Exploiting novel strategies for the production of surfactin in Bacillus subtilis cultures

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WHAT WE KNOW IS A DROP,

WHAT WE DON'T KNOW IS AN OCEAN.

Isaac Newton

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Authors' contributions

The theoretical background and the manuscripts included in this thesis have been published previously with the knowledge and approval of the supervisor Prof. Dr.-Ing. Rudolf Hausmann. All further co-authors read and approved the manuscripts.

Chapter 1.2: Theoretical background

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MG performed literature review, created tables and figures and drafted the manuscript. KMH and MH contributed writing the section "Regulation of lipopeptide biosynthesis". RH substantially contributed to conception and design of the chapter. All authors proof-read the manuscript.

Chapter 2.1: 1st publication

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MG planned and executed the experiments, collected data, created the graphs and drafted the manuscript. IK performed part of the experiments and collected and evaluated corresponding data. KMH and JA constructed the strains and contributed to interpretation of the experiments. MH significantly contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

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Chapter 2.2: 2nd publication

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MHo planned and executed the experiments, collected data, created the graphs, and drafted the whole manuscript. DF, LS, and KR performed part of the experiments and collected and evaluated corresponding data. SX under supervision of KH constructed the reporter strains. LL supported in interpretation of results. MHe contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

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MHo planned and executed the experiments, collected data, created the graphs and drafted the whole manuscript. AB and DSFC performed part of the experiments and collected and evaluated corresponding data. KR, PB, CT and PK were involved in strain engineering. MHe supported in interpretation of results. LL significantly contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

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Summary

Biosurfactants, also often termed microbial surfactants, are synthesized by various microorganisms. These surface-active molecules are a promising alternative to petrochemically and oleochemically produced surfactants. Advantageously, biosurfactants are reported to be better biodegradable and less toxic. The cyclic lipopeptide surfactin synthesized by *Bacillus subtilis* displays one interesting biosurfactant. Many studies report on the outstanding physico-chemical characteristics and add on benefits such as antimicrobial properties. Hence, surfactin has the potential to be used in a variety of industrial sectors. Nevertheless, processes ensuring both robustness and high titers are rare, especially as conventional aerobic bioreactor cultivations share one major disadvantage, namely excessive foaming. To approach industrial processes, different methods are applied, which can be categorized in three practices. These are (1) media and process parameter optimization, (2) strain engineering, and (3) investigating novel process strategies. For the latter category, the anaerobic growth by nitrate respiration poses an interesting foam-free alternative. In this sense, the anaerobic cultivation of *B. subtilis* to produce surfactin coupled with the three afore mentioned practices was addressed in this thesis targeting at a foam-free surfactin production process.

In the **1st publication**, the genome reduced strain *B. subtilis* IIG-Bs20-5-1, a derivative of the laboratory strain 168 able to synthesize surfactin, was evaluated with respect to its suitability as surfactin producer at various temperatures under both aerobic and anaerobic conditions. It was hypothesized that a deletion of 10% of the genome, e.g., non-essential genes synthesizing prophages or the antibiotic bacilysin, saves metabolic resources and hence results in increased surfactin titers. Strains *B. subtilis* JABs24, a 168 derivative able to synthesize surfactin but without genome reduction, and the surfactin producer *B. subtilis* DSM 10^T served for comparison. Although strain IIG-Bs20-5-1 was superior regarding specific growth rate μ and biomass yield $Y_{X/S}$, the strain was inferior with respect to surfactin titers, product related yields $Y_{P/S}$ and $Y_{P/X}$, and specific productivity q. Indeed, compared to others in literature described strains, *B. subtilis* JABs24 was emphasized as promising target strain for further process development, reaching high surfactin titers of 1147 mg/L aerobically and 296 mg/L anaerobically as well as exceptionally high product yields $Y_{P/X}$ under anaerobic conditions.

Accordingly, iterative process optimization was hypothesized to improve anaerobically achieved surfactin titers. However, several aspects to consider of anaerobic growth of *B. subtilis* by nitrate respiration were described in the **2nd publication.** Amongst others, increasing ammonium concentrations, resulting from nitrate reduction to ammonium via nitrite, were shown to have no impact on growth of strain JABs24, but surfactin titers and expression of nitrate reductase promoter P_{narG} were reduced. Nitrite was shown to peak within the first hours of cultivation and concentrations up to 10 mmol/L resulted in prolonged lag-phases. Moreover, acetate accumulated drastically during the time course of cultivation independent of glucose availability, thus decreasing the glucose flux into biomass. Acetate additionally influenced both specific growth rate μ and P_{narG} expression negatively. Concluding, the general feasibility of anaerobic fed-batch cultivations to synthesize surfactin was shown, but several aspects must be addressed in future works to make this strategy an equated process with aerobic cultivations.

In the **3rd publication,** a self-inducible surfactin synthesis process was presented where expression of the surfactin operon in *B. subtilis* JABs24 was induced under oxygen limited conditions. The native promoter of the *srfA* operon P_{srfA} was replaced by anaerobically inducible nitrate reductase promoter P_{narG} and nitrite reductase promoter P_{nasD}. Shake flask cultivations with varying oxygen availabilities demonstrated that both P_{narG} and P_{nasD} can serve as auto-inducible promoters. At high oxygen availability, surfactin was not produced in the promoter exchange strains. At lowest oxygen availability, the strain carrying P_{narG} reached lower surfactin titers than the native JABs24 strain, although expression levels of P_{narG} and P_{srfA} were similar. However, strain *B. subtilis* MG14 with P_{srfA}::P_{nasD} reached 1.4-fold higher surfactin titers with 696 mg/L and an exceptionally high $Y_{P/X}$ of 1.007 g/g with overall lower foam levels. Though, bioreactor cultivations have illustrated that the anaerobic induction must be performed slowly as to avoid cell lysis, resulting in so-defined aerobic-anaerobic switch processes. With further appropriate process optimization, a simple and robust surfactin production process with highly reduced or even no foam formation can be achieved employing strain *B. subtilis* MG14.

To sum up, this thesis demonstrated that the anaerobic production of surfactin is indeed feasible and represents a promising approach. However, several challenges were depicted that must be addressed in future works by, e.g., applying strain engineering, making this approach a laborious research topic. On the contrary, the self-inducible process strategy with a strain carrying an anaerobically inducible promoter system combined with aerobic-anaerobic switch-processes has great potential and should be addressed in future research.

Zusammenfassung

Biotenside, oft auch mikrobielle Tenside genannt, werden von verschiedenen Mikroorganismen synthetisiert und stellen eine Alternative zu petrochemisch und oleochemisch hergestellten oberflächenaktiven Molekülen dar. Biotenside weisen eine bessere biologische Abbaubarkeit auf und besitzen eine geringere Toxizität. Ein interessantes Biotensid ist das von *Bacillus subtilis* gebildete zyklische Lipopeptid Surfactin. Dieses besitzt vielversprechende physikalisch-chemische Eigenschaften und wirkt zudem antimikrobiell. Daraus resultieren zahlreiche Anwendungsmöglichkeiten im Lebensmittel-, Kosmetik-, Pharma-, Agrar- und Umweltbereich. Dennoch gibt es wenige in der Literatur beschriebene stabile Prozesse, in welchen hohe Surfactinkonzentrationen erreicht werden. Zudem sind konventionelle aerobe Kultivierungen von einer starken Schaumbildung geprägt. Optimierung von Medien- und Prozessparametern, Stammentwicklung und Untersuchung neuartiger Prozessstrategien stellen drei Ansätze dar, um industriell umsetzbare und lukrative Prozesse zu entwickeln. Die anaerobe Kultivierung von *B. subtilis* mittels Nitratatmung ist hierbei eine interessante schaumfreie Strategie, welche im Rahmen dieser Thesis die Grundlage bildete und weiter untersucht wurde.

