later process stages from day 5 to 8 as it is the case for experiment #2, #6 and #14, but with critical viabilities. In general it must be said that specific productivities after day 6 and 7 may not be reliable due to a negative viable specific growth rate or low viability. The obtained osmolality values also show the effect of the supplementation. Osmolalities up to 700 mOsm×kg^{-1} were obtained by freezing point depression, while the negative control only showed an osmolality of 300 mOsm×kg^{-1} in the course of the process.

Experimental-ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Center points			NC
DMSO	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	0	0	0	-1
Betaine	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	0	0	0	-1
Maltodextrin	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	0	0	0	-1
Valproic acid	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	0	0	0	-1
4-PBA	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1	0	0	0	-1

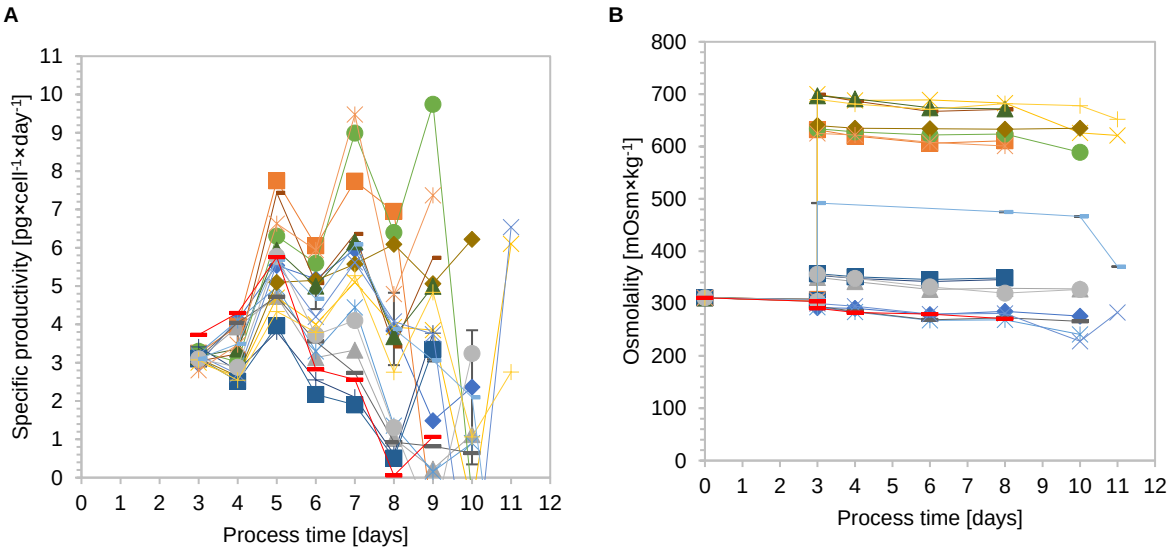


Figure 30: The specific productivity (A) and osmolality (B) in course of the process time of the fractional factorial screening design of resolution V+.

The metabolic data also show a major impact of different culture supplements. Figure 31A and B show the glucose concentration and the corresponding glucose consumption rate in the course of the process. The graph shows that the glucose concentration is always the lowest in the negative control until day 5 because of higher cell numbers and is depleted one day later. In contrast, most of the supplemented cultures still had glucose on day 6 of the cultivation. Furthermore, some cultures did not consume all the available glucose, so that at the end of the screening residual glucose was measured. Highly variable lactate profiles were determined for the different experiments (Figure 31 C, D). Especially cultures supplemented with DMSO (Experiment #2, #4, #6, #8, #10, #12, #14 and #16) tend to consume lactate faster so that lactate depleted on day 7. This effect can also be seen in Figure 31D, which shows that some supplements tend to trigger the lactate consumption, whereas others improve the lactate production rate (indicated by the arrow).

Experimental-ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Center points			NC
DMSO	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	0	0	0	-1
Betaine	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	0	0	0	-1
Maltodextrin	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	0	0	0	-1
Valproic acid	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	0	0	0	-1
4-PBA	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1	0	0	0	-1

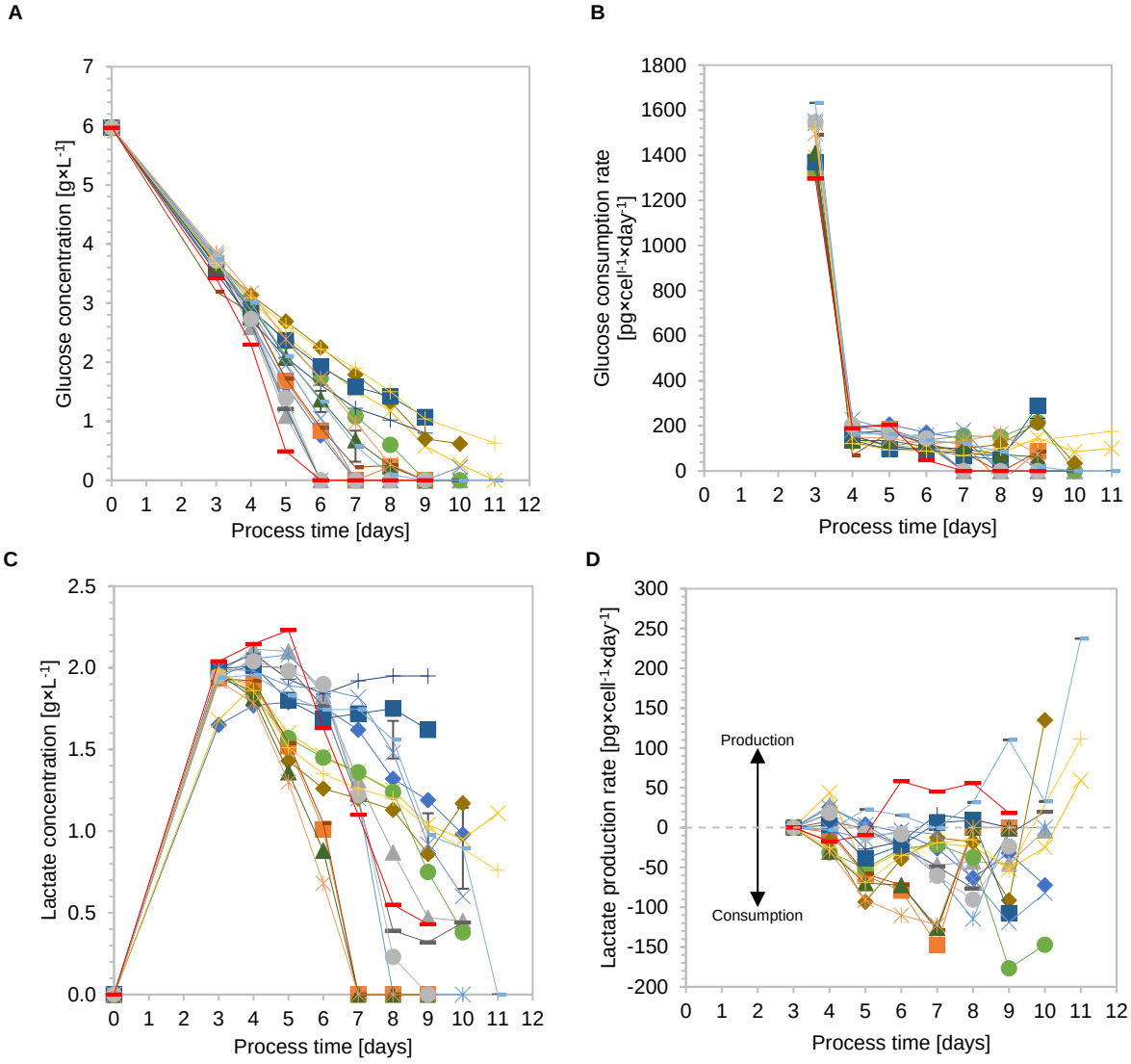


Figure 31: Glucose concentration (A) and the corresponding glucose consumption rate (B) and the lactate concentration (C) and the corresponding lactate consumption rate (D). The direction of the arrow indicates whether the cells specifically consume or produce the metabolite.

The L-glutamine profiles show again a different pattern (Figure 32A). L-glutamine completely depletes in the negative control on day 4 of the cultivation, whereas other cultures still provide a sufficient concentration. Furthermore, some supplemented cultures seem to stabilize the L-glutamine concentration after day 4, which is also shown in the L-glutamine consumption rate over the process time. Most of the cultivation experiments still had L-glutamine available until the end of the cultivation. In DMSO supplemented cultures glutamine depletion is not observed.

Experimental-ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Center points			NC
DMSO	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	0	0	0	-1
Betaine	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	0	0	0	-1
Maltodextrin	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	0	0	0	-1
Valproic acid	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	0	0	0	-1
4-PBA	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1	0	0	0	-1

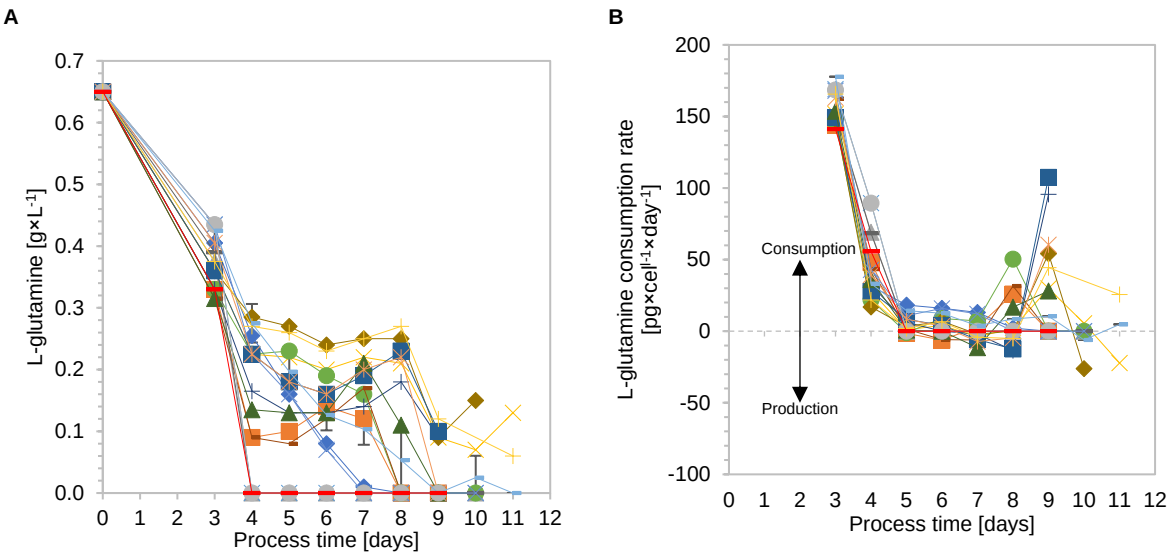


Figure 32: The L-glutamine concentration (A) and the corresponding L-glutamine consumption rate (B) in the course of the process.

The L-glutamic acid level of differently supplemented cultures did also show different L-glutamic acid concentrations over the process time. Again the DMSO supplemented cultures seem to accumulate the L-glutamic acid, while other cultivation experiments like the negative control consume the L-glutamic acid (Figure 33A). This observation is also manifested in the L-glutamate production rate over the process time (Figure 33B). The ammonia level in the differently supplemented cultures seem to be similar and only start to deviate at the end of the cultivation (Figure 33C).

Experimental-ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Center points			NC
DMSO	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	1	0	0	0	-1
Betaine	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	-1	-1	1	1	0	0	0	-1
Maltodextrin	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1	1	1	0	0	0	-1
Valproic acid	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1	1	1	1	1	0	0	0	-1
4-PBA	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1	0	0	0	-1

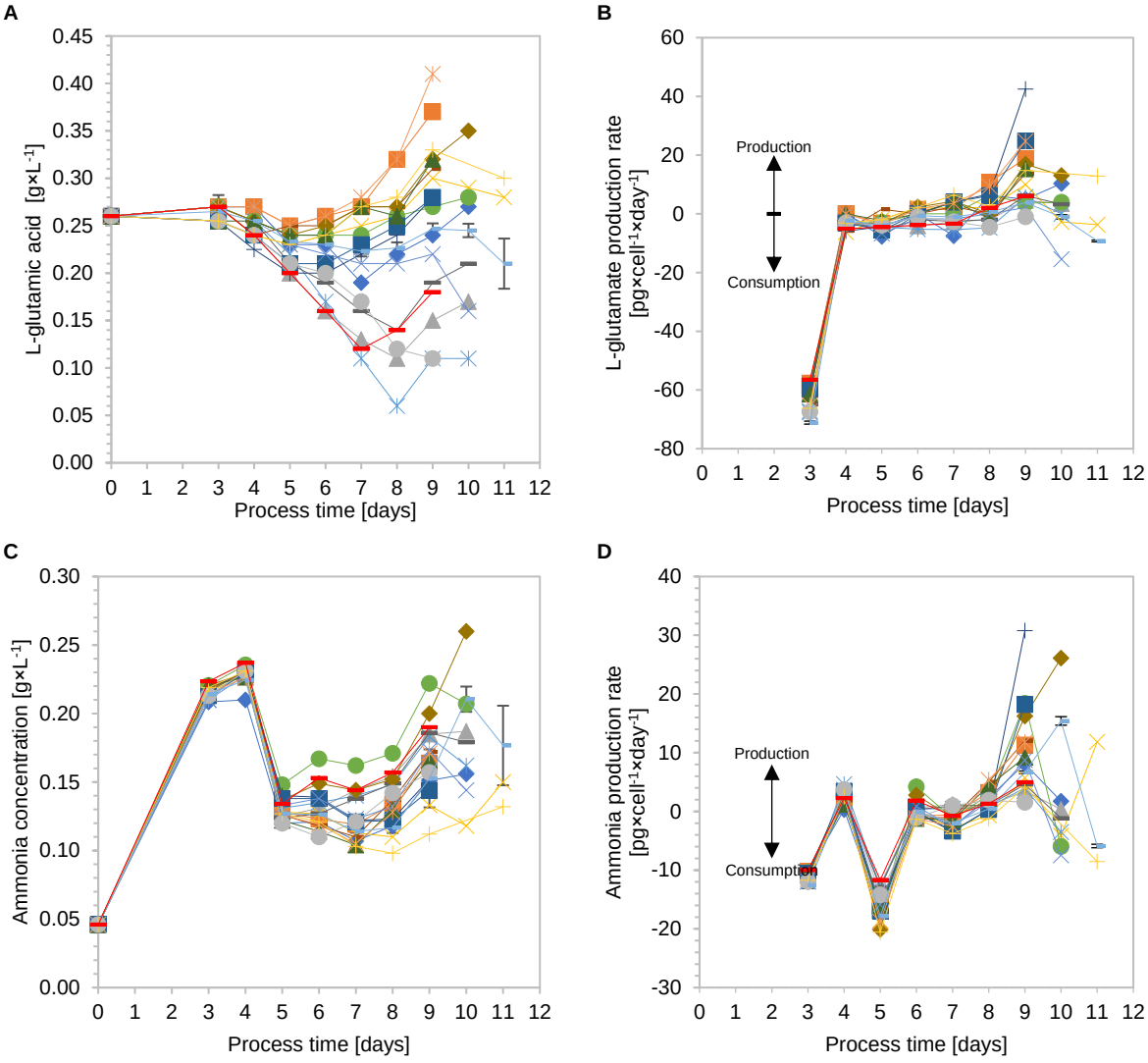


Figure 33: The course of the L-glutamic acid concentration (A), L-glutamate production rate (B), ammonia concentration (C) and ammonia production rate (D) over the process time in the fractional factorial design resolution V+ screening.

3.4.3 Statistical evaluation of the main effects

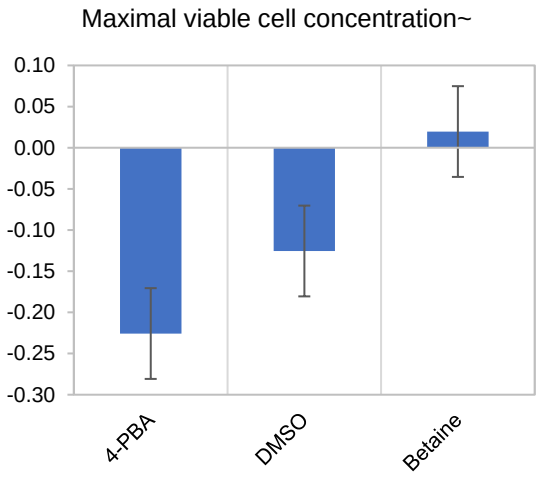
The evaluation of the obtained responses yielded in various results. Only the most significant effects are shown and pointed out later in this section. A tilde (~) in the heading of the graph indicates that the data set needed to be transformed to ensure a proper statistical evaluation, which was the case for the maximal viable cell concentration (mVCC) and the maximal antibody titer (mTiter). All other data did not need any transformation prior the evaluation. Again the model quality is summarized in the table on the right hand side. In this case the mean specific productivity was considered as the maximal specific productivity did not show a normal distribution and a too high variation in the center points. A '*' between two factors indicates an interaction of the two factors. The effect of the obtained interactions was only minor or negative so that a visual characterization is not shown here. A summary of the obtained results taken for the evaluation is given in Table 13. In general maximal viable cell concentrations ranging from 4.6×10^6 cells×mL^{-1} to 11.7×10^6 cells×mL^{-1} were reached. In comparison the negative control did reach a maximal viable cell concentration of 11.8×10^6 cells×mL^{-1}. Maximal antibody titers (mTiter) range from 73 µg×mL^{-1} to 207 µg×mL^{-1}. The process lactate consumption rate (P_{lac}Con) varied from 5 pg×cell^{-1}×day^{-1} up to 58 pg×cell^{-1}×day^{-1} indicating a very different lactate metabolism. Another response taken into account was the mean specific productivity (øqP), which ranged from 3 pg×cell^{-1}×day^{-1} to 6 pg×cell^{-1}×day^{-1}, which corresponds to a 2-fold increase. Maximal specific productivity ranged from 4 pg×cell^{-1}×day^{-1} to 10 pg×cell^{-1}×day^{-1}. Furthermore, a maximal process duration of 11 days could be achieved, in comparison the negative control only reached a process duration of 9 days.

Table 13: Summary table of the obtained responses and chosen factor settings for the fractional factorial screening of resolution V+. The obtained responses have been the maximal viable cell concentration (mVCC), maximal antibody titer (mTiter), Process lactate consumption rate (P_{lac}Con) and the mean specific productivity (øqP). Additional parameters are summarized in the category "performance", like the maximal specific productivity (mqP) and the process duration. Experiment #16 is indicated as "all supplements" in the following graphs.

	Factors					Responses				Performance	
Experimental-ID	DMSO	Betaine	Maltodextrin	Valproic acid	4-Phenylbutyric acid	mVCC [× 10^6 c×mL^{-1}]	mTiter [µg×mL^{-1}]	P_{lac}Con [pg×$cell^{-1}$×day^{-1}]	øqP [pg×$cell^{-1}$×day^{-1}]	mqP [pg×$cell^{-1}$×day^{-1}]	Process duration [days]
1	-1	-1	-1	-1	1	5.33	151.40	18.38	4.07	5.95	10
2	1	-1	-1	-1	-1	6.75	206.90	53.10	5.20	7.75	9
3	-1	1	-1	-1	-1	10.81	160.20	23.81	2.50	4.73	10
4	1	1	-1	-1	1	5.33	142.95	19.95	3.35	6.10	11
5	-1	-1	1	-1	-1	10.85	173.90	30.99	2.75	4.98	10
6	1	-1	1	-1	1	4.99	170.55	50.78	5.65	9.75	10
7	-1	1	1	-1	1	5.60	80.35	5.30	2.61	3.81	9
8	1	1	1	-1	-1	8.35	199.65	49.64	5.21	7.43	9
9	-1	-1	-1	1	-1	11.67	163.35	25.51	2.49	4.72	10
10	1	-1	-1	1	1	4.64	149.20	34.08	5.12	6.22	10
11	-1	1	-1	1	1	5.43	73.15	12.93	2.40	3.96	9
12	1	1	-1	1	-1	6.97	186.45	50.08	4.85	6.16	9
13	-1	-1	1	1	1	6.16	164.25	34.87	3.23	6.54	11
14	1	-1	1	1	-1	6.40	196.35	58.09	6.28	9.48	9
15	-1	1	1	1	-1	11.58	178.40	30.04	2.83	5.78	10
16	1	1	1	1	1	5.06	125.60	28.98	3.51	5.27	11
17	0	0	0	0	0	6.62	166.20	25.95	3.71	6.02	11
18	0	0	0	0	0	6.04	177.15	29.70	4.33	6.15	11
19	0	0	0	0	0	5.90	166.05	22.20	4.37	6.09	11
NC	-1	-1	-1	-1	-1	11.75	162.55	27.77	2.76	5.76	9

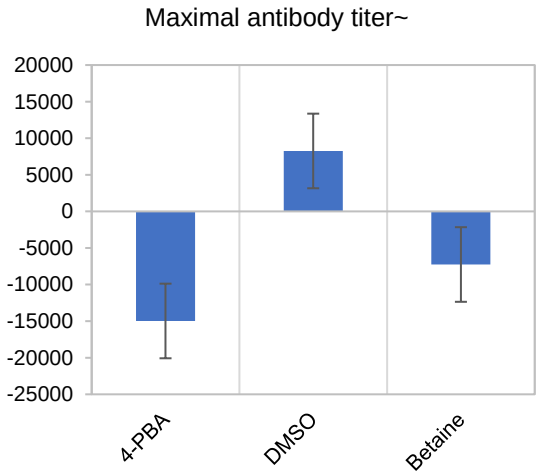
The effect plots display the effect of the selected chemical compound on defined screening parameters. Figure 34A shows the effect on the maximal viable cell concentration and the quality of the model summarized by the R^2 and Q^2 value. No significant positive effect on the maximal viable cell concentration could be observed during this fractional factorial screening. Only betaine had a low positive effect on the maximal cell concentration, but this effect was not significant. 4-PBA and DMSO had a significant negative effect as shown in the plot. The obtained results are reliable due to a good model quality indicated by a R^2 and Q^2 at about 0.8. Figure 34B describes the effect of different supplements on the maximal antibody titer. A high R^2 describes a good fitting of the model, but a rather low Q^2 indicates a poor predictability. DMSO was the only substance with a positive and significant impact on the maximal antibody titer, whereas betaine and 4-PBA showed a significant negative impact. The strong effect of the DMSO supplementation on the maximal antibody titer was mainly due to an enhanced specific productivity in course of the process time. Effect analysis show that the supplementation of DMSO had a significant positive effect on the mean specific productivity. This would explain the enhanced maximal antibody titers with lower cell concentrations. Unlike DMSO, no other substance positively influenced the mean specific productivity (Figure 34C). Combinations of the substances also contributed significantly negatively on the mean specific productivity and are therefore not further characterized. Again a high model quality ensure reliable data interpretation. The quality parameters R^2 and Q^2 are both above 0.8 indicating a high score for this model.

A



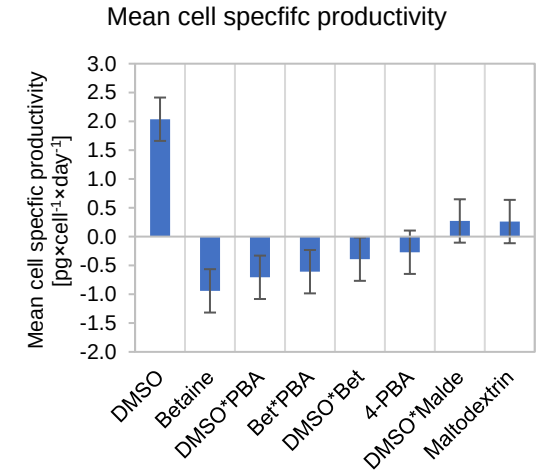
Parameter	Value
R^2	0.87
Q^2	0.79

B



Parameter	Value
R^2	0.80
Q^2	0.66

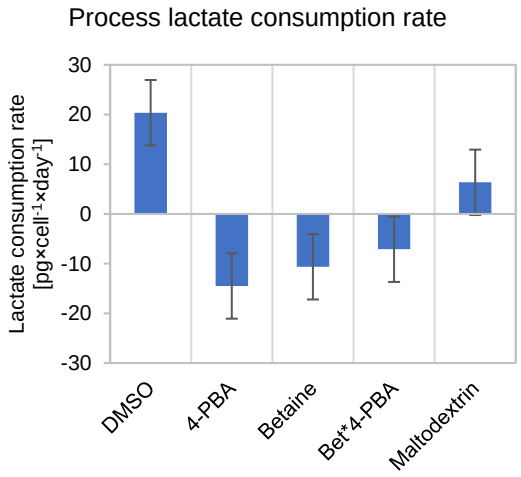
C



Parameter	Value
R^2	0.96
Q^2	0.84

Figure 34: Summary of the effect analysis. Significant terms are indicated by the error bars which do not cross the 0 line. Error bars represent a 95% confidence interval. A term is considered to be significant if the 0 bar is not crossed. Maximal viable cell concentration and the maximal antibody titer needed to be transformed, which is indicated by the tilde (~).

In addition to the previous fractional factorial screening experiments also metabolite concentrations have been chosen as screening parameters. Especially lactate formation is critical in bioprocessing of mammalian cells. The effect analysis yielded information about supplementations that affected the process lactate consumption rate. The supplemented DMSO showed a significant positive effect on the process lactate consumption rate. Furthermore the obtained model quality parameters show a high score indicating a reliable model for the evaluation.



Parameter	Value
R^2	0.87
Q^2	0.73

Figure 35: The effect analysis of the process lactate consumption rate of differently supplemented cultivation experiments. The error bars represent a 95% confidence interval.

3.4.4 DMSO inhibits cell growth but maintains the specific productivity

Previous screening experiments already showed the effect of the DMSO supplementation. The maximal viable cell concentration was significantly negatively affected, which can be seen in Figure 36A. Already two days after DMSO supplementation on day 3, decreased total cell concentrations were observed. Furthermore, the viability in supplemented cultures started to decrease at the same time point as the total cell concentration deviates (Figure 36C). The described observation can already be seen in the dot plot, in which the DMSO supplemented cultures show a reduced maximal viable cell concentration (Figure 36B).

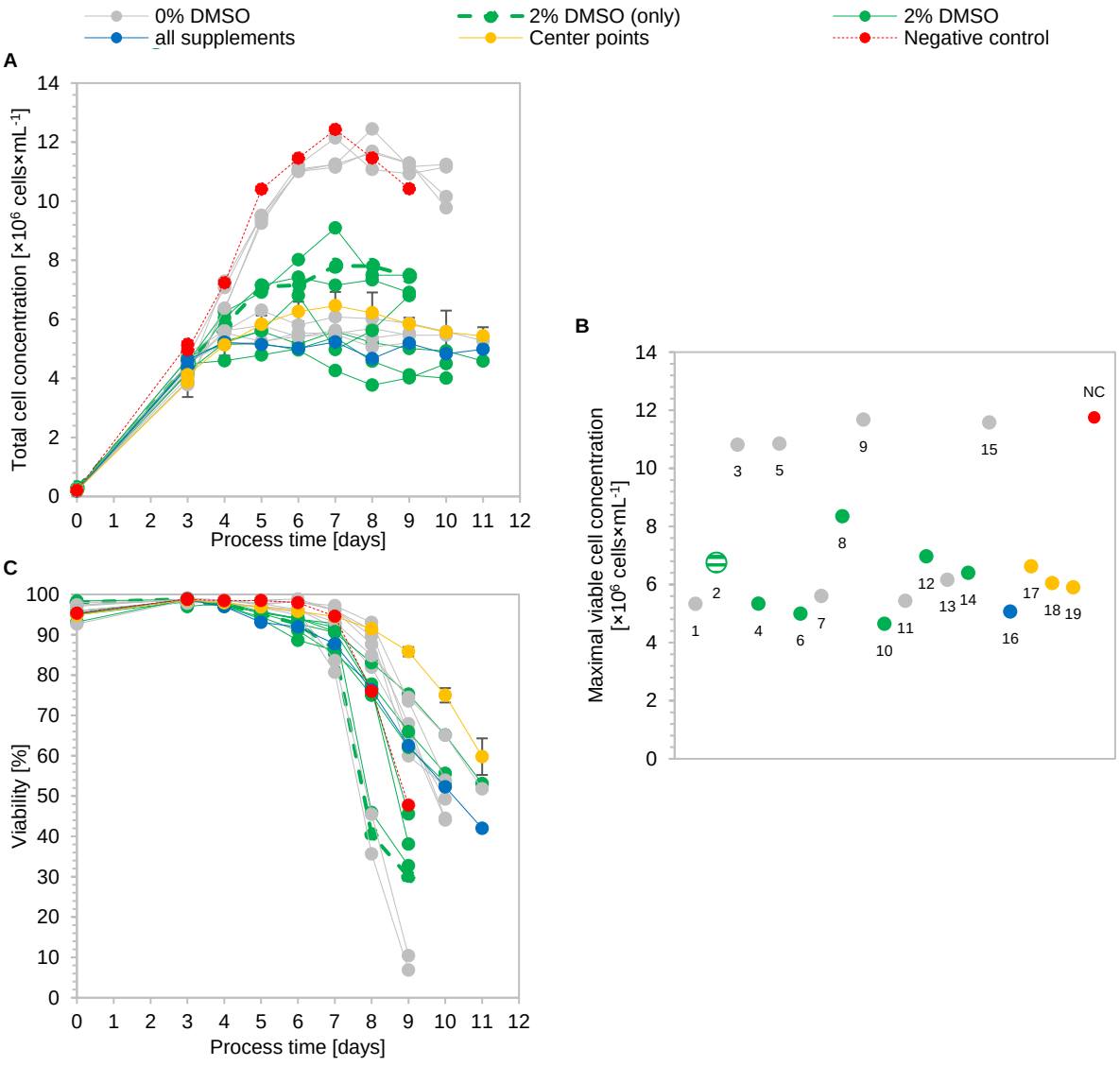


Figure 36: The impact of DMSO supplementation on the maximal viable cell concentration (B) and the total cell concentration (A) and viability (C) in the course of the process time. Error bars in the left graphs represent the standard deviation of three biological replicates of the center points. “2% DMSO (only)” in the figure legend describes the experiment only supplemented with 2% DMSO, this corresponds to experiment number 2. In contrast the “2% DMSO” term describes that DMSO was supplemented in 2%, but also other substances used for this screening experiment could be present. Experiment 16 have been supplemented with all supplements and is indicated in blue.

Supplementation of DMSO further led to a significantly enhanced maximal antibody titer. Especially experiment #2, only supplemented with DMSO showed the highest maximal antibody titer among all conducted experiments (Figure 37A, B dashed line). The maximal antibody titer of experiment #2 could be enhanced by 1.27-fold in comparison to the negative control.