In der **1. Publikation** wurde *B. subtilis* IIG-Bs20-5-1, ein Stamm mit einer Genomreduzierung von 10 %, hinsichtlich seiner Eignung als Surfactinproduzent untersucht. Der nicht genomreduzierte Stamm *B. subtilis* JABs24 sowie *B. subtilis* DSM 10^T dienten als Referenzen. Es wurden aerobe und anaerobe Kultivierungen bei verschiedenen Temperaturen durchgeführt. Es wurde vermutet, dass eine Deletion von nicht-essenziellen Genen metabolische Ressourcen einspart und zu höheren Surfactinkonzentrationen führt. Der Stamm IIG-Bs20-5-1 war sowohl bei der Wachstumsrate μ als auch in der Biomasse-Ausbeute $Y_{X/S}$ überlegen, wies aber geringere Surfactinkonzentrationen, produktbezogene Ausbeuten $Y_{P/S}$ und $Y_{P/X}$, und spezifische Produktivitäten q auf. *B. subtilis* JABs24 wurde allerdings aufgrund seiner hohen Surfactinkonzentrationen, 1147 mg/L aerob und 296 mg/L anaerob, und der unter anaeroben Bedingungen vergleichsweise hohen Produktausbeuten $Y_{P/X}$ als vielversprechender Produktionsstamm für weitere Entwicklungen identifiziert. Entsprechend sollten weitere Optimierungen auf Basis des anaeroben Wachstums mit *B. subtilis* JABs24 folgen, um diese Strategie zu aeroben Prozessen konkurrenzfähig zu machen.

Das anaerobe Wachstum mittels Nitratatmung hat allerdings einige Nachteile gegenüber aeroben Prozessen, welche in der **2. Publikation** beschrieben wurden. Es konnte gezeigt werden, dass durch die Nitratatmung steigende Ammoniumkonzentrationen keinen Einfluss auf das Wachstum von *B. subtilis* JABs24 haben, allerdings zu verringerten Expressionen des Promotors P_{narG}, welcher für die Nitratatmung essenziell ist, und Surfactinkonzentrationen führten. Nitrit reicherte sich kurzzeitig an und bis zu 10 mmol/L führten zu verlängerten Lag-Phasen. Unabhängig von den Glucoseverfügbarkeiten akkumulierte Acetat kontinuierlich. Es zeigte sich, dass steigende Acetatkonzentrationen sowohl die Wachstumsrate μ als auch die Promotorexpression von P_{narG} reduzierten. Die anaerobe Kultivierung ist somit eine interessante Prozessführung, jedoch müssen verschiedene Aspekte in zukünftigen Arbeiten untersucht werden, um diesen Prozess zu aeroben Kultivierungen konkurrenzfähig zu machen.

In der **3. Publikation** wurde ein Prozess beschrieben, bei welchem die Surfactinsynthese in *B. subtilis* JABs24 durch sauerstofflimitierte Bedingungen induziert wird. Der native Promotor des *srfA*-Operons P_{srfA} wurde durch P_{narG} und P_{nasD} ersetzt, welche unter anaeroben Bedingungen induziert werden. Kultivierungen mit variierendem Sauerstoffgehalt zeigten, dass P_{narG} und P_{nasD} als autoinduzierbare Promotoren verwendet werden können. Bei hoher Sauerstoffverfügbarkeit wurde in den Promotoraustauschstämmen kein Surfactin detektiert. Bei niedrigsten Sauerstoffgehalten erreichte der Stamm mit P_{srfA}::P_{narG} Promotoraustausch geringere Surfactintiter als der native JABs24 Stamm, obwohl die Expressionslevel von P_{narG} und P_{srfA} ähnlich waren. *B. subtilis* MG14 mit P_{srfA}::P_{nasD} erreichte dagegen 1,4-fach höhere Surfactinkonzentrationen von 696 mg/L und einen vergleichsweise hohen $Y_{P/X}$-Wert von 1,007 g/g. Bioreaktorkultivierungen zeigten zudem, dass die Induktion durch Sauerstoffreduktion über einen größeren Zeitraum durchgeführt werden muss, um eine Zelllyse zu vermeiden, was zu sogenannten aerob-anaeroben Switch-Prozessen führte. Mit weiteren Prozessoptimierungen kann unter Verwendung von *B. subtilis* MG14 ein einfacher und robuster Prozess mit stark reduzierter oder gar ohne Schaumbildung ermöglicht werden.

Diese Arbeit hat gezeigt, dass die anaerobe Synthese von Surfactin realisierbar ist und einen vielversprechenden Ansatz darstellt. Es wurden jedoch mehrere limitierende Faktoren aufgezeigt, die z. B. mittels Stammentwicklung in zukünftigen Arbeiten untersucht werden müssen. Aerob-anaerobe Switch-Prozesse in Kombination mit einem selbstinduzierbarem P_{srfA}::P_{nasD} kodierendem Promotoraustauschstamm stellen demgegenüber einen aussichtsreichen Ansatz dar und sollten in zukünftigen Arbeiten weiter untersucht werden.

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Chapter 1 Introduction

1.1 General introduction

Surfactants are an important class of chemical compounds that can be found in our everyday lives. Amongst others, they are used in detergents, personal care products, food systems, medicines, automotive fluids, paints or even in firefighting foams (Myers 2006, Schramm et al. 2003). The numerous applications are also reflected in the estimated increase in the Global Surfactants Market by 5.8% by 2025 to USD 40 billion from USD 30.75 billion in 2019 (marketdataforecast.com 2021).

Structurally, these molecules consist of a hydrophobic and hydrophilic moiety, resulting in an amphiphilic character and hence surface-active properties. Chemically produced surfactants derived from crude oil (petrochemical surfactants) or vegetable / animal oil (oleochemical surfactants) account for the largest volume of the global surfactant market. However, especially petrochemically derived surfactants are under strong scrutiny due to their production based on limited fossil resources, impact on the environment such as reduced biodegradability, and existing toxicity. In addition, also the trend towards sustainability and “green label” drives the market of surfactants towards more environmentally friendly alternatives (marketdataforecast.com 2021).

Accordingly, research in the field of natural and bio-based surfactants is increasing steadily. In this field, microbial derived surfactants, in the following referred to as biosurfactants, are a promising alternative due to their biotechnological production on renewable resources at moderate pH values and temperatures (Banat et al. 2012). Moreover, biosurfactants share characteristics such as very good surface and interfacial activity, high tolerance towards changes in pH, temperature and ionic strength, biodegradability and being low or even non-toxic (Santos et al. 2016). Besides, many biosurfactants have antimicrobial activities which furthermore expands their field of applications. Biosurfactants can be classified according to their structure into lipopeptides, glycolipids, lipopolysaccharides, fatty acids and phospholipids (Sobrinho et al. 2013, Santos et al. 2016). In the field of lipopeptides, especially those synthesized by *Bacillus* species are declared as next generation biosurfactants with surfactin being the most researched and promising (Coutte et al. 2017; Henkel et al. 2017).

1.2 Lipopeptide biosurfactants from *Bacillus* species

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CHAPTER

6

Lipopeptide Biosurfactants From *Bacillus* Species

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6.1 INTRODUCTION

Bacillus species, especially *Bacillus subtilis*, are important microorganisms in industrial biotechnology due to their natural secretion of diverse proteins (van Dijl and Hecker, 2013). Among others, *B. subtilis* is used to produce enzymes such as proteases, cellulases, amylases, and laccases (Singh et al., 2017). The natural secretion of these products into the medium offers advantages in their respective purification procedures (van Dijl and Hecker, 2013). In addition, as a well-studied or even the best-studied gram-positive bacterium, genetic manipulation of *B. subtilis* is well established, and therefore, it is a common host for large-scale production (Harwood, 1992). For instance, genetically engineered *B. subtilis* is used for the industrial production of riboflavin, an essential vitamin for growth and reproduction in both humans and animals (Schwechheimer et al., 2016). Furthermore, *B. subtilis* is a convenient industrial strain due to its excellent cultivation performances, high product titers, and the lack of toxic by-product formations (van Dijl and Hecker, 2013).