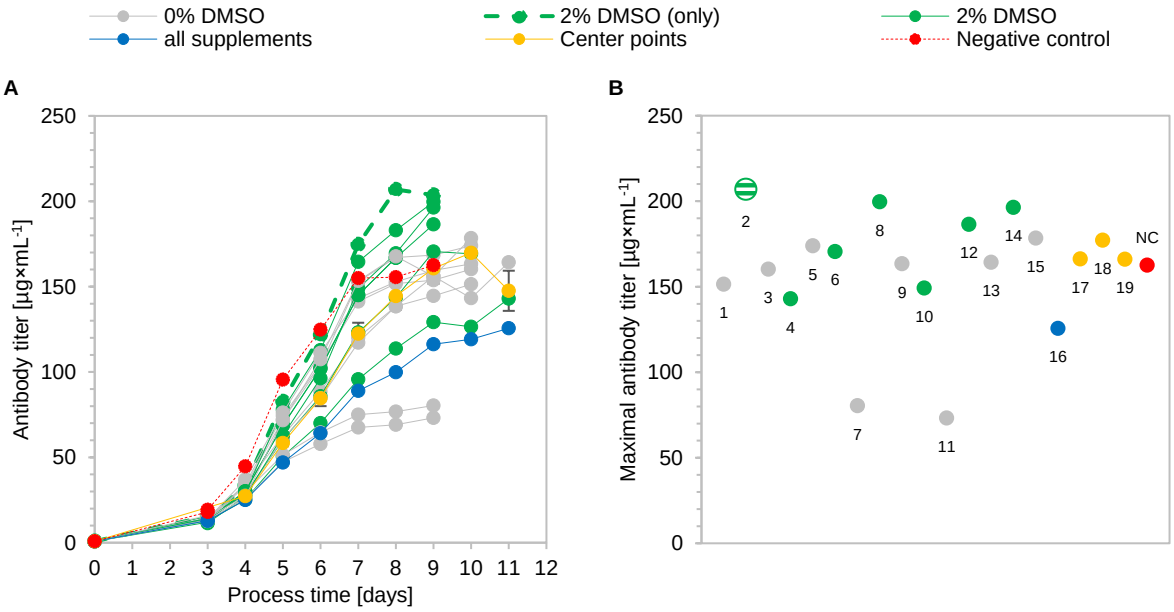


Figure 37: The impact of DMSO supplementation on the maximal antibody titer (B) and the course of the antibody titer over the process time (A). The error bars in the left graph display the standard deviation of three biological replicates.

An enhanced antibody titer with a reduced maximal viable cell concertation can only be maintained by an enhanced cell specific productivity. As mentioned in the section describing the effect plot the mean specific productivity was considered as screening parameter. Nevertheless an enhanced specific productivity in the course of the process time could be observed, shown in Figure 38A and by the obtained maximal values in Figure 38B. The viable specific growth rate is steadily falling and only valid until day 5 of the cultivation for most of the cultures. Especially experiment #6 and #14 show a different viable specific productivity, which is defined as the productivity at which the viable specific growth rate is still positive.

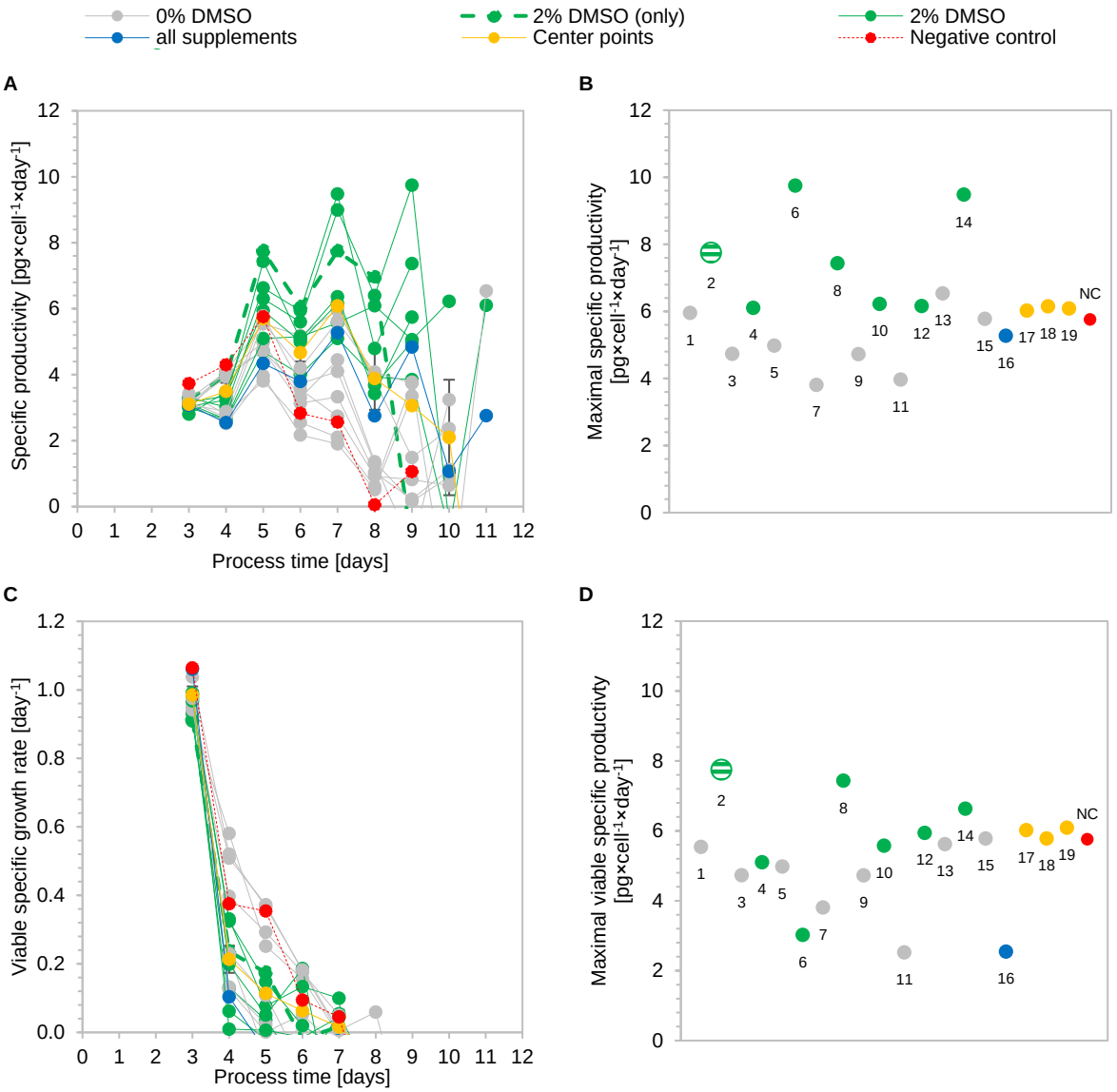


Figure 38: DMSO supplementation and the impact on specific productivity in course of the process time (A) and the obtained maximal specific productivities (B). The maximal viable specific productivity (D) shows a slight difference for experiment #6 and #14. This measure is characterized in that only positive viable specific growth rates are considered. Figure C shows the course of the viable specific growth rate. At day 7 all viable specific growth rates (C) have been negative, which may bias the maximal specific productivity.

As mentioned in the beginning of this section also metabolite data was taken into account. Figure 39B describes the glucose and L-glutamine concentration over the process time. An effect analysis was not performed as the observations have been complicated to express in numerical values. Figure 39A shows that the DMSO supplemented cultures seem to consume less glucose, so that glucose depletion occurs in the later process stage. In contrast the glucose depletes already on day 6 in the negative control. Experiment #2 shows that as soon as the glucose and lactate deplete on day 7 a steep decrease in the viability can be observed. Similar findings have been observed for the L-glutamine concentrations in course of the process time. L-glutamine never depletes in the course of the process time. Furthermore a stabilization of the L-glutamine concentration can be seen between day 4 and 7.

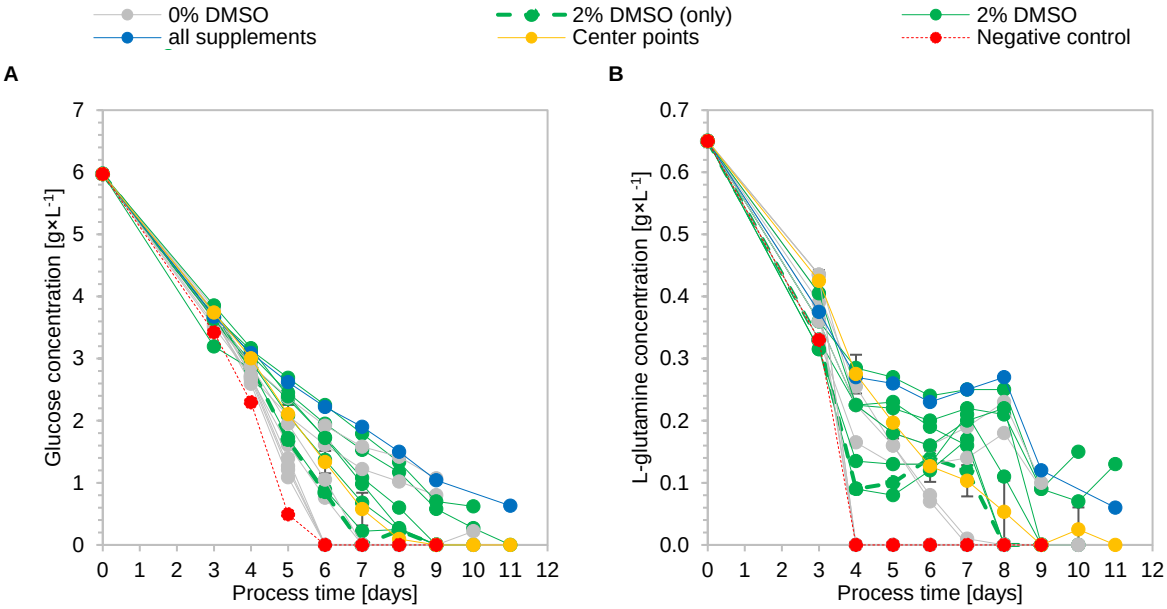


Figure 39: The concentration of glucose (A) and L-glutamine (B) in the course of the process time of DMSO supplemented cultures.

Supplementation of DMSO impacted on the metabolism of the CHO cells. Lactate depleted significantly faster in DMSO supplemented cultures as shown in Figure 40A. Already the graph indicate that lactate completely depleted on day 7 of the cultivation in DMSO supplemented cultures (Figure 40D). Only cultures supplemented with DMSO and 4-PBA show an enhanced lactate concentration. Furthermore the dot plot in part B of the figure elucidates the maximal lactate concentration in the course of the process time is in most of the DMSO supplemented cultures lower. Only experiment #1 show a comparable low maximal lactate concentration. The prior mentioned observations are also manifested in the cell specific lactate consumption rate. DMSO supplemented cultures already consume lactate on day 4, which is indicated by a negative lactate production rate (Figure 40C).

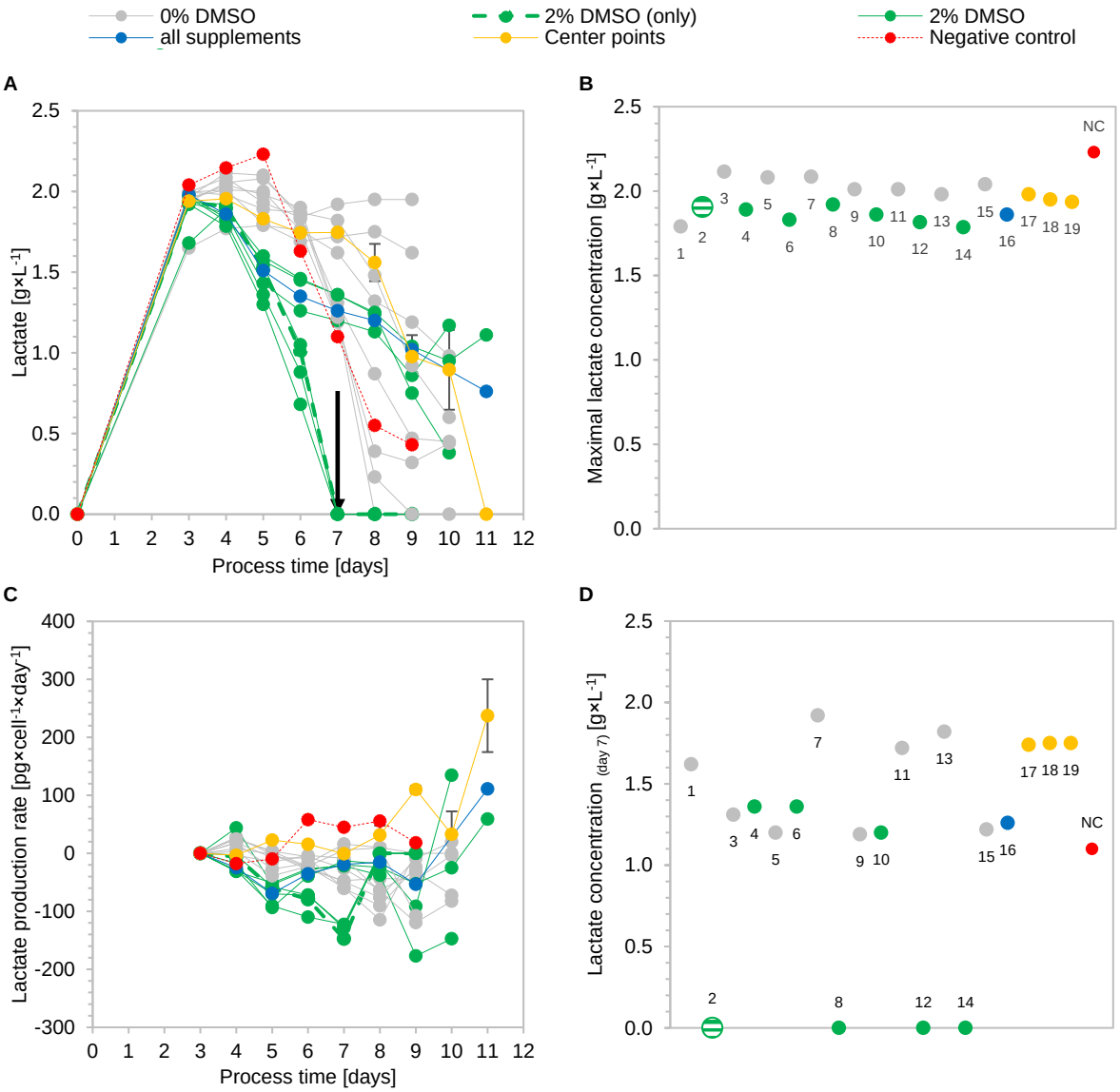


Figure 40: Overview of the impact of DMSO supplementation on the lactate metabolism. Part B summarizes the maximal lactate values of all experiments. The black bold arrow in part A indicates the day at which lactate complete depleted in course of the process time, the depletion is further characterized in part D. In addition, a graph representing the lactate production rate is shown here, see part C.

Another impact of DMSO supplementation was on the glutamate metabolism. DMSO supplemented cultures tend to accumulate L-glutamate in a greater extend in the course of the process than other cultures. L-glutamate production rates as well as process L-glutamate production rates were positively impacted and therefore highlighted in the Figure 41A and B. Effect analysis did not yield in a sufficient regression model to characterize the substances. The culture supplemented only with DMSO (#2), enhanced the initial concentration of 0.26 g×L^{-1} by 1.4-fold.

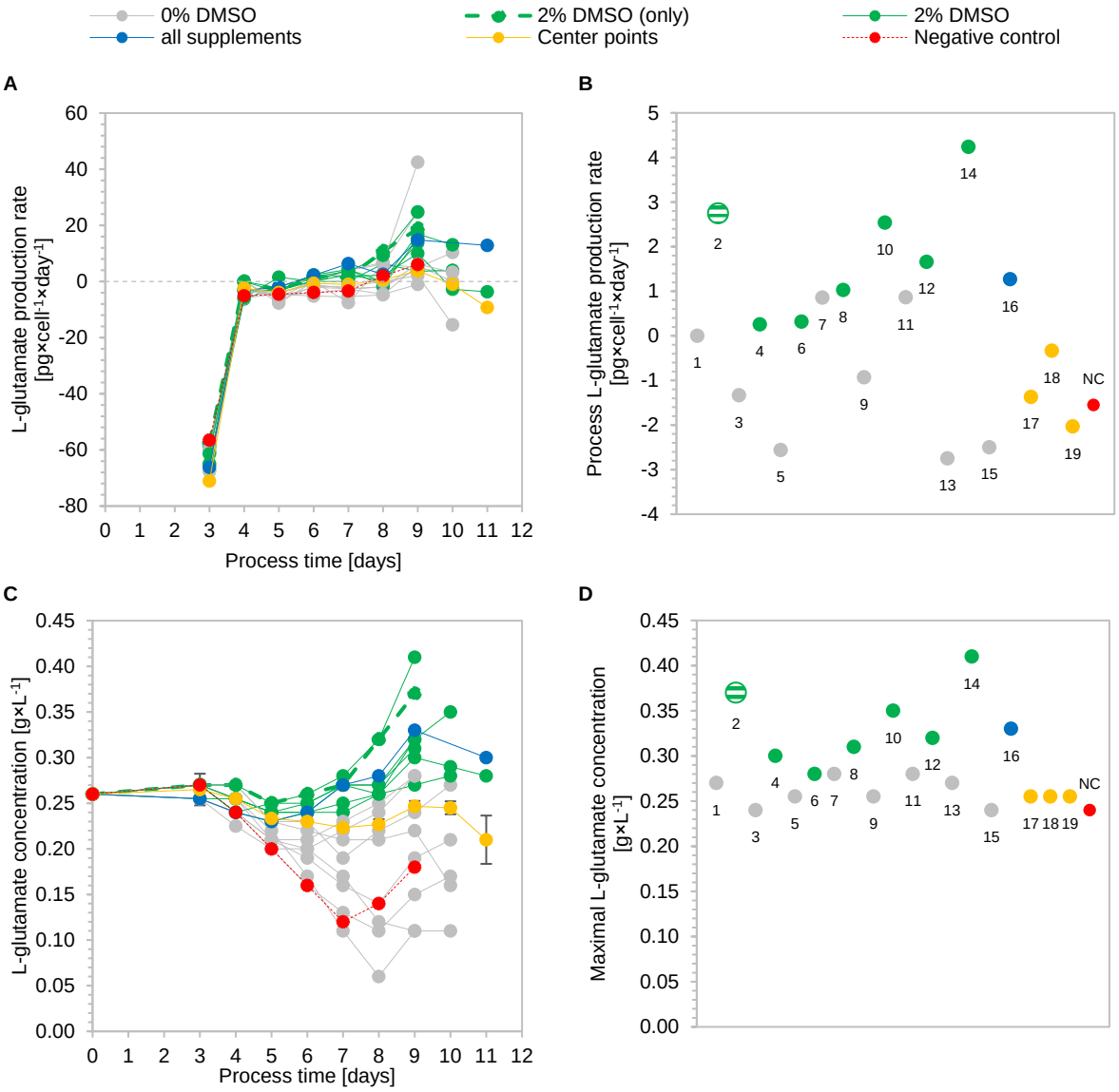


Figure 41: Overview of the L-glutamate metabolism of DMSO supplemented cultures. Part A elucidates the L-glutamate production rate in the course of the process time, whereas the Part B characterizes the process specific production rate of L-glutamate. Part C depicts the L-glutamate concentration during the process time, with the corresponding maximal L-glutamate concentrations in part D.

3.4.5 Betaine alters the viable cell concentration but reduce the antibody titer and the specific lactate consumption rate

Supplementation of betaine impacted on the maximal viable cell concentration, which was slightly improved by the supplementation, but not significantly. Pure supplementation of betaine resulted in comparable cell concentrations as the negative control, who reached the highest maximal cell concentration among all experiments. Only the viability was kept higher in comparison to the negative control over the process time in the culture supplemented only with betaine (experiment #3).

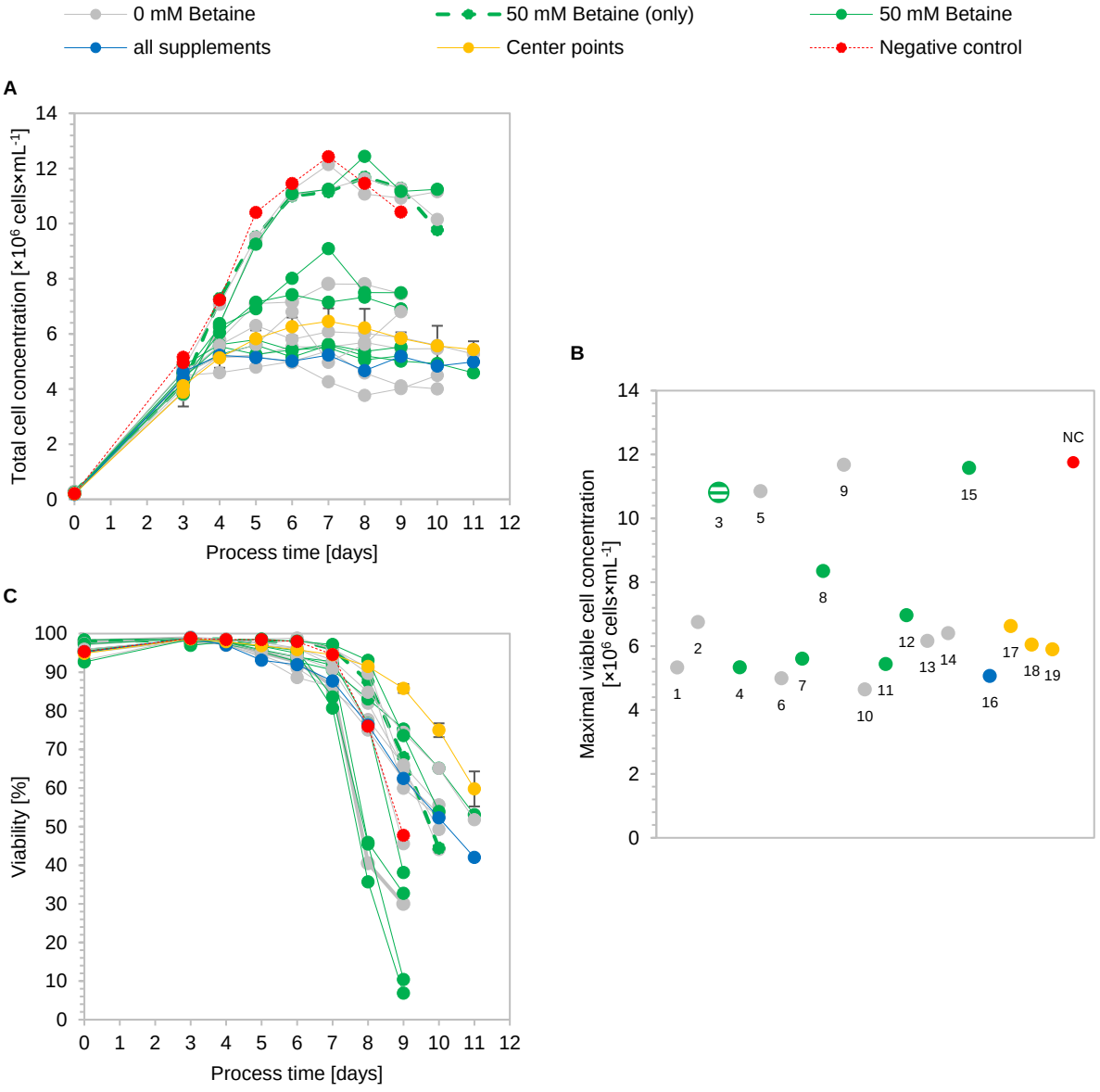


Figure 42: The impact of betaine supplementation on the total cell concentration and the viability over the process time (A and C) and the obtained maximal viable cell concentration (B).

As already elucidated in the effect analysis, the antibody titer was significantly reduced by the supplementation of betaine. Figure 43A characterizes the betaine supplemented cultures and as indicated no improvement in comparison to the negative control could be obtained. Experiment #3, only supplemented with betaine showed a comparable antibody titer like the negative control. Only experiment #8 and #12 resulted in similarly high antibody titers, but have been supplemented with DMSO in addition which is not indicated in Figure 43.

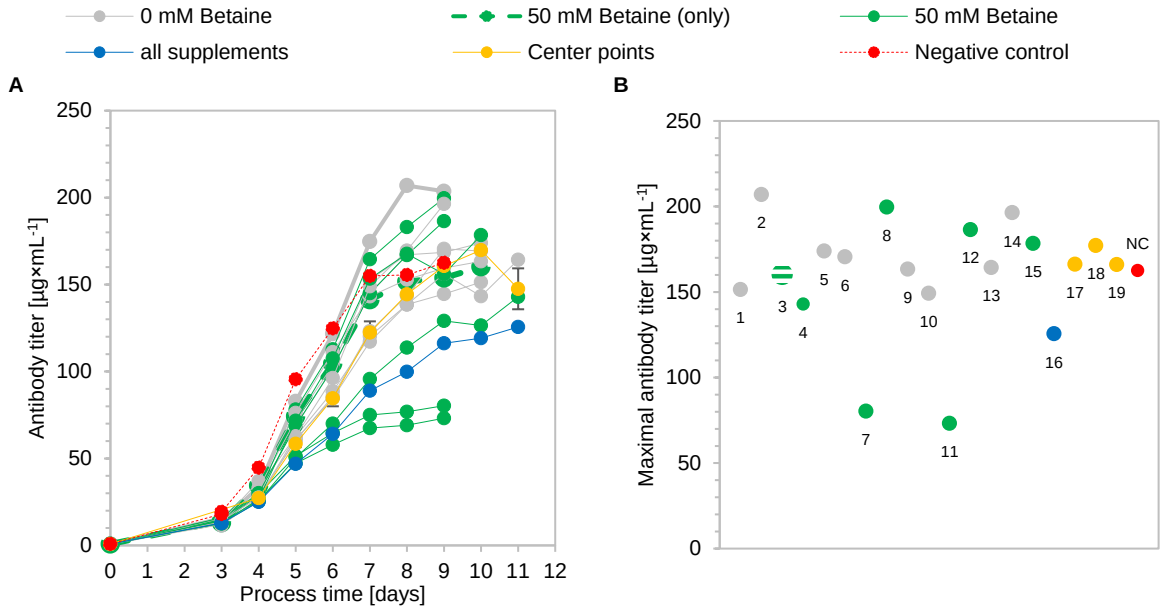


Figure 43: The impact of betaine supplementation on the antibody titer in the course of the process time (A) and the corresponding obtained maximal antibody titers taken for the effect analysis (B).

Supplementation of betaine impacted also on the lactate metabolism. According to the effect analysis, a significantly depressed process lactate consumption rate could be observed. Especially experiment #3, only supplemented with betaine showed the negative impact. Already the course of the lactate concentration over the process time (Figure 44A) shows that the betaine supplemented cultures had an enhanced level of lactate among all other cultures. The culture supplemented with only betaine showed similar lactate profiles as the negative control. The dot plot characterizing the maximal lactate concentration in Figure 44B again shows the effect of DMSO. All even number were also DMSO supplemented and therefore show a lower maximal lactate concentration. Furthermore, Figure 44D shows the process consumption rate which was according to the effect analysis significantly negatively affected, which is shown in the dot plot as well. All supplemented cultures showed a lower process consumption rate. Only even numbers show a higher consumption rate due to the DMSO supplementation as mentioned prior.

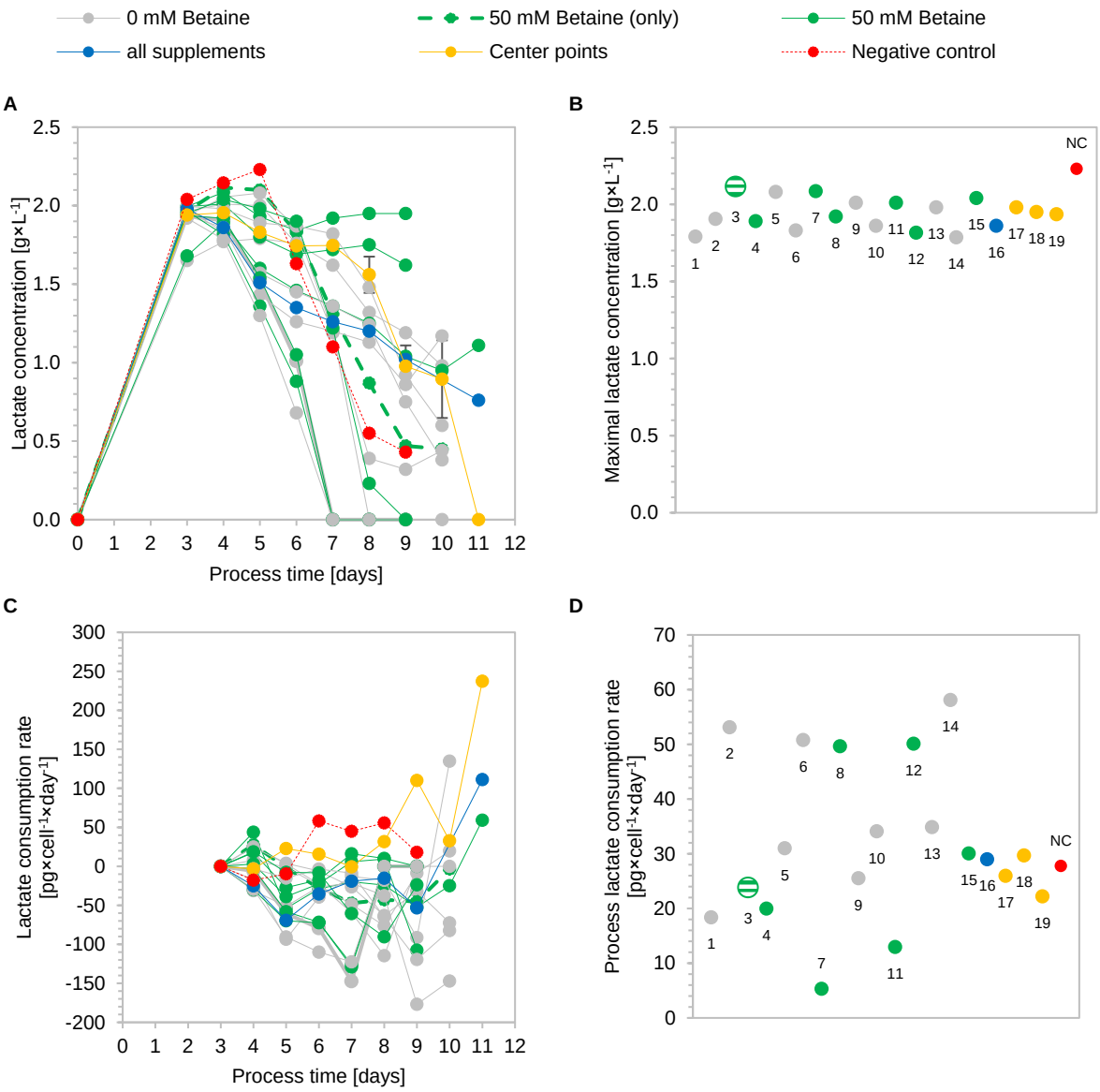


Figure 44: The course of the lactate concentration (A) and lactate consumption rate (C) over the process time and the obtained maximal values for the lactate concentration (B) and the process lactate consumption rate (D) for betaine supplemented cultures.

3.4.6 Maltodextrin and valproic acid

According to the evaluation, supplementation of maltodextrin and valproic acid did not yield in any significant effect, therefore a detailed characterization is not shown here. The observed effects from the prior screening experiments could not be confirmed in this fractional factorial resolution V+ screening experiment.