Next to the already implemented industrial processes, great interest in *Bacillus* spp. has grown due to their ability to synthesize and secrete cyclic lipopeptides. *Bacillus* spp. produce five families of lipopeptides, with surfactin as the most relevant, followed by iturin and fengycin (Coutte et al., 2017). Each family of lipopeptides consists of different congeners, and although all lipopeptide families possess structural similarities, their chemical and physicochemical characteristics vary both inter- and intrafamily (Beltran-Gracia et al., 2017). The broad spectrum of these molecules and their characteristics ranging from surface-active properties to antimicrobial activities make them exceptional candidates for a variety of applications. However, in comparison with well-established industrial processes employing *Bacillus* strains to produce, for example, enzymes, the yields obtained for lipopeptides are currently

Biobased Surfactants
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comparably low. Hence, although often claimed as promising biobased surfactant, a stable and reliable process with high yields has not been realized so far. This chapter will therefore primarily give a firm background on the respective lipopeptide families, their synthesis by nonribosomal peptide synthetases (NRPS), and the underlying quorum-sensing mechanisms. Furthermore, the physicochemical properties and the concomitant potential applications of these promising biosurfactants will be given. In addition, this chapter evaluates current approaches toward large-scale production of lipopeptides that includes both up- and downstream processing. In the end, several challenges that must be considered in future research will be presented.

6.2 TYPES AND CLASSIFICATION OF LIPOPEPTIDES

Cyclic lipopeptides are chemical compounds that generally possess an amphiphilic structure. The hydrophilic part, which is formed by an oligopeptide ring, is connected to a fatty acid chain. The huge structural variety of these compounds is based on the variation of the oligopeptide composition, the sequence of the polar head, or the diversity of the fatty acid tail (Mnif and Ghribi, 2015b). The fatty acid can be either linear or branched and varies in the length or degree of oxidation. Furthermore, lipopeptides are outstanding with respect to their structural diversity by often containing D-amino acids and iso- or anteiso-hydroxy fatty acids.

Both *Bacillus* spp. and *Pseudomonas* spp. are known to produce the currently most interesting lipopeptides; however, also yeast and fungi can synthesize cyclic lipopeptides. Based on a survey in the database NORINE, Coutte et al. (2017) counted 263 different lipopeptides synthesized by 11 microbial genera. Within these genera, *Pseudomonas* spp., *Bacillus* spp., and *Streptomyces* spp. represent the most abundant lipopeptide producers with 78, 98, and 40 different lipopeptides, classified in 11, 5, and 6 lipopeptide families, respectively. An overview of these lipopeptide families and the subfamilies and the number of known lipopeptides within these subfamilies is given in Table 6.1. The data shown are based on a research in the database NORINE with the annotation search terms "lipopeptide" for "category" and the respective organism for "organism name" (Caboche et al., 2008; Flissi et al., 2016). The table furthermore illustrates the structure of the lipopeptides and is divided in cyclic, partial cyclic, and linear. In comparison with other lipopeptide producers, the amounts synthesized by natural *Bacillus* spp. are generally higher, and thus, these lipopeptides are more commonly in focus of research (Coutte et al., 2017). However, the non-*Bacillus* lipopeptides should also not be disregarded as they expand the structural diversity and potential application areas of *Bacillus* lipopeptides.

6.2.1 Excursion to Non-*Bacillus* Lipopeptides

An overview of structures, characteristics, and biosynthesis of *Pseudomonas* lipopeptides is given by Raaijmakers et al. (2010). The *Pseudomonas* lipopeptides were first divided into four groups, namely, viscosin, amphisin, tolaasin, and syringomycin. As shown in Table 6.1, up to date, 11 families of *Pseudomonas* lipopeptides were described. A purified viscosin was reported to have excellent surface-active properties since it had a low critical micelle concentration (CMC) of 54 mg/L, which corresponds to 0.048 mmol/L, and reduced the surface tension

TABLE 6.1 Overview of Lipopeptide Families, Subfamilies, and Number of Known Lipopeptides Within These Subfamilies

Organism Name	Lipopeptide Families	Number of LP Within Family	Subfamilies	Peptide Structure
Pseudomonas	Orfamide	3	Orfamide	Cyclic
	Amphisin	9	Arthrofactin	Cyclic
			Tensin	
	Corrugatin	1	Corrugatin	Linear
	Plusbacin[a]	8	Plusbacin	Cyclic
	Putisolvin	3	Putisolvin	Partial cyclic
	Pyoverdin[b]	1	Pyoverdin	Linear
	Syringafactin	6	Syringafactin	Linear
	Syringomycin	11	Syringomycin	Partial cyclic
			Pseudomycin	
	Syringopeptin	11	Syringopeptin	Partial cyclic
	Tolaasin	11	Tolaasin	Partial cyclic
			Corpeptin	
	Viscosin	14	Viscosin	Partial cyclic
			Massetolide	
Bacillus[c]	Kurstakin	7	Kurstakin	Partial cyclic
	Fengycin	3	Fengycin/plipastatin	Partial cyclic
	Iturin	32	Iturin	Cyclic
			Bacillomycin	
			Mycosubtilin	
	Polymyxin	19	Polymyxin	Partial cyclic
	Surfactin	37	Surfactin	Cyclic
			Lichenysin	
Streptomyces	A54145	8	A54145	Cyclic
	Ca-dependent antibiotic	10	CDA	Cyclic
	Arylomycin[a]	12	Arylomycin	Partial cyclic
	Daptomycin	4	Daptomycin	Partial cyclic
			A21978	
	Enduracidin	2	Enduracidin	Partial cyclic
	Friulimicin	4	Amphomycin	Partial cyclic

Data shown are based on a search in the database NORINE with the annotation search terms "lipopeptide" for "category" and respective organism for "organism name" (Caboche et al., 2008; Flissi et al., 2016).
[a] *Putative NRPS product.*
[b] *Only peptide structure, no fatty acid moiety, but NRPS product.*
[c] *Locillomycin as possible further family of LP.*

at the air/water interface to 28 mN/m (Saini et al., 2008). Ma et al. (2017) evaluated the effectiveness of a new class of *Pseudomonas* lipopeptides, namely, orfamide-like lipopeptides, synthesized by *P. protegens*, against the fungus *Cochliobolus miyabeanus*. This fungus causes brown spot disease on rice, and the lipopeptide was reported to trigger induced systemic resistance in rice plants and consequently was described as a promising biological agent in the agricultural area.

The antibiotics daptomycin and colistin are lipopeptides already approved to be used in medicine. Daptomycin, for example, synthesized by *S. roseosporus*, showed potent antibacterial activities against many gram-positive organisms (Steenbergen et al., 2005).

Although less studied, also several fungi can synthesize lipopeptides nonribosomally. Echinocandins are representative fungal lipopeptides produced by *Aspergillus nidulans* and were described to have a high potential of being implemented in antifungal drugs (Hüttel et al., 2016).