3.4.7 4-Phenylbutyric acid inhibits cell growth but maintains the specific productivity and enhances the lactate concentration

The effect analysis yielded information about significant negative effect on the maximal viable cell concentration, which can be seen in Figure 45A. Already one day after the supplementation on day 3, a growth inhibition can be observed. No 4-PBA supplemented culture exceed a total cell concentration of 6.5×10^6 cells×mL^{-1} as shown in the dot plot in Figure 45B. For example experiment #13 only showed approximately 50% of the maximal viable cell concentration in the negative control. The course of the viability in 4-PBA cultures had a similar decrease like the negative control, only experiment #7 and #11 performed worse than the negative control. The plain supplemented 4-PBA culture prolonged the process duration by one day (dashed line).

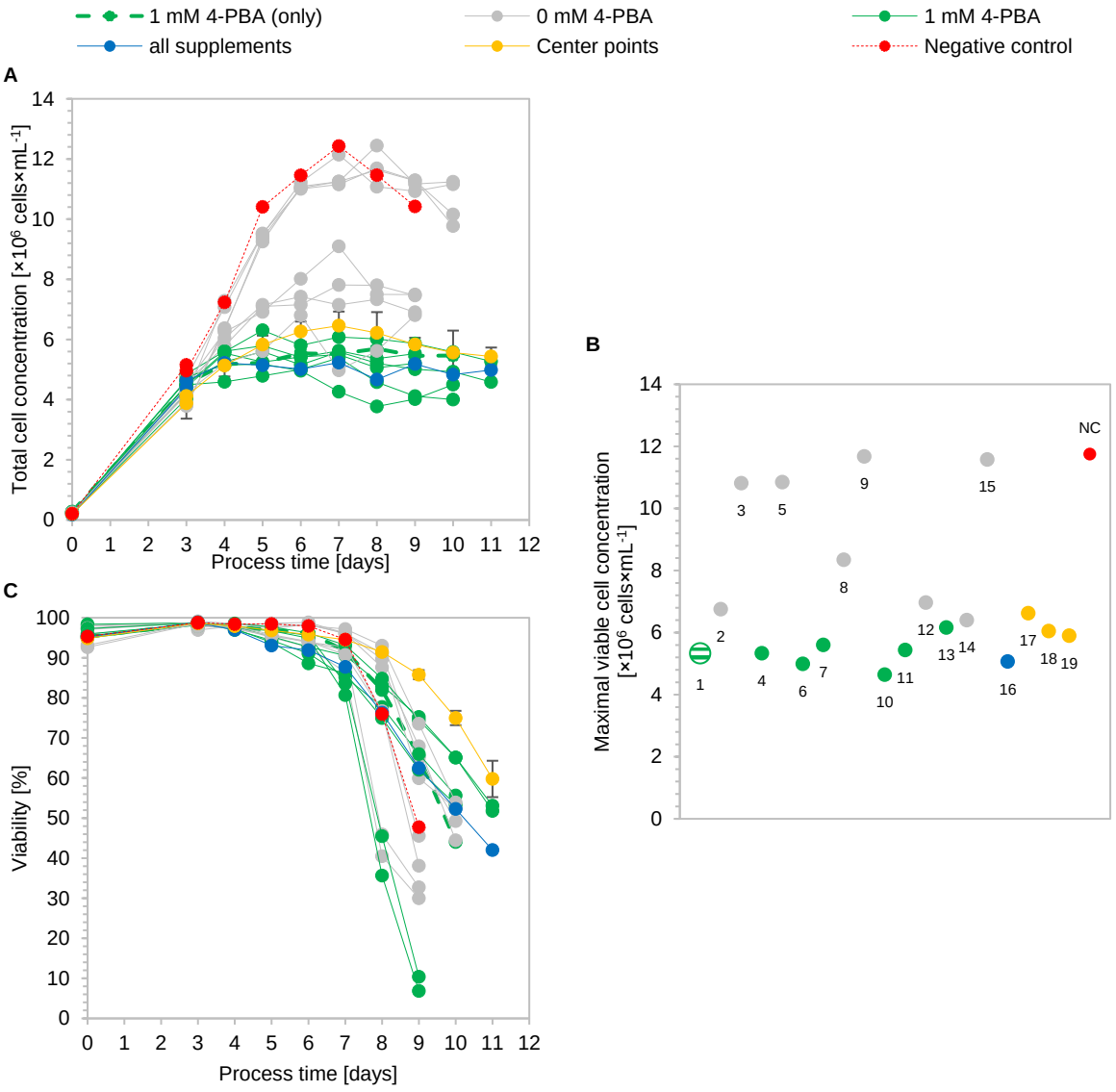


Figure 45: The impact of 4-PBA supplementation on the total cell concentration (A) and viability (C) over the process time and the obtained maximal viable cell concentrations (C) used for the effect analysis.

The effect analysis elucidated that the supplementation of 4-PBA impacted significantly negative on the maximal antibody titer. No supplemented culture exceeded the maximal antibody titer of the negative control to a greater extent. Furthermore the culture supplemented only with 4-PBA (experiment #1) had an approximately 30% reduced maximal antibody titer than the culture supplemented only with DMSO (experiment #2), which had the highest maximal antibody titer among all experiments.

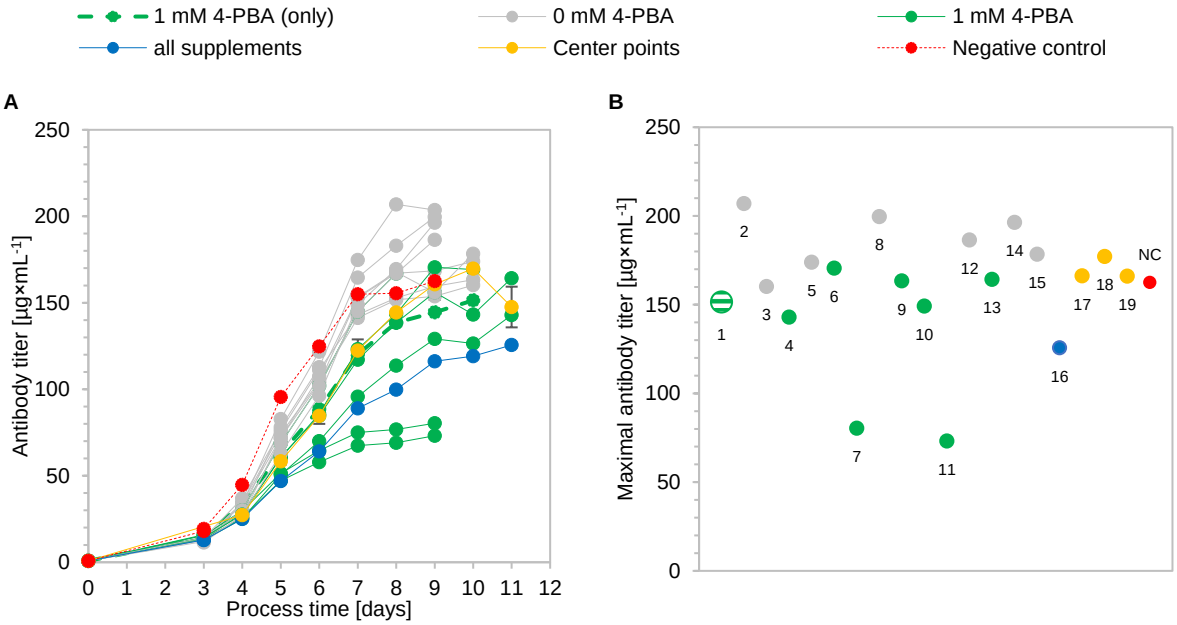


Figure 46: The course of the antibody titer over the process time (A) and the obtained maximal antibody titers (B) taken for the effect analysis.

The effect analysis yielded also information about the significant negative impact on the process lactate consumption rate. Already the lactate concentration in course of the process time show only minor changes in the lactate concentration between day 3 and 7. No lactate depletion could be observed in cultures supplemented with 4-PBA (Figure 47A). Furthermore the lactate consumption rate was always negative indicating that lactate was formed and not consumed during the process.

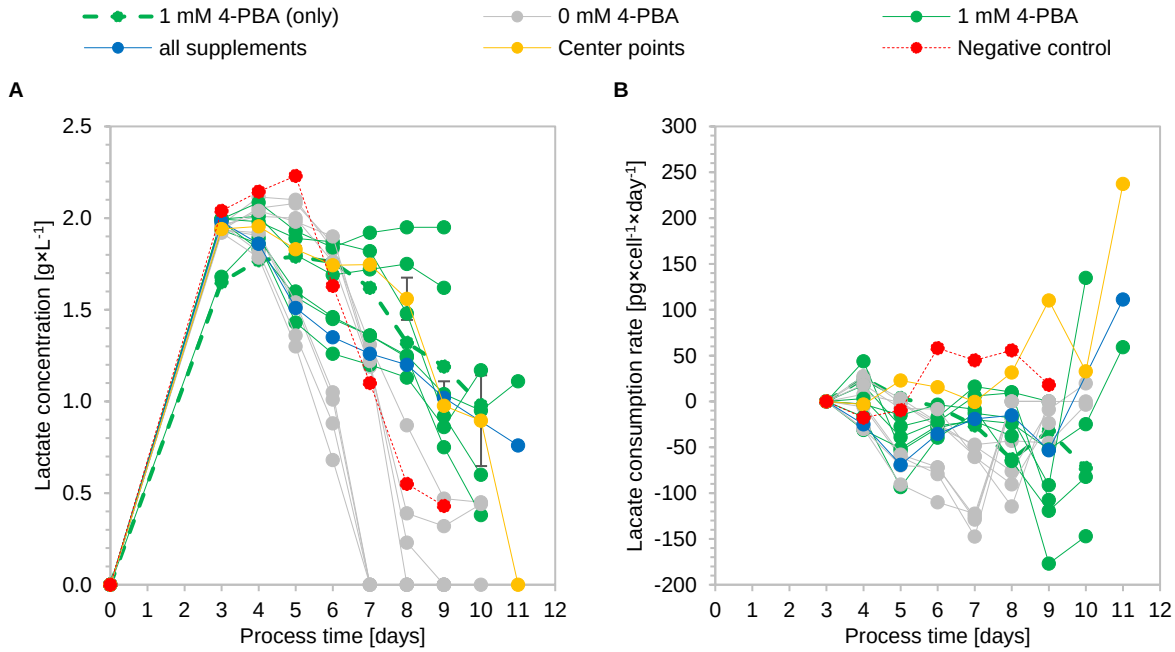


Figure 47: Lactate concentration (A) and lactate consumption rate (B) in the course of the process time influenced by the 4-PBA supplementation.

3.5 Application of DMSO in a semi-perfusion cultivation

Experimental data obtained during the first screening experiment yielded information about the positive impact of DMSO on the antibody titer by increasing the cell specific productivity. In order to verify and further investigate the impact of DMSO on the specific productivity of the cell, a semi-perfusion experiment was performed. Two cultures were supplemented with 2% DMSO and in parallel, two cultures were adjusted with HQ-H_2O to obtain the same dilution of the basal media as in the DMSO supplemented ones. The aim of the experiment was to produce a sufficient amount of recombinant protein for protein A purification and biophysical characterization by DSC. In addition, metabolite concentrations were also monitored to further characterize the impact of DMSO on the metabolism of the CHO cells. The obtained results show that there is almost no difference in the total cell concentration between supplement and volume adjusted cultures. A maximal total cell concentration of about 24×10^6 cells$\times$mL^{-1} was achieved in both cultures as shown in Figure 48A. The DMSO supplemented culture reached the maximal value on day 7 and the non-supplemented one day later on day 8. Already on day 5 the viability of the DMSO supplemented culture dropped and reached the termination criteria of a viability below 60% on day 9 (Figure 48B). In contrast the non-supplemented culture showed a slower decrease in the viability, which resulted in a process duration of 12 days. On day 5 of the cultivation the antibody titers of both experimental set ups started to deviate from each other (Figure 48C). DMSO supplemented cultures achieved an approximately 20% enhanced antibody titer by an enhanced cell specific productivity (Figure 48D). The average diameter of the cells do not to deviate from each other at the beginning. Only after day 5, at which the viability dropped in DMSO supplemented cultures a reduced average diameter is observed (Figure 48E). Between day 4 and 5 the viable specific growth rate is stabilized by the DMSO supplementation in contrast to the non-supplemented cultures as shown in Figure 48F.

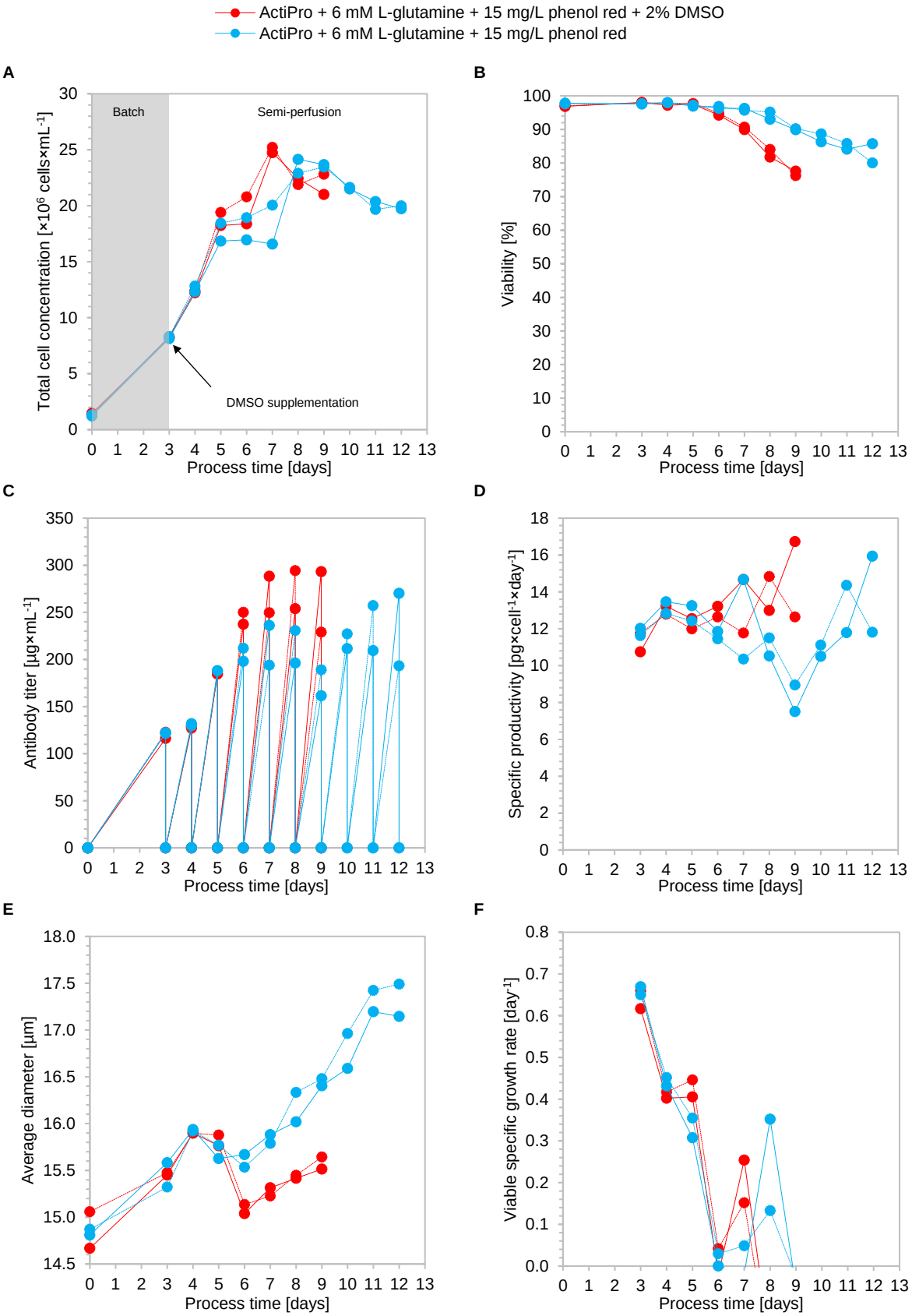


Figure 48: Different cultivation parameters in the course of the semi-perfusion process time. The total cell concentration (A) over the process time, with additional highlighting of the different cultivation phases. Day 0 to 3 were conducted as batch and from day 3 until the termination criteria of a viability below 80% was reached. The additional indication is only shown in (A), but represents also the phases for the viability (B), antibody titer (C), specific productivity (D), average diameter (E) and the viable specific growth rate (F) over the process time.