In the recent past, interest in new natural isolates and their respective production of secondary metabolites has increased. Janek et al. (2010), for example, extracted a cyclic lipopeptide from *P. fluorescens* BD5 and named it pseudofactin. Further techniques such as oil displacement test and drop collapse method were employed as analytic tools to screen for lipopeptides. With these techniques, new lipopeptides such as pontifactin from *Pontibacter korlensis* SBK-47 (Balan et al., 2016) or cystargamide from *Kitasatospora cystarginea* (Gill et al., 2014) were described. Kiran et al. (2017) identified a lipopeptide produced by *Nesterenkonia* sp. MSA31 and showed its potential application in the food industry. In their study, egg yolk and baking powder in a muffin recipe were replaced by different concentrations of this lipopeptide, and the texture was evaluated. Another lipopeptide interesting to the food industry was investigated by Li et al. (2018). The lipopeptide paenibacterin synthesized by *Paenibacillus thiaminolyticus* was evaluated to be able to control *Listeria monocytogenes*, a foodborne pathogen. To be more specific, paenibacterin downregulated biofilm formation in *L. monocytogenes*, combatting a major initiator of pathogenicity.

6.2.2 Bacillus Lipopeptides—Discovery and Structural Diversity

Among all known microbial lipopeptides, lipopeptides produced by *Bacillus* species are the most studied. So far, *Bacillus* spp. are known to produce five different families of lipopeptides, among Fig. 6.1 displays the research articles published dealing with either surfactin, iturin, or fengycin alone or all three lipopeptides together, highlighting the increasing interest in the past decade.

As illustrated in Fig. 6.1, research on the most studied *Bacillus* lipopeptide surfactin started in 1968, and in 2015, almost 100 articles were published dealing with this biosurfactant. For iturin and fengycin, research started 10 and 20 years later, respectively, and the number of articles counted was almost 40 for each of them in 2015. Mostly reported and studied producers of lipopeptides belong to the species *B. subtilis*. But also other strains such as *B. circulans* (Hsieh et al., 2008; Sivapathasekaran et al., 2010), *B. licheniformis* (Li et al., 2008), *B. amyloliquefaciens* (Borriss et al., 2011; Hsieh et al., 2008), and *B. methylotrophicus* (Jemil et al., 2017) are able to synthesize either one or more of the lipopeptides belonging to the surfactin, iturin, or fengycin family. As illustrated in Table 6.1, based on the data search, polymyxins were displayed as another family of *Bacillus* lipopeptides. Polymyxins were discovered in 1947

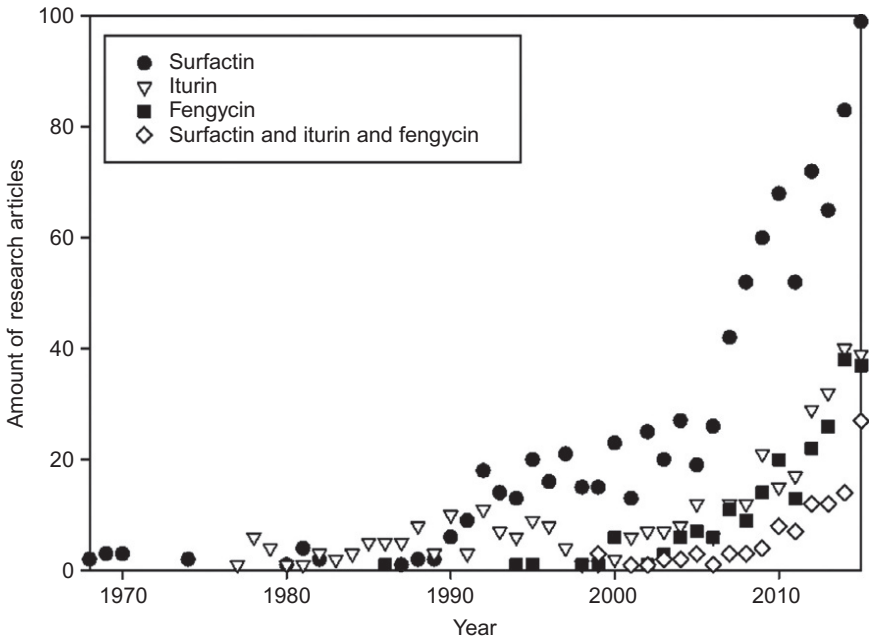

FIG. 6.1 Sum of articles per year from 1968 to 2016 taken from the peer-reviewed literature database Scopus (www.scopus.com) with respect to the keywords "surfactin," "iturin," "fengycin," and "surfactin AND iturin AND fengycin." Results limited to "article" and keywords mentioned in "article title, abstract, and keywords."

and were used as antibiotics in hospitals between the 1950s and 1970s. Due to toxicological concerns, the application was reduced. Nowadays, polymyxins are again used in therapeutic applications for patients where other antibiotic treatments failed due to an increasing antibiotic resistance observed (Rabanal and Cajal, 2017). However, a study conducted in 1991 splits the order Bacillales into several families, creating among others the genus *Paenibacillus*. The strain *B. polymyxia* was afterward assigned as *P. polymyxa* (Grady et al., 2016). As this chapter focuses on lipopeptides synthesized by the genus *Bacillus*, polymyxins are not further described. Interestingly, in the recent years, two new families of *Bacillus* lipopeptides were discovered, namely, the kurstakin (Hathout et al., 2000), also displayed in Table 6.1, and the locillomycin (Luo et al., 2015) families. The next section will individually describe the respective lipopeptide families. Chemical structures of representatives of the three main families are given in Fig. 6.2. A summary of further different lipopeptide variants is given by Cochrane and Vederas (2016) and Mnif and Ghribi (2015b).

6.2.2.1 The Surfactin Family—Surfactin, Pumilacidin, and Lichenysin

In 1968, Arima et al. (1968) described surfactin, whose chemical structure was assigned as a lipopeptide consisting of L-aspartic acid, L-glutamic acid, L-valine, and two L-leucine and D-leucine residues in its peptide ring. Kakinuma et al. (1969a,b) published a more detailed structure of the first surfactin structure discovered 1 year later. This surfactin congener was determined to possess a 3-hydroxy-13-methyltetradecanoic (C15) fatty acid as lipid residue, which is connected to the first amino acid of the heptapeptide ring, namely, L-Glu.

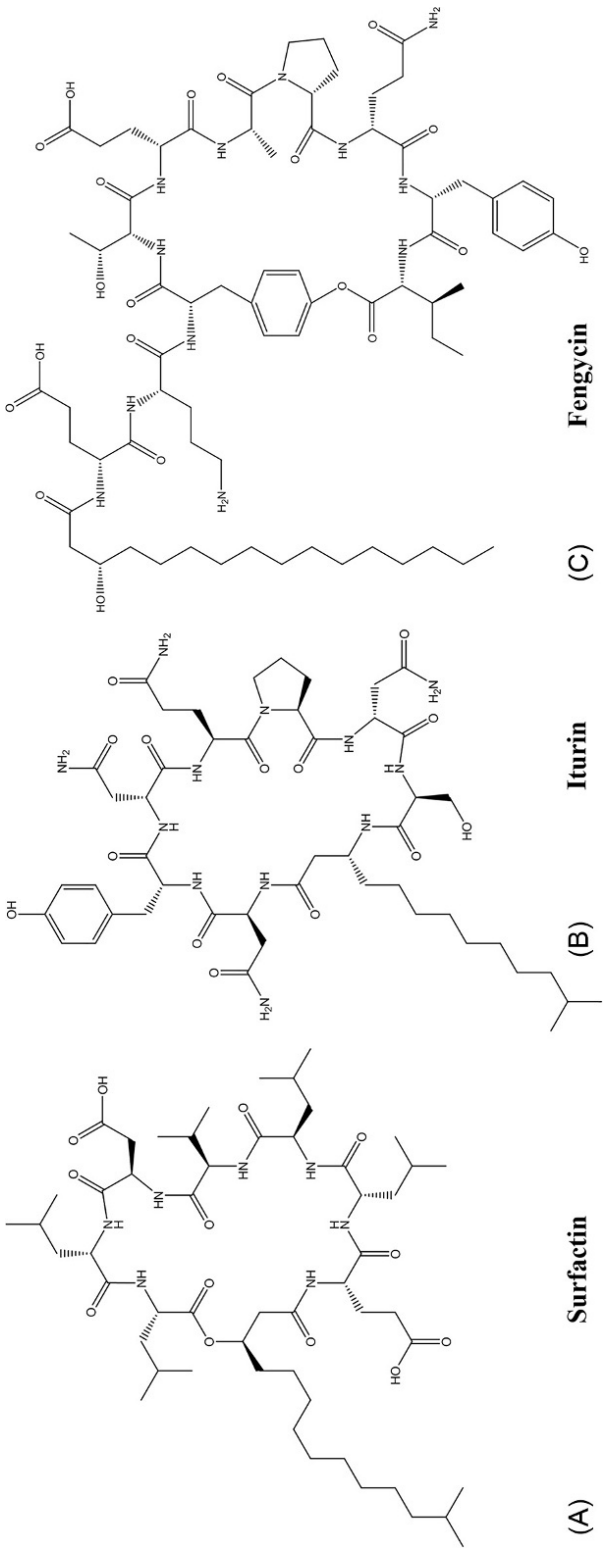

FIG. 6.2 Chemical structure of representative congeners of (A) surfactin, (B) iturin, and (C) fengycin lipopeptides.

The peptide ring itself is composed of the amino acids in the sequence L-Glu, L-Leu, D-Leu, L-Val, L-Asp, D-Leu, and L-Leu. The first and last amino acids L-Glu and L-Leu form the lactone ring by an ester linkage. A main congener of surfactin is given in Fig. 6.2A. Up to now, more than 30 different surfactin congeners were reported that differ either in their amino acid composition or in their fatty acid residues. However, the chiral sequence for all surfactin molecules remains identical with LLDLLDL (Cochrane and Vederas, 2016).

Pumilacidins were first discovered in 1990 in the strain *B. pumilus* with the variants A–F (Naruse et al., 1990). In these variants, the amino acid at position 7 is substituted by either L-Val or L-Ile. Peypoux et al. (1991) discovered a surfactin variant with L-Val at position 7 in the strain *B. subtilis* S499. Due to reported structural similarities, pumilacidin variants can be assigned to the surfactin family. Lichenysins were discovered in 1995 and named after their strain of origin, namely, *B. licheniformis* (Yakimov et al., 1995). Structurally, lichenysins were reported to be closely related to surfactin, with the main difference being the substitution of L-glutamic acid with L-glutamine at position 1.

6.2.2.2 The Iturin Family—Iturin, Bacillomycin, and Mycosubtilin

The discovery of iturin dates back to 1952, and the structure of iturin A was elucidated in 1978 by Peypoux et al. (1978). Similar to surfactin, iturins are cyclic heptapeptides. The amino acid sequence of the iturin A molecule is L-Asn, D-Tyr, D-Asn, L-Gln, L-Pro, D-Asn, and L-Ser. The lipid moiety of the first reported iturin A molecule was reported to be a mixture of 3-amino-12-methyltridecanoic acid and 3-amino-12-methyltetradecanoic acid (Peypoux et al., 1978). Similar to surfactin, the fatty acid is attached to the first amino acid, and the cyclization occurs between the first and the last amino acid. However, in contrast to surfactin, the cyclization between the first and the last amino acid occurs by an amide bond as depicted in Fig. 6.2B. In addition to iturin, also mycosubtilin and bacillomycin were assigned to this family of lipopeptides due to structural similarities.

6.2.2.3 The Fengycin Family—Fengycin and Plipastatin

While surfactin and iturin show structural similarities, fengycins, as illustrated in Fig. 6.2C, differ more. They are decapeptides, and the cyclization occurs between a phenol side chain of the third amino acid D-Tyr and the C-terminus of the amino acid at position 10 (Cochrane and Vederas, 2016). Hence, fengycin lipopeptides can be considered as partial cyclic. Fengycin A and B were first mentioned in 1986. Vanittanakom and Loeffler (1986) explored these molecules in the strain *B. subtilis* F-29-3 as antifungal lipopeptides. The amino acid sequence of fengycin A was determined as L-Glu, D-Orn, D-Tyr, D-Thr, L-Glu, D-Ala, L-Pro, L-Gln, L-Tyr, and L-Ile. For fengycin B, D-Ala at position 6 is replaced by D-Val (Schneider et al., 1999).

Another lipopeptide, closely related to fengycin, was named plipastatin. While fengycin A is composed of D-Tyr at position 3 and L-Tyr at position 9, plipastatin A constitutes of L-Tyr and D-Tyr at positions 3 and 9, respectively (Cochrane and Vederas, 2016). However, up to now, fengycin and plipastatins are also often used as synonyms.

6.2.2.4 The Kurstakin and Locillomycin Families

Lately, two novel lipopeptide families were discovered, namely, the kurstakin family (Hathout et al., 2000) and the locillomycin family (Luo et al., 2015). Hathout et al. (2000) investigated the structures of three representatives of the kurstakin family, isolated from a culture

of the strain *B. thuringiensis* subs. *Kustaki* HD-1. Kurstakins are partial cyclic heptapeptides. The fatty acid with varying chain length is connected via the first amino acid. The residues of kurstakin are Thr, Gly, Ala, Ser, His, Gln, and Gln with the cyclization between the fourth and seventh amino acid (Cochrane and Vederas, 2016). The presence of histidine is rare in the structural diversity of lipopeptides explored so far (Hathout et al., 2000).

Locillomycins were extracted from a culture of the strain *B. subtilis* 916. These molecules are nonapeptides with the amino acid sequence of Thr, Gln, Asp, Gly, Asn, Asp, Gly, Tyr, and Val (Luo et al., 2015). For both kurstakin and locillomycin family lipopeptides, the complete structural identification, for example, with respect to the presence of D- or L-amino acids, still needs to be further investigated.

6.3 LIPOPEPTIDE BIOSYNTHESIS

6.3.1 Nonribosomal Peptide Synthetases

Lipopeptides from *Bacillus* species are encoded by nonribosomal peptide synthetases (NRPSs) or hybrid NRPSs. The synthesis of peptide structures by NRPSs has several unique features. For example, also noncanonical amino acids can be incorporated into peptides synthesized by NRPSs, which allows for a high structural diversity. However, NRPSs catalyze very specific reactions, and modifications with genetic engineering methods are currently not established compared with ribosomally synthesized peptides. The NRPSs encoding for the lipopeptides surfactin, iturin, and fengycin share some specific features. NRPSs are large multienzyme complexes organized in modules that possess iterative functions (Hamdache et al., 2013). Each of these multidomain modules is responsible for a reaction cycle and is composed of different domains such as adenylation domain (A); peptidyl carrier domain (PCP), also often referred to as thiolation domain; and condensation domain (C). The operons encoding for surfactin, iturin A, and fengycin, with the respective arrangement of modules and domains, are illustrated in Fig. 6.3.

The adenylation domain selects the amino acid and activates it to an aminoacyl adenylate by the consumption of adenosine triphosphate (ATP). The PCP domain is a transport unit where the amino acid adenylate is tethered by a thioester bond and binds to a conserved serine residue on the carrier protein domain. The condensation domain forms a new peptide bond between two adjacent modules and their respective aminoacyl substitutes (Finking and Marahiel, 2004; Hamdache et al., 2013; Strieker et al., 2010). While a linear arrangement of these domains is necessary to ensure for elongation of the oligopeptide structures, several modules hold further modifying domains, for example, for epimerization, hydroxylation, methylation, or cyclization (Finking and Marahiel, 2004). The last module for both surfactin, iturin, and fengycin synthetases comprises another domain, the thioesterase domain (TE). This domain initiates product release by cleaving the last thioester bond and cyclization of the oligopeptide chain (Finking and Marahiel, 2004; Wu et al., 2017). The following sections give a more detailed overview of the synthesis of the lipopeptides surfactin, fengycin, and iturin.