Metabolite concentrations show a different pattern among the two differently supplemented cultures. Figure 49A describes the glucose concentration over the process time. During the first 6 days glucose concentrations in both culture conditions are similar, but then differ on day 7, at which DMSO supplemented cultures did utilize less glucose so that the residual glucose level is higher in DMSO supplemented cultures than in non-supplemented cultures. This observation is also shown in Figure 49B as the glucose consumption rate decreases in DMSO supplemented cultures. L-glutamine concentrations show similar patterns as the glucose concentration in the course of the process time as shown in Figure 49C. On day 8 of the cultivation residual L-glutamine remains in the supernatant in DMSO supplemented cultures and was not utilized in comparison to the non-supplemented negative control. This observation is confirmed in the L-glutamine consumption rate, which already deviates on day 5 in DMSO supplemented cultures from non-supplemented cultures (Figure 49C). Another aspect is the concentration of L-glutamic acid over the process time as shown in Figure 49E. Already on day 7 DMSO supplemented cultures accumulate L-glutamic acid, shown in Figure 49E. Non-supplemented cultures do also accumulate L-glutamic acid, but to a lower extend. Both cultures consume L-glutamic acid until day 6 and then switch to production indicated by L-glutamic acid accumulation. An increase of about 30% of the initial L-glutamic acid concentration was reached in DMSO supplemented cultures.

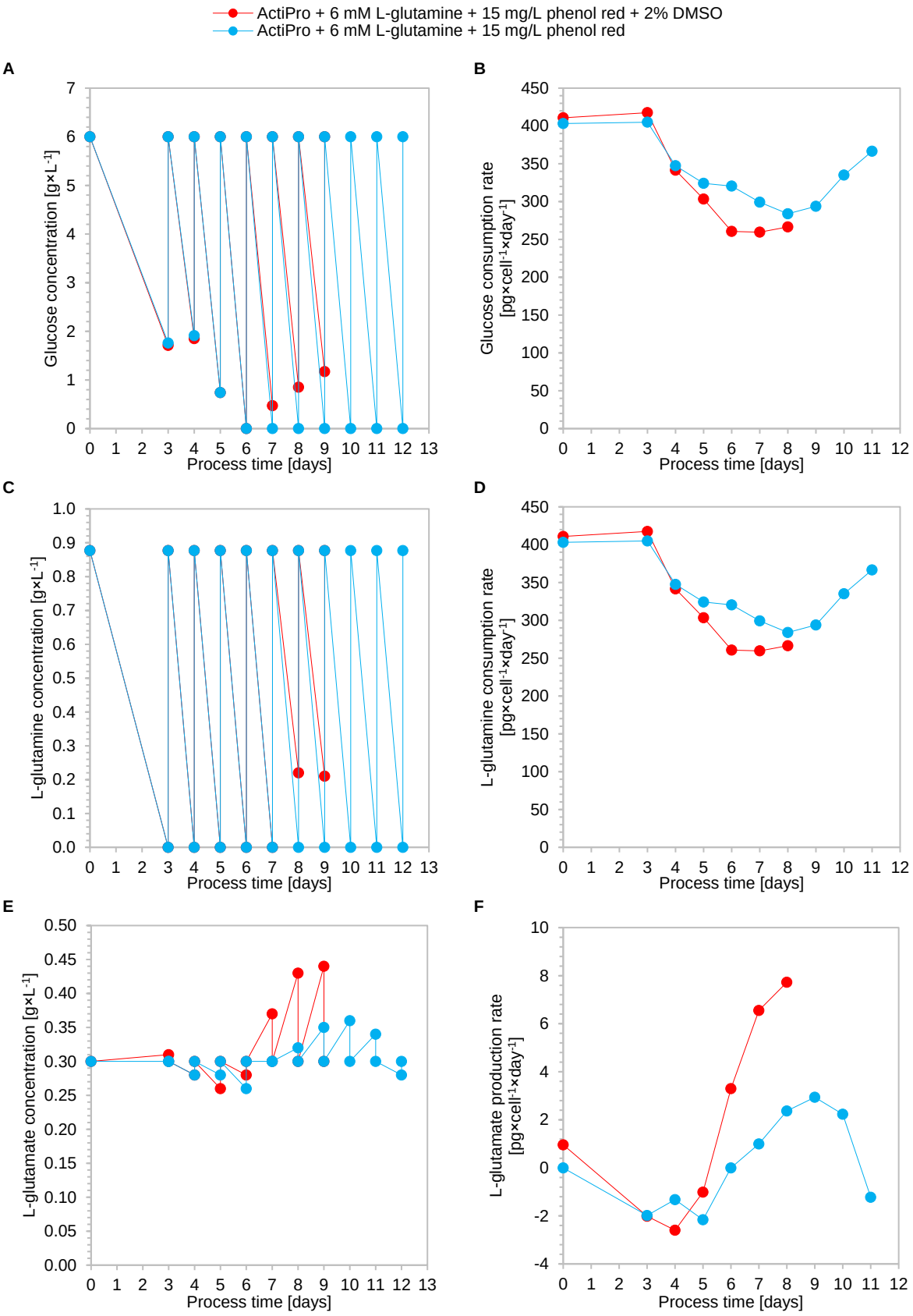


Figure 49: Metabolite concentrations and their corresponding production or consumption rates. Section A and B describes the glucose and the corresponding consumption rate in the course of the time. The L-glutamine concentration and the corresponding consumption rate is shown in section C and D. Metabolite profiles of L-glutamic acid is elucidated in section E and F.

Figure 50A describes the lactate concentration over the process time. Both cultures show the same lactate concentration until day 6 at which the concentration starts to deviate in both cultures. DMSO supplemented cultures show a lower concentration of lactate in comparison to the non-supplemented negative control as shown in Figure 50A on day 6. Furthermore the lactate production rate do also decrease after day 4 in DMSO supplemented cultures to a greater extent than in the non-supplemented negative control as shown in Figure 50B. The ammonia concentration of both cultures deviates only minor from each other. DMSO supplemented cultures show a slightly higher concentration of ammonia than non-supplemented as shown in Figure 50C, but the ammonia specific productivity is lower from the beginning of the cultivation.

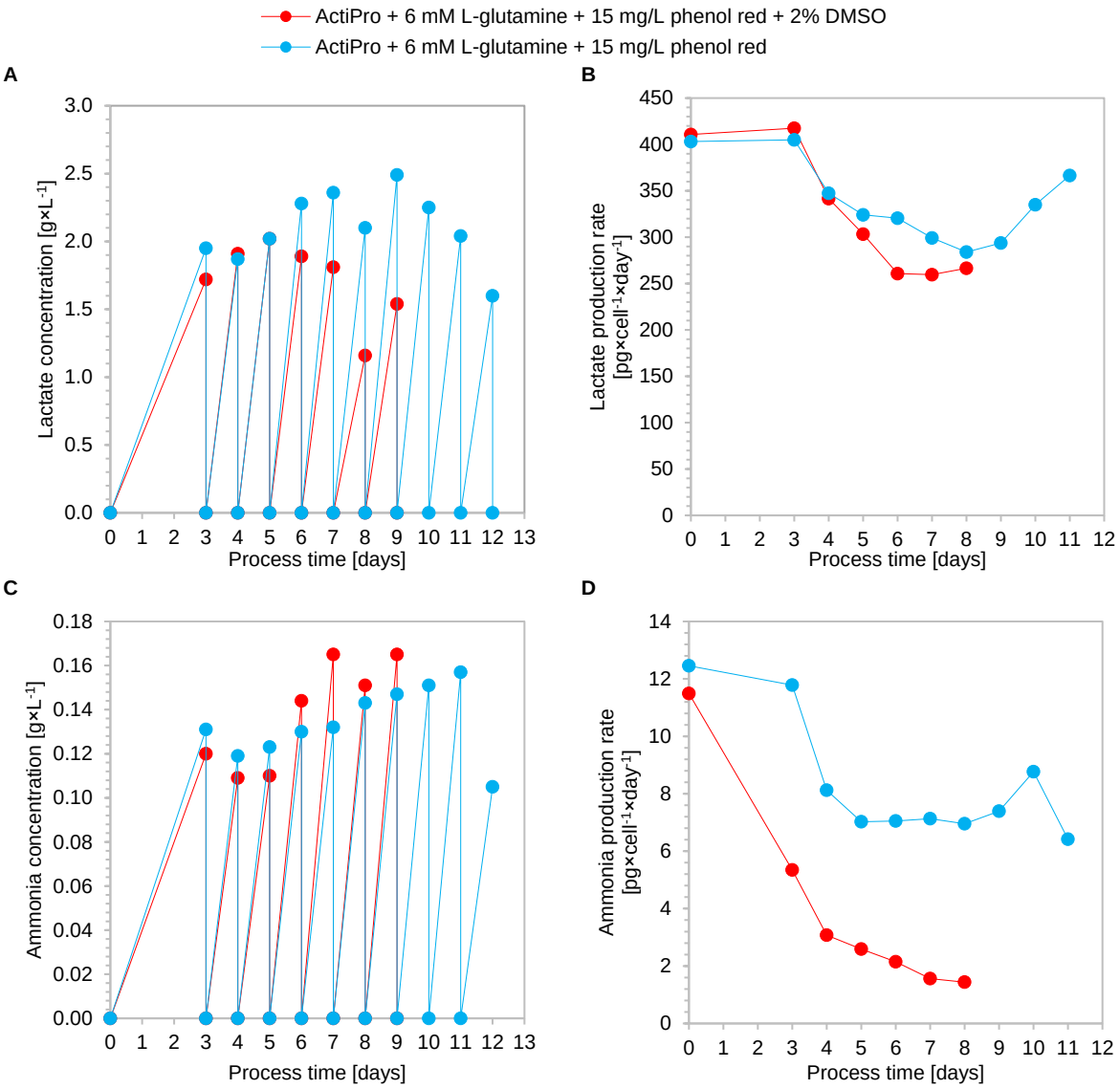


Figure 50: Lactate and ammonia concentration over the process time (A and C) and the corresponding production rates (B, D).

3.6 Characterization of DMSO in a glucose controlled batch to further investigate the impact on the metabolism and product formation

For further characterization of DMSO supplemented cultures a new cultivation mode was performed. Earlier experiments showed that DMSO supplemented cultures terminate probably due to a carbon source depletion. Glucose as well as lactate reached lower critical levels so that cells die due to a lack of carbon source. A glucose controlled batch was performed to circumvent the carbon source limitation. The feeding was set according to the lactate and glucose concentration during the cultivation. Only if the glucose and lactate concentration reached a threshold of 2 $g \times L^{-1}$, an adjustment with a 250 $g \times L^{-1}$ glucose stock solution was conducted, so that the glucose concentration after the feeding procedure was set to 3 $g \times L^{-1}$. The aim of this experiment was not only to prolong the process time, but also to monitor changes in the metabolism and the ability to switch between different carbon sources like glucose and lactate. Previous experiments showed that the supplementation of 2% DMSO had an impact on the lactate metabolism. Already the total cell concentration over the process time shown in Figure 51A show the effect of the DMSO supplementation. Shortly after the supplementation on day 3, DMSO supplemented cultures showed a reduced cell concentration, but an affection of the viability could not be observed (Figure 51B). The viability only significantly decreased on day 6 in the DMSO supplemented cultures as shown in Figure 51B. On the same day the antibody titers also start to deviate from the non-supplemented negative control. DMSO supplemented cultures showed a higher antibody titer and maintain it until the termination criteria of a viability below 60% was reached. The maintenance and enhancement of the antibody titer is due to an enhanced cell specific productivity on day 5 shown in Figure 51D. From day 4 to 6 the specific productivity was steadily increased in DMSO supplemented cultures. The non-supplemented cultures showed a stabilized specific productivity, but no enhancement. Almost no differences could be observed in the viable specific growth rates, both cultures grow to the same maximal cell concentration in a similar time interval.

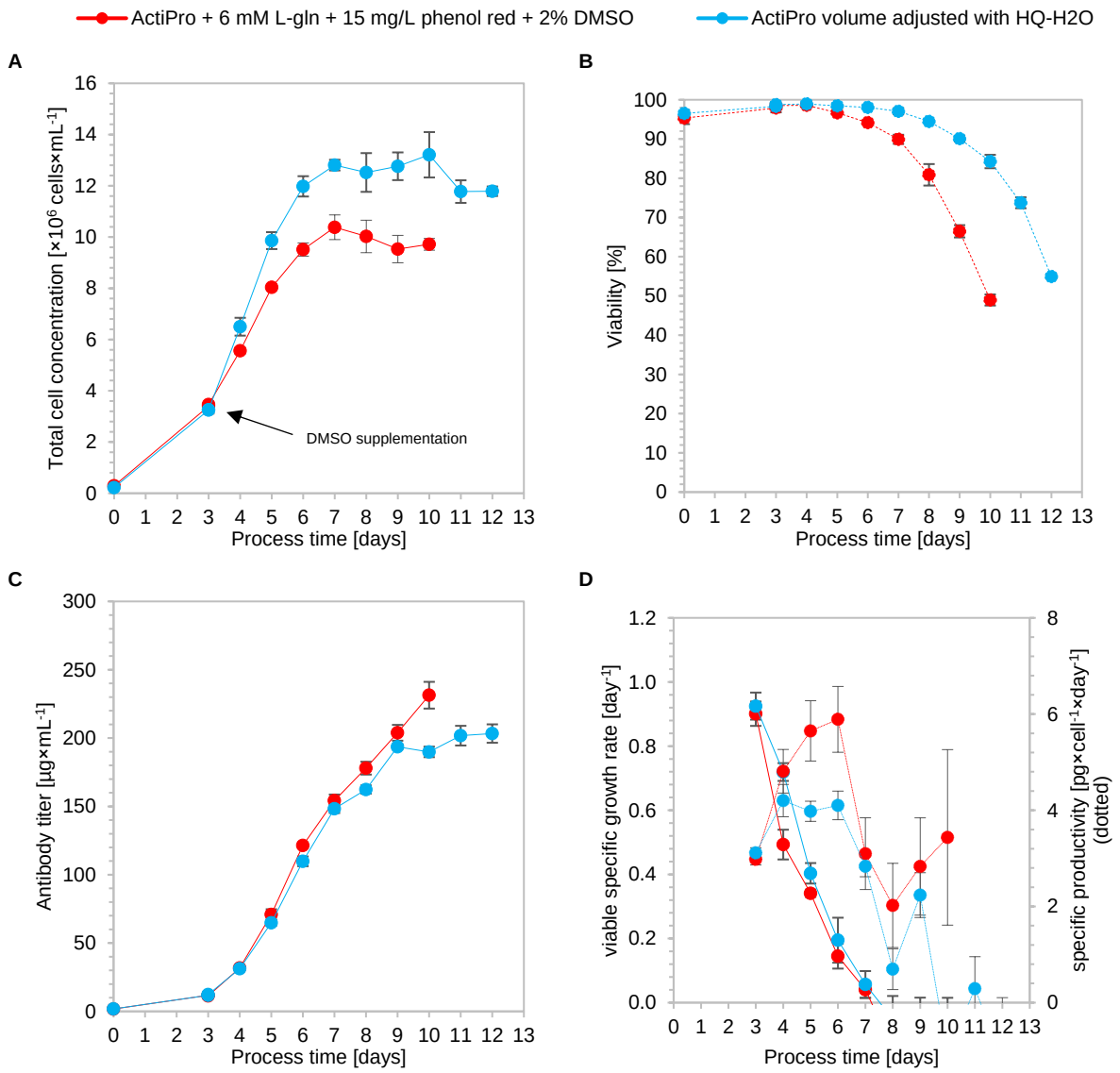


Figure 51: The total cell concentration (A), viability (B), antibody titers (C), viable specific growth rate (D) and specific productivity (D) in the course of the process time.