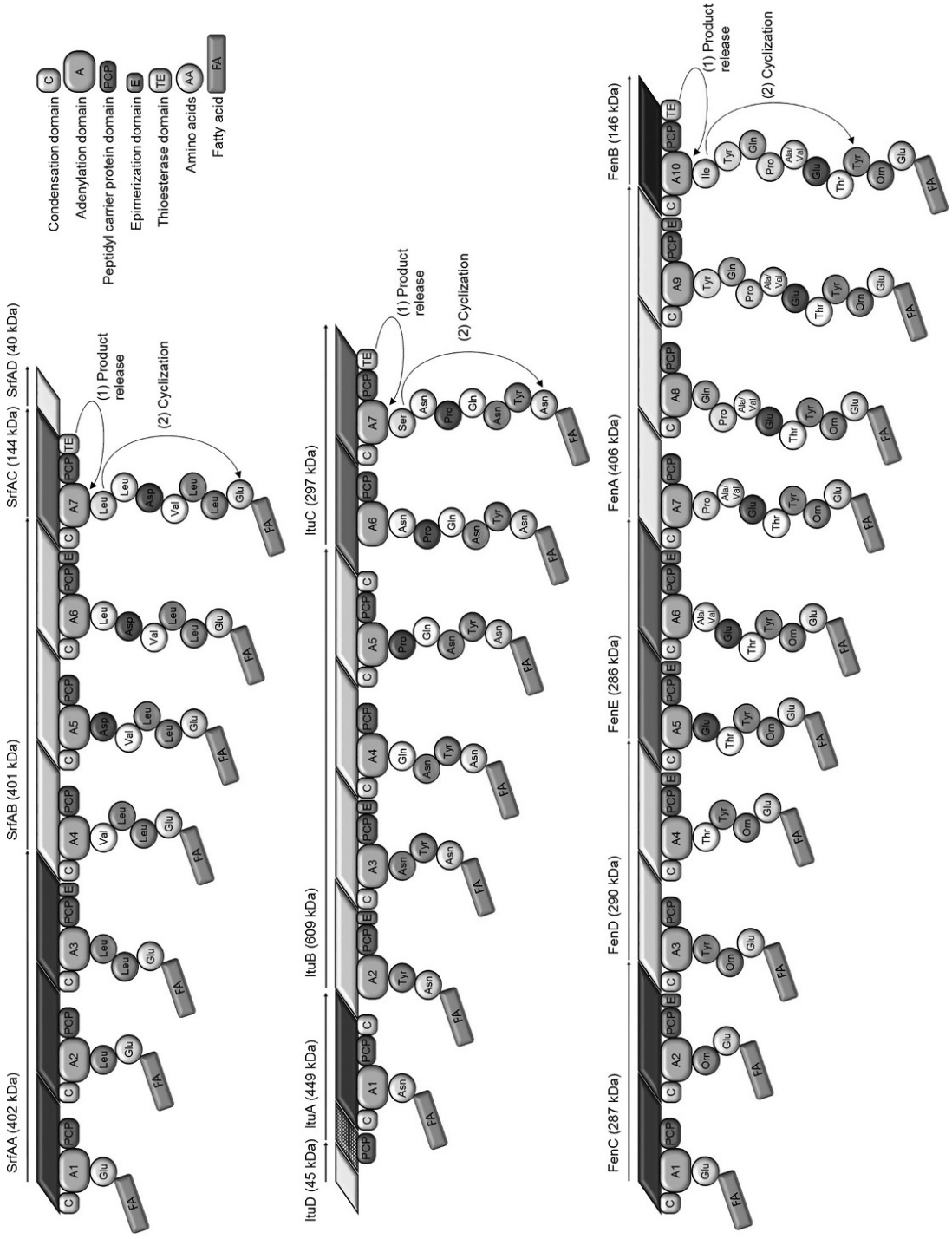

FIG. 6.3 Schematic overview of the operons encoding for the nonribosomal peptide synthetases for the lipopeptides surfactin, iturin, and fengycin. The modules with the respective domains and the amino acids incorporated into the growing peptide chain by each module are illustrated.

6.3.2 Surfactin Synthesis

The NRPS responsible for surfactin synthesis is encoded by *srfA*, which consists of four open reading frames (ORF). The first three ORFs *srfAA* (402 kDa), *srfAB* (401 kDa), and *srfAC* (144 kDa) encode the NRPS subunits that elongate the oligopeptide chain (Kraas et al., 2010). As illustrated in Fig. 6.3, each of these subunits consists of modules with condensation, adenylation, and peptidyl carrier domains. The last modules of SrfAA and SrfAB additionally contain an epimerization domain that converts L-Leu to D-Leu. However, the subunits are only able to form a mature complex after posttranslational modification of the PCP domains. This is performed by a phosphopantetheinyl transferase encoded by the *sfp* gene, which is crucial for the surfactin biosynthesis. The *sfp* gene is located 4 kb downstream of the *srfA* operon and is responsible for the posttranslational phosphopantetheinylation of the PCP domains to convert them from the inactive apoform into the active holoform (Das et al., 2008; Reuter et al., 1999; Roongsawang et al., 2010; Wu et al., 2017). Sfp phosphopantetheinylates the PCP domains by transferring the phosphopantetheine group from CoA and thereby introduces a reactive thiol terminus to each PCP domain. This enables both to load the amino acid on the domain and to form the peptide bond (Quadri et al., 1998). The forth ORF, *srfAD* (40 kDa), which is not directly involved in the elongation process of the peptide chain, is reported to synthesize an external type II thioesterase, with the function of recycling misprimed peptidyl carrier protein (PCP) domains (Koglin et al., 2008).

Surfactin synthesis begins with a CoA-activated fatty acid. However, the NRPS cluster itself does not contain an acyl-CoA-ligase. Kraas et al. (2010) reported that two out of four identified CoA ligases, namely, LcfA and YhfL, play a major role in fatty acid activation. Interestingly, a mutant lacking all four identified ligases still produced surfactin, indicating the presence of further pathways that provide the activated fatty acid. The activated fatty acid is recognized by the C-domain of the first module of the *srfAA* subunit, which catalyzes the acylation with the amino group of the first amino acid to be incorporated, namely, L-Glu (Kraas et al., 2010; Steller et al., 2004; Wu et al., 2017). This amino acid itself was activated through adenylation by the adenylation domain within its respective module. Next, the peptide chain is elongated by the addition of two L-Leu within the SrfAA subunit. The PCP domains thereby allow the traveling of activated amino acids between the catalytic centers, and the condensation domain catalyzes the reaction of two neighboring amino acids and forms a peptide bond. The last module of SrfAA additionally contains an epimerization domain, which converts the second L-Leu to D-Leu. Afterward, three amino acids, namely, L-Val, L-Asp, and L-Leu, are incorporated in the nascent oligopeptide within the SrfAB subunit that is similar to SrfAA. Here, L-Leu is also converted to D-Leu by an epimerization domain located in the last module of SrfAB. The last subunit SrfAC is composed of the L-Leu peptide chain elongation domains and a thioesterase domain. This type I thioesterase catalyzes the reaction between the first and the last amino acid of the peptide chain by forming a macrolactone (Koglin et al., 2008). Release of the surfactin molecule from the enzyme complex then finalizes the biosynthesis of surfactin. Since no active transporter has been identified so far, Tsuge et al. (2001) postulated a passive diffusion of surfactin into the extracellular milieu.

6.3.3 Fengycin Synthesis

As illustrated in Fig. 6.3, the biosynthesis of fengycin is encoded by five ORFs and is also initiated by the attachment of a β-hydroxy fatty acid to the first amino acid of the first module within FenC. The other ORFs encoding for fengycin are, in the following order, *fenC* (*ppsA*) (287 kDa), *fenD* (*ppsB*) (290 kDa), *fenE* (*ppsC*) (286 kDa), *fenA* (*ppsD*) (406 kDa), and *fenB* (*ppsE*) (146 kDa) (Tapi et al., 2010; Wu et al., 2007). The general organization of the functional domains of the respective modules is similar to the surfactin NRPS, with the linear arrangement of the peptidyl carrier protein domain, adenylation domain, condensation domain, and several epimerization domains. Each of FenC, FenD, and FenE adds two amino acids to the oligopeptide chain, whereas FenA and FenB add three and one amino acid, respectively (Ongena and Jacques, 2008; Wu et al., 2007). As reported for surfactin, the release of the molecule is initiated after macrocyclization, which is catalyzed by the TE-domain located in FenB. Fengycin lipopeptides are partial cyclic, and the lactone bond formation occurs between the third amino acid L-Tyr and the last amino acid L-Ile (Samel et al., 2006).

Similar to surfactin, *sfp* is required for the biosynthesis of fengycin. As a result, a frameshift mutation in the *sfp* ORF hampered the biosynthesis of both surfactin and fengycin in *B. subtilis* 168 although it carries both the surfactin and fengycin biosynthesis operons (Coutte et al., 2010a).

6.3.4 Iturin Synthesis

The biosynthesis of lipopeptides belonging to the iturin family differs from both surfactin and fengycin lipopeptides. Hitherto, Duitman et al. (1999) elucidated the structural arrangement of the mycosubtilin synthetase; the iturin A operon was investigated by Tsuge et al. (2001); and Moyne (2004) elucidated the structural arrangement of the operon encoding for bacillomycin D. All biosynthesis operons consist of four ORFs, namely, *ituD*, *ituA*, *ituB*, and *ituC* for the iturin A operon and *fenF*, *mycA*, *mycB*, and *mycC* for mycosubtilin. Similar to the surfactin operon, *ituABC*/*mycABC* encodes the NRPS. Unlike the synthesis of surfactin and fengycin, *ituA*/*mycA* combines several functions. *ituA*/*mycA* are hybrids of a polyketide synthase and NRPS and have an additional PCP and C domain (Aron et al., 2005). The *ituA*/*mycA* subunits possess several functions. Duitman et al. (1999) proposed a model that illustrates the incorporation of the fatty acid into the assembly line of mycosubtilin. First, in a reaction dependent on ATP, CoA is coupled to a long-chain fatty acid by the acyl-CoA-ligase domain. This activated fatty acid is transferred to the 4-phosphopantetheine cofactor of the first PCP domain. A malonyl-CoA transacylase, which is encoded by *fenF*, catalyzes the reaction of a malonyl-CoA to the second PCP domain. In the next step, the malonyl and acyl thioester condensate. This reaction is catalyzed by the β-ketoacyl synthetase domain and results in a β-ketoacyl thioester. This thioester is then converted into a β-amino fatty acid. A condensation domain further catalyzes the transfer to a PCP domain prior to coupling to asparagine by another condensation domain. The subsequent elongation process is similar to the biosynthesis of surfactin and fengycin. However, there are differences in the arrangement of the subunit domains. For example, while both *srfB*/*fenB* and *srfC*/fenC start at the condensation domain, this domain is integrated in the respective afore subunit for *ituB*/*mycB* and *ituC*/*mycC* (Tsuge et al., 2001).

6.4 REGULATION OF LIPOPEPTIDE BIOSYNTHESIS

The surfactin synthetase is encoded by the tetracistronic *srfA* operon (*srfAA*, *srfAB*, *srfAC*, and *srfAD*) (Nakano et al., 1991). Expression of the *srfA* operon is directly linked to the cell density and the growth phase. During exponential growth, the *srfA* operon is repressed by AbrB, the transition state regulator, and its homolog Abh (Chumsakul et al., 2011). Upon entrance of *B. subtilis* into the stationary phase, the cells begin to differentiate into different subpopulations including cannibals, biofilm or protease producers, sporulators, and competent cells. The competent cells, approximately 10%–20%, can take up extracellular DNA and produce surfactin (Hamoen et al., 2003). This connection is due to the presence of the *comS* ORF, which encodes an antiadaptor protein protecting ComK, the master regulator of competence, from posttranslational degradation, within the *srfAB* ORF (D'Souza et al., 1994; Hamoen et al., 2003). Induction of the *srfA* operon and *comS* only takes place in competent cells by a phosphorylated activator, ComA (Fig. 6.4). The ComA response regulator belongs to the ComPA two-component signal transduction system in which ComP is a sensor kinase that autophosphorylates and afterward phosphorylates ComA (Hamoen et al., 2003). This phosphorylation happens when ComP senses the presence of a short extracellular quorum-sensing peptide, ComX (Magnuson et al., 1994; Pottathil et al., 2008). The pre-ComX protein is intracellularly processed by ComQ and secreted into the medium. During the stationary phase, the cell density increases that results in a higher concentration of ComX and stimulation of ComPA, thereby activation of P_{srfA} by ComA~P (Hamoen et al., 2003). Hence, the surfactin production depends on quorum sensing (Hamoen et al., 1995). In addition to ComX-dependent quorum-sensing regulation, phosphatase regulator (Phr) peptides and their cognate response regulator aspartyl phosphatase (Rap) systems are involved in the regulation of P_{srfA} (Shank and Kolter, 2011). So far, 11 Rap enzymes have been identified in *B. subtilis* dephosphorylating their target proteins. Among them, RapC, RapD, RapF, RapH, RapK, and RapP are known to dephosphorylate ComA~P and thereby prevent its function (Omer Bendori et al., 2015; Auchtung et al., 2006). Here, the regulation of PhrC-RapC will be discussed in more detail as an example. PhrC, also known as CSF for competence and sporulation stimulating factor, is produced and processed intracellularly and secreted to the extracellular milieu. The mature form of PhrC is then taken up via an ATP-binding cassette (ABC) transport system, oligopeptide permease (Opp) (Perego et al., 1991). Transcription of *phrC* is activated by the sigma factor H (σ^H) (Hamoen et al., 2003). The *sigH* gene is regulated by the sporulation master regulator, Spo0A (Weir et al., 1991). Therefore, the physiological state of the cells is important for the expression of quorum-sensing factor, PhrC. The quantity and phosphorylation state of Spo0A determine its targets. Upon entrance to stationary phase, Spo0A activates the expression of *sigH*. Consequently, surfactin production depends on quorum sensing influenced by cell density of *B. subtilis*.