The course of metabolite concentration over the process time describes the metabolic status of the cells. Already the glucose concentration over the process time indicates an effect caused by DMSO. Figure 52A shows a similar course of the glucose concentration in both cultures until day 5 at which the DMSO supplemented cultures stopped to consume glucose. The increase in glucose concentration in the graph is due to glucose supplementation indicated by the dashed line representing the accumulated glucose over the process line. Glucose has been fed from day 5 to 10 in the DMSO supplemented culture, whereas the non-supplemented culture was fed from day 5 to 12. As soon as the glucose concentration reach a critical low level, DMSO supplemented cultures switch to lactate consumption indicated in Figure 52C on day 5. The lactate production rate on day 5 also indicates a change as the measure is negative at this time point, which shows that lactate is consumed not produced. This

can also be observed for the corresponding negative control but to a lower extent. Especially day 6 indicates that the DMSO supplemented cultures consume lactate, whereas the non-supplemented negative control produce lactate.

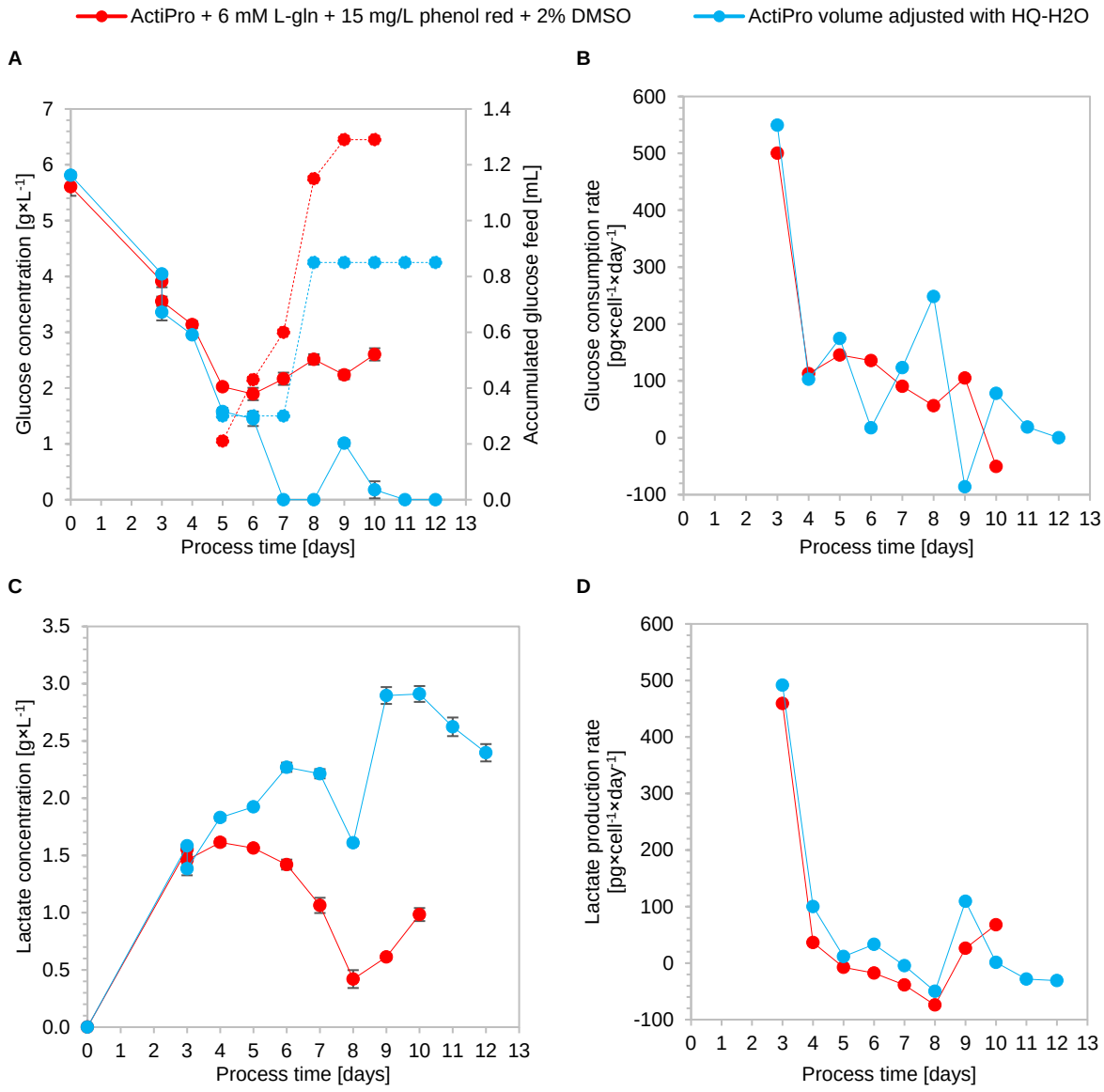


Figure 52: The glucose concentration in the course of the process time (A), the accumulated glucose feed (A) and the corresponding glucose consumption rate (B). Section C and D characterizes the lactate concentration and the corresponding lactate production rate respectively.

The metabolite profile of L-glutamine shown in Figure 53A show hardly a difference between the two cultures. Only L-glutamine depletion takes place two days earlier in the non-supplemented negative control, namely on day 5. In contrast the L-glutamate concentrations in both supplemented cultures deviate immensely. In the DMSO supplemented culture an accumulation of L-glutamate can be observed in the course of the process time. An approximately 20% increase of the initial concentration can be

observed in supplemented cultures. L-glutamate depletes in the negative control on day 8 only a minor amount of L-glutamate is accumulated on day 12. This observation is also manifested in the L-glutamate consumption rate. DMSO supplemented cultures show a small or negative consumption rate indicating that L-glutamate is produced. The ammonia profiles show similar pattern indicating that there is no difference between the non-supplemented and DMSO supplemented culture.

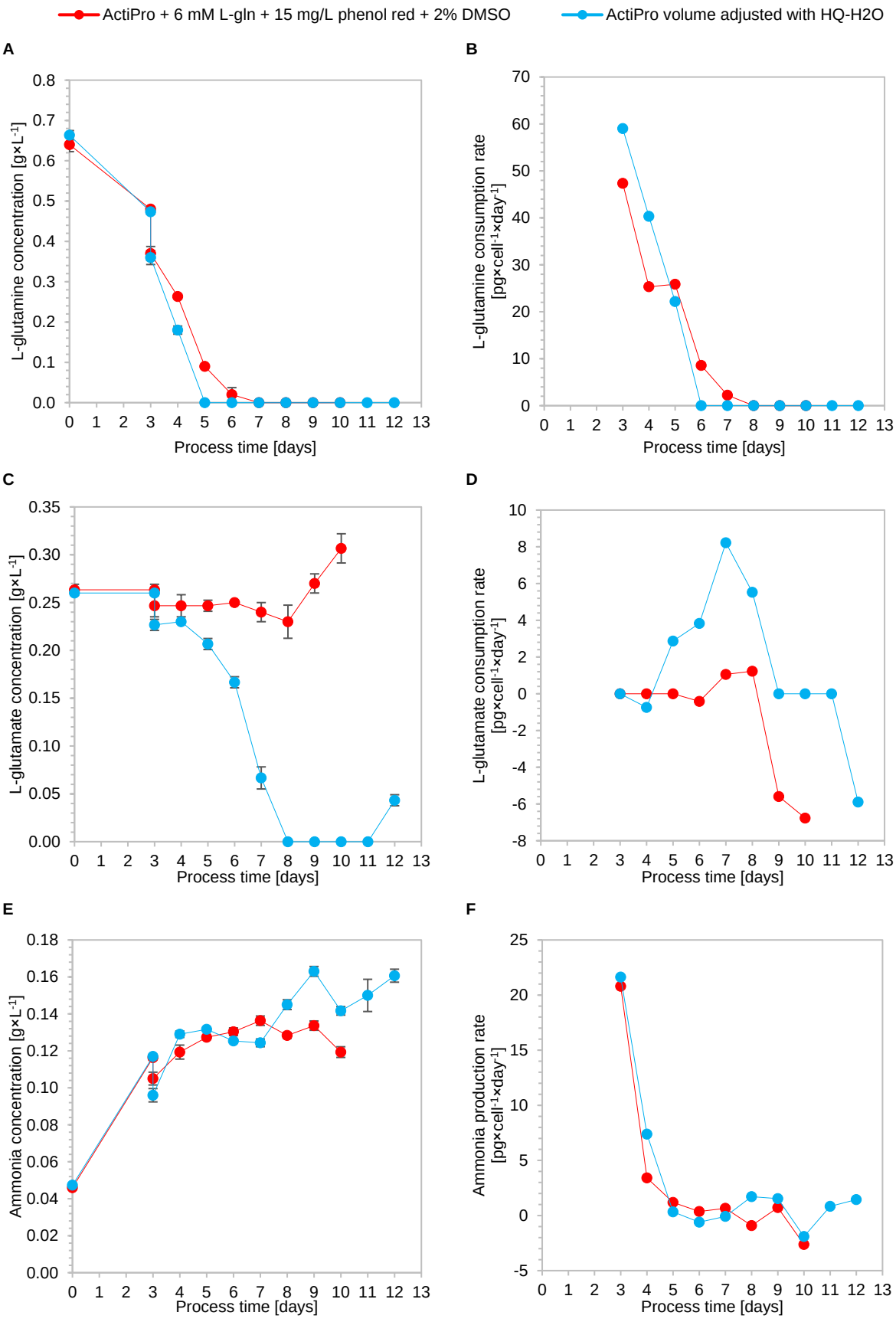


Figure 53: Overview of the L-glutamine (A), L-glutamate (C), ammonia (E) concentration and their corresponding consumption and production rates (B, D and F) in the course of the process time.

Figure 54 characterizes the cell diameter over the process time. After the supplementation on day 3, DMSO supplemented cells swell to a greater extent than the non-supplemented negative control as indicated in Figure 54A. Only a minor difference could be observed, but simultaneously with the change in cell size also the antibody titer of the supplemented cells exceed the antibody titer of the negative control. The surface to volume ratio is a function of the cell diameter and show similar patterns as the average cell size. A lower value indicates a greater ability to interact with the environment, which is the case for DMSO supplemented cultures.

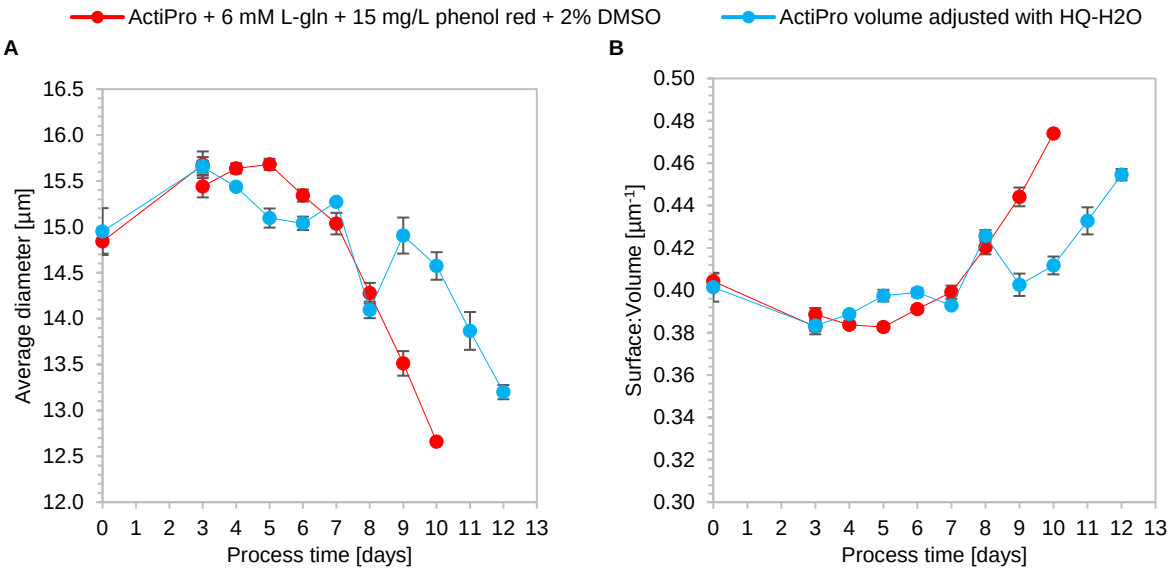


Figure 54: The impact of DMSO supplementation on the average cell diameter and thereby on the surface to volume ratio.

3.7 Impact of different buffer systems on thermal stability of human IgG_1 PG9 antibodies produced in DMSO supplemented and non-supplemented semi-perfusion cultivations

Thermal stability of biomolecules is an essential quality attribute. Buffer systems as well as culture conditions determine the resistance of individual biomolecular subunits to endothermic unfolding. Differences in melting temperatures (T_m) were measured of a purified IgG_1 antibody using DSC. One semi-perfusion was supplemented with 2% DMSO, whereas the other culture did not contain any supplement and have been adjusted with HQ-H_2O instead. The antibodies derived from two different semi-perfusion experiments (see section 3.5) and were purified via protein A chromatography by a sodium acetate buffer system as described in section 2.14. After the purification, a part of the eluate was re-buffered into a phosphate buffer system. At the end of the purification there were 4 different samples, an antibody from the

2% DMSO semi-perfusion cultivation in sodium acetate buffer and in phosphate buffer and the non-supplemented semi-perfusion also in the two buffer systems. Table 14 describes the different obtained T_m values obtained from the DSC measurement shown in Figure 55. Highest stability with a typical T_m value of 82°C and 79°C in phosphate or acetate buffer, respectively, were observed for the antibody CH3 domain in accordance with values from literature. Additionally, CH2 unfolding was also higher at 67°C in phosphate buffer compared to only 60°C in the acetate buffer system. For the F_{ab} fragment two peaks indicating non-cooperative unfolding were observed. The lowest unfolding temperature of 60°C or 47°C was assigned to the F_v region and a second peak at 75°C or 71°C was assigned to the CH1 or CH1/CL unfolding in phosphate or acetate buffer, respectively. The obtained results show that melting temperatures within one buffer system are highly similar, independent if the protein was produced from a DMSO supplemented or non-supplemented culture. Therefore it can be concluded that DMSO did not alter the intrinsic structural stability of the produced antibody. Comparison of the two used buffer systems showed that proteins in phosphate buffer had always higher melting temperature than in sodium acetate buffer.

Table 14: Overview of different melting temperatures and the corresponding structural element. Culture conditions describe the supplements added during cultivation. Purification was performed via protein A and a buffer exchange was performed afterwards in order to monitor different impacts on thermal stability.

		T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	T_{m4} (°C)
Culture conditions	**Buffer system**	F_v	CH2	CH1/CL	CH3
Negative control	PO_4^{3-}	60.11	67.62	74.96	82.06
2% DMSO	PO_4^{3-}	59.59	67.10	74.91	82.09
Negative control	NaAc	46.91	60.00	70.54	79.42
2% DMSO	NaAc	47.21	60.16	70.72	79.43

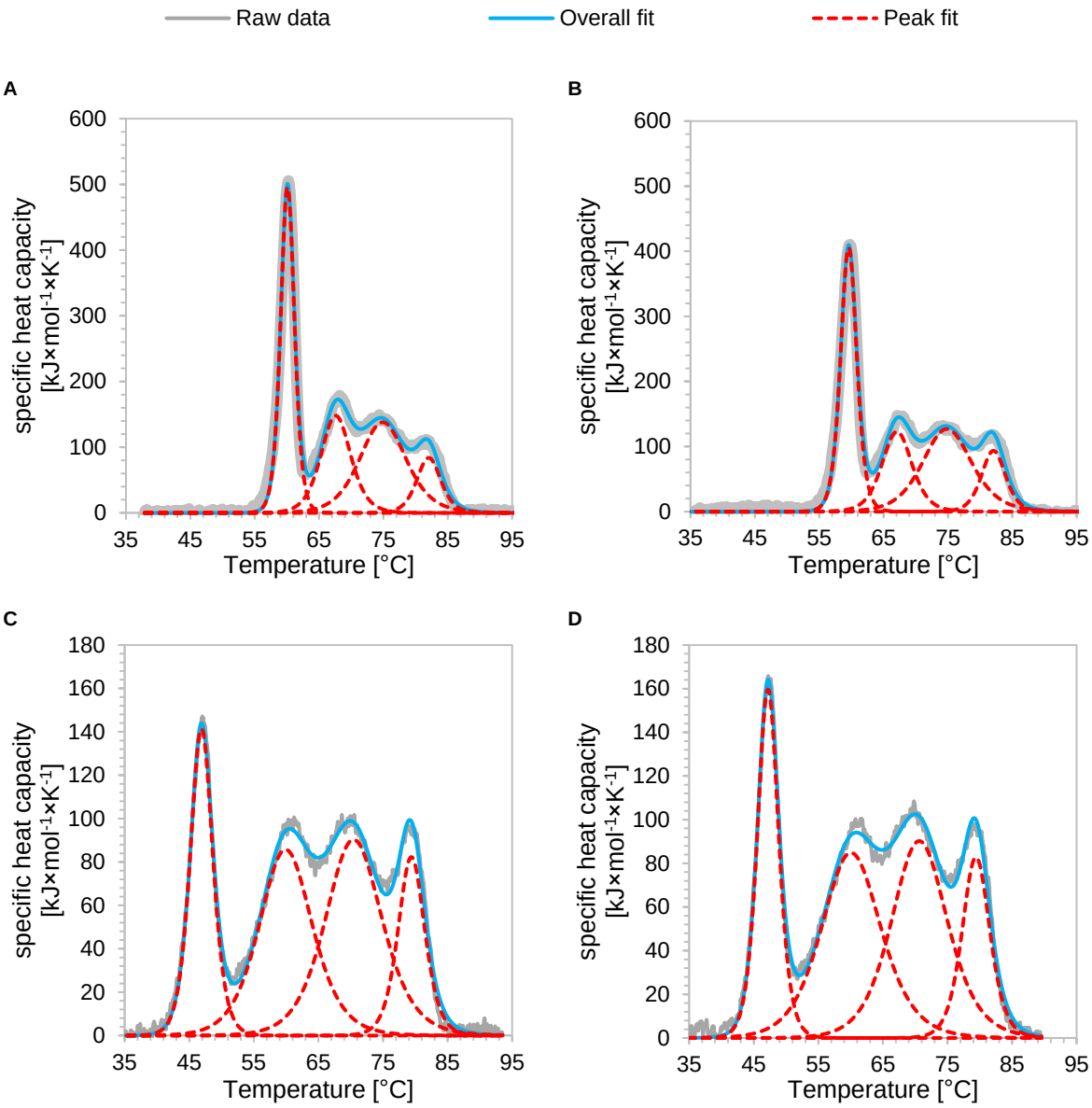


Figure 55: DSC thermograms of PG9 IgG_1 antibody in phosphate buffer (A and B) or sodium acetate buffer (C and D). A and C derive from the same semi-perfusion cultivation, which was not supplemented with DMSO, whereas B and D derive from 2% DMSO supplemented semi-perfusion cultivation.

4 Discussion

In this project, experimental design was applied to screen 21 different substances in order to identify promising cultivation supplements. The application of the fractional factorial screening designs allowed a reduction of individual experiments compared to the traditional one-factor-at-a-time (OFAT) approach. Furthermore the screening yielded information about the impact of the supplementation on different culture parameters, including antibody titer, specific productivity, viable cell concentration and changes in the metabolism.

4.1 Comparison of experimental screening design to the traditional one-factor-at-a-time approach

It is well known that experimental design reduces the number of required experiments while improving statistical confidence. In the first screening 14 different substances have been screened and evaluated based on their effect on defined responses (Ritacco, Wu, and Khetan 2018). In such a fractional factorial design, 19 cultures were defined including one triplicate center point in order to check the linear correlation of the observed responses between different factor levels. Center points can be considered as additional quality control data, but must not be included in a quick screening experiment, so that the resulting number would be reduced to 16 experiments. In addition a negative control was performed, which did not contribute to the evaluation, but was necessary to compare the cultures with the plain basal media. The final evaluation of an experimental design gives detailed information about the significance of different factors on the defined responses. In order to obtain a significant result at least three independent experiments would be required to calculate a statistical relevant standard deviation. So in order to obtain the same result by a traditional OFAT approach as with the fractional factorial design at least 3×14=42 experiments would be necessary. Table 15 summarizes and compares the consumables necessary to perform the screenings with the fractional factorial design (Experimental design) and the one-factor-at-the-time approach (Traditional approach). Beside economic benefits also environmental issues should be considered at this point. Experimental design is, based on the simple calculation performed here, more environmentally friendly and less cost intensive than the traditional approach. As a conclusion it can be said that the traditional approach is 2-fold expensive.

Table 15: This table should summarize the used consumables necessary to perform the screenings. Center points (CP) and the negative control (NC).

	Experimental design	**Traditional approach**
Cultivation tubes	16 + 3 CP + 1 NC	42
Basal media (á 30 mL)	600 mL	1260 mL
Serological pipettes (10 day batch)	200 pieces	420 pieces

4.2 The influence of a strong effect on the quality of the effect analysis in fractional factorial screening experiments of resolution III

Both initial screening experiments were based on a fractional factorial screening design of resolution III. This design is characterized in that it is not able to discriminate between main effects and two factor interactions, but suitable for a screening experiment. Consequently, the regression models only contain linear (main) coefficients. Both screenings identified substances with a significant effect on multiple responses. The two most prominent substances were DMSO and 4-PBA, that significantly impacted on the viable cell concentration, antibody titer and cell specific productivity. The resolution in general is defined as the ‘degree of mixing’ in the experimental set up, describing the ability to discriminate the effect of different factors. Factors with a large effect, like the 4-PBA and DMSO in this example, can bias the evaluation in a way, that substances with only a minor impact are shielded by them (Siebertz, Bebber, and Hochkirchen 2010). For example the effect of betaine in the first fractional factorial screening experiment could have been significant if the negative effect of DMSO would not have been so strong and omnipresent in the response analysis. Another example also from this screening was the polyol mannitol, which had a positive effect on the antibody titer, but again no significant effect could be obtained by a 95% confidence interval, probably also due to the strong effect of DMSO. A negative effect on viable cell concentration was observed for 4-PBA supplementation. Maltodextrin and ß-alanine showed a positive effect on the viable cell concentration, but again no significance according to the confidence intervals also probably due to the strong effect of 4-PBA. The linear (main) effect describes the difference in the response value when changing the factor level from -1 to +1. 4-PBA had the highest linear (main) effect, indicating the dominant effect of this substance. For the evaluation of the maximal specific productivity in the fractional factorial screening the prior stated assumptions could not be confirmed, but especially maximal values are prone to

measurement errors and the specific productivity even more as the cell concentration determination and the antibody titer determination contribute to the calculation. Furthermore, poor model quality for maximum specific productivity prevents the use of this regression model for future response prediction.

4.3 The effect of DMSO and possible applications

One of the most promising substances identified in this study was the solvent compound DMSO. Traditionally, DMSO is used as cryoprotectant in cell culture to prevent crystal formation during freezing of cell bank cultures. This aspect also explain the enhanced osmolality caused by the supplementation. Furthermore, DMSO was already used as supplement in CHO culture (N. Kumar, Gammell, and Clynes 2007) for reversible cell cycle arrest in the G1 phase (Fiore 2002), for enhancement of cell specific mAb production in hybridoma cultures (Ling et al. 2003) or for recombinant protein production from transient cultures. In another report the CHO expression of interferon-beta (Rodriguez et al. 2005), fusion protein and beta-galactosidase (Liu, Chu, and Hwang 2001) was increased, but the latter study indicated that mAb production was not increased by DMSO in the used CHO cell line or additional three tested hybridoma cell lines (Liu, Chu, and Hwang 2001). DMSO was reported to fulfil the requirements for ease of use, safety, cost-effectiveness, ample supply and unaltered product quality assessed by mAb glycosylation and final bioactivity (Ling et al. 2003). It was reported that DMSO acts as a chemical chaperone by stabilizing proteins in their native conformation (Rodriguez et al. 2005; Yoshida et al. 2002) and that DMSO caused CHO cell growth arrest, prevent apoptosis and promotes a differentiated phenotype (Rodriguez et al. 2005).

In this thesis we identified significant effects of DMSO on various screening parameters. The maximal viable cell concentration was reduced and also the viability decreased earlier in supplemented cultures, so that the cultivation termination criteria was reached one day before the non-supplemented cultures. Furthermore, the maximal specific productivity was enhanced and thereby reached higher final antibody titers. Interestingly the antibody titer increases as soon as the viability decreases, which may indicate that only the F_c fragment is properly processed as this domain is the first part of the antibody which fully evolves. A prerequisite for the determination of the antibody titer is a functional F_c fragment, which would be the case, but the BLI quantification do not yield in any information about the overall folded state of the protein (Feige and Buchner 2014). Beside the impact on productivity related parameters,

which are already reported in literature as in Johari et al. 2015, also impacts on the cell metabolism and cell size could be observed. Already Pan et al. 2017 reported a correlation between cell size and the cell specific productivity during fed-batch cultivation, which could also be reported in this study. Up to our knowledge an impact on the cell size caused by the supplementation of DMSO is so far not known. During the fractional factorial screening of resolution III an increase in cell size could be seen and similar observations could be observed in the glucose controlled batch experiment, but the effect was not as pronounced in the resolution V+ fractional factorial screening. The semi-perfusion cultivation did not show an impact on cell size during cultivation. Interestingly, during the fractional factorial screening and the glucose controlled batch the antibody titer and the cell specific productivity of the non-supplemented culture and the DMSO supplemented culture start to deviate as soon as the average cell diameter increases of the DMSO supplemented cultures, confirming the assumption that there is a correlation between the cell size and the cell specific productivity. Another important aspect not reported in literature up to our knowledge is the effect of DMSO on the lactate metabolism of CHO cells. Supplemented CHO cultures tend to consume lactate faster than non-supplemented ones. DMSO is widely used as detergent in biochemical assays. It has been reported that the supplementation of 5% to a glyceraldehyde-3-phosphate dehydrogenase (GA3P-DH) assay enhanced the activity twofold (Wiggers et al. 2007). This enzyme plays a major role in various cellular pathways beside its activity in the glycolysis. The prior mentioned study was carried out by a purified enzyme obtained from *Trypanosoma cruzi*, but it is known that this enzyme is highly conserved and show only little variance from species to species so that a comparison is valid in this case (Sirover 2011). As already mentioned GA3P-DH is a glycolytic enzyme, so that the modulation by DMSO supplementation would explain the altered lactate metabolism. Another review describes GA3P-DH as glucose sensor mediating the cell growth via mechanistic target for rapamycin (mTOR). This would explain the deviating cell concentration in the glucose controlled batch on day 4, as the glucose level in the DMSO supplemented culture and in the negative control fell below 2.5 $g \times L^{-1}$ and the DMSO supplemented culture stopped growing. Similar observations could be observed for the DMSO supplemented cultures in the fractional factorial screening design of resolution V+, in which the cells stopped growing when the glucose level reached 2.5 $g \times L^{-1}$ (Nicholls, Li, and Liu 2012). Another aspect concerning the metabolism of CHO cells is the fact that DMSO supplemented cultures

accumulated L-glutamic acid, indicating that the non-essential amino acid is produced by endogenous enzymes and not consumed. At the end of every DMSO supplemented cultivation the L-glutamic acid concentration was higher than the initial concentration. This can be due to the switch to lactate consumption, which requires NAD^+ to convert the lactate to pyruvate by the lactate dehydrogenase. In order to generate NAD^+ alternative oxidases have to be considered to obtain a favorable redox status of the cell. The malate-aspartate shuttle is a membrane bound enzyme in the mitochondria able to regenerate NAD^+ via an antiporter system. By this reaction also L-glutamic acid is accumulated, which could explain the lactate consumption (Locasale and Cantley 2011). One problem with DMSO is its toxicity. Cells tend to die faster in supplemented cultures, so that the process time is reduced. In order to use DMSO for other cultivation modes an adaption to these culture conditions is necessary, by that the tolerance against DMSO toxicity could be enhanced and longer process times be achieved. The experiments showed that DMSO adapted cells can be used in all cultivation modes. Especially, batch and fed-batch cultivation are of special interest as they performed best in this study. The semi-perfusion cultivation supplemented with DMSO did not yield in an improved performance, as the process terminated earlier and the total amount of produced antibody at the end of the process was comparable to the negative control.

4.4 4-Phenylbutyric acid as a suitable candidate to inhibit growth but maintain specific productivity

The effect of 4-PBA supplementation was also exceptionally high, similar to the DMSO supplementation. In the fractional factorial screening of resolution III a positive impact on the cell specific productivity could be observed, which was not confirmed in the fractional factorial screening of resolution V+. Immediately after supplementation cells stopped growing, but kept producing protein, which yielded in an enhanced cell specific productivity. The combined effect of limiting specific and maximal viable cell concentration but maintained cell specific productivity would be a promising approach for a perfusion culture, in which the cell concentration is normally set by a defined cell bleeding rate, which is often complicated to operate and also results in extensive product loss. By the supplementation of 4-PBA these issues can be circumvented and a desired cell concentration can be set by a defined supplementation of 4-PBA. Another cultivation mode, which can be considered for the application of 4-PBA supplementation is a fed batch cultivation mode. A fed-batch in general is

characterized in that a high cell concentration is reached and thereby high product titers. By the supplementation of 4-PBA the cell concentration can be limited to a desired lower value and the cells can be growth arrested and serve as “plain” expression platforms for recombinant protein production. A limited cell concentration in fed-batch cultivation would also mean a reduced accumulation of toxic by-products, which would also steadily increase with the cell concentration. Furthermore, the application of different feeds would be more economic as the feed components are directly utilized and not invested for biomass formation. Already Johari et al. 2015 reported beneficial aspects of 4-PBA formation to cultivation experiments. The cell specific productivities could be enhanced and the aggregate formation reduced. Furthermore they applied the supplementation strategy to a fed-batch cultivation and could thereby increase the product titer by 5.9-fold compared to their negative control.

4.5 The impact of different buffer systems on the thermal stability of the recombinant produced human IgG_1 PG9 antibody

Differences in melting temperatures of DSC thermograms already show a clear impact of different buffer systems on the thermal stability of the protein. Proteins suspended in the sodium acetate buffer system initiated the first F_{ab} unfolding event at lower temperatures than proteins formulated in the phosphate buffer system. The T_{m1} values of both preparations irrespectively of the DMSO supplementation deviate by approximately 13°C. A similar pattern can be seen for the other T_m values. The obtained T_m values are comparable to Wu et al. 2010, in which they used the MabVII, a human IgG_1, with an additional N-linked glycosylation in H-CDR2 region as in the human IgG_1 PG9 antibody. But after all it can be said that the overall thermal stability was not affected by the supplementation of DMSO, which underlines the potency of chemical chaperones as media additives to improve the process performance but maintain consistent product quality. Measurement of thermostability by DSC was used as the primary method to assess the impact of DMSO on structural integrity of the expressed protein. However, the impact of DMSO on other product quality attributes was not assessed in this project and should be analyzed in more detail in follow-up experiments.

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6 Appendix

6.1 List of Tables

6.2 List of Figures