In addition to the ComA(~P)-dependent pathway, other regulators are also involved in the regulation of P_{srfA} including DegU, CodY, Abh, PhoP, PerR, and Spx. Each of these regulators links the surfactin biosynthesis to one of the physiological states of *B. subtilis*. DegU is a global regulator that belongs to the DegS-DegU two-component signal transduction system. DegU is mainly known for its function as an activator for the genes encoding degradation enzymes, such as *aprE*. However, DegU has other roles, for instance, in the formation of biofilm or swarming. Depending on the physiological state of *B. subtilis*, the amount and

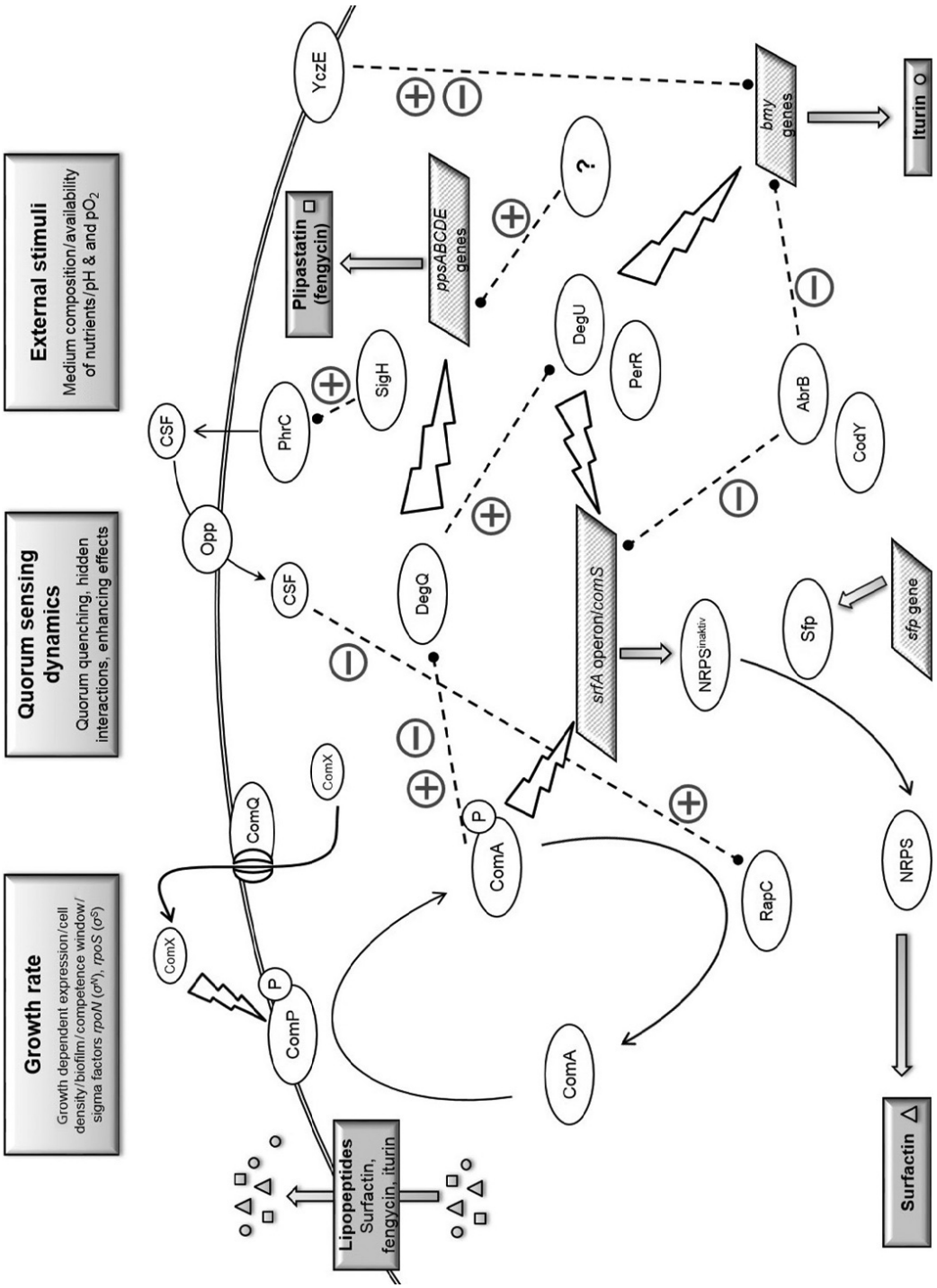

FIG. 6.4 Overview of molecular interactions in regulation of genes related to lipopeptide biosynthesis. Main regulatory proteins and transcription factors involved in regulation of the ComX-dependent quorum-sensing network are shown along with external and growth-dependent effects (top boxes). The connection to lipopeptide biosynthesis is given by expression of the *srfA* operon (surfactin biosynthesis), *bmy* genes (iturin biosynthesis), and *pps* operon (fengycin biosynthesis).

phosphorylation state of DegU vary. DegU is phosphorylated by DegS(~P) in a reaction facilitated by DegQ (Marlow et al., 2014; Murray et al., 2009). In the undomesticated *B. subtilis* NCIB3610, it has been recently shown that deletion of *degS* has a positive effect on P_{srfA}. As a result, it is assumed that DegU(~P) represses the *srfA* promoter (Miras and Dubnau, 2016). On the other hand, P_{degQ} is activated by ComA(~P) (Msadek et al., 1991) showing that increasing the concentration of DegU(~P) is one step after phosphorylation of ComA via quorum sensing. Therefore, in a model suggested by Miras and Dubnau (2016), activation of the ComA(~P) results in the expression of *degQ* that results in higher concentration of DegU(~P). As a result, DegU(~P) shuts down P_{srfA} and activates other differentiation pathways.

Apart from the mentioned quorum-sensing pathways, expression of the *srfA* operon is also influenced by the availability of nutrients, such as phosphate and amino acids. PhoP belongs to the two-component signal transduction system PhoPR, which is activated during phosphate starvation conditions. In this signal transduction system, PhoR is the sensory histidine kinase, while PhoP is the response regulator. This system activates the production of enzymes and transporters dealing with the assimilation of extracellular phosphate (Pragai et al., 2004). Recently, genome-wide ChIP-on-chip analysis indicated that *srfAA* is activated by PhoP(~P). Surprisingly, *comQ* was also found as a new member of the PhoP(~P) regulon (Salzberg et al., 2015). This means that phosphate limitation activates surfactin biosynthesis directly via PhoP(~P) and indirectly via ComQ that finally activates a ComA(~P)-dependent pathway. Depletion of amino acids or GTP also upregulates the *srfA* operon because of CodY derepression (Serror and Sonenshein, 1996). CodY is a global regulator affecting different pathways in *B. subtilis*. CodY mainly represses genes that help the cell to grow during poor nutritional availability, and its affinity for DNA is enhanced in the presence of high intracellular GTP or branched-chain amino acids (isoleucine and valine) (Sonenshein, 2007). Therefore, during the exponential phase and in the presence of sufficient nutrients, P_{srfA} is repressed, whereas upon entrance to stationary phase, P_{sfrA} is released from repression by CodY due to the low concentration of intracellular GTP and depletion of branched-chain amino acids.

Finally, the presence of oxidative agents downregulates the expression of the *srfA* operon (Mostertz, 2004). This is due to the structural changes of peroxide response regulator (PerR), which positively regulates P_{srfA} (Hayashi et al., 2005). In the presence of H_2O_2, oxidation of the Fe atoms results in a structure of PerR that likely dissociates from the P_{srfA} DNA (Zuber, 2009). Moreover, the presence of diamide also induces the encoding gene of Spx, which inhibits the interaction between RNA polymerase and ComA(~P); thereby, it inhibits the activation of P_{srfA} (Nakano et al., 2003).

6.5 LIPOPEPTIDE PHYSICOCHEMICAL PROPERTIES AND CONCOMITANT COMMERCIAL ASPECTS

Lipopeptides synthesized by *Bacillus* spp. are attractive to diverse industrial sectors. Several review articles (Banat et al., 2000; Kanlayavattanakul and Lourith, 2010; Geetha et al., 2018; Gudiña et al., 2013; Mnif and Ghribi, 2015b; Nitschke and Costa, 2007; Nitschke and Silva, 2018; Ongena and Jacques, 2008; Shafi et al., 2017; Shaligram and Singhal, 2014; Zhao et al., 2017) give an overview of chemical attributes and their possible applications such as in the petroleum industry; environmental applications; agricultural applications; laundry

products; and the pharmaceutical, cosmetic, and food industry. The following chapter will give a brief overview of several physicochemical properties of lipopeptides. In addition, selected application fields will be described in more detail.

6.5.1 Physicochemical Properties

Surfactin is often claimed as a very efficient surface-active molecule. High-purity surfactin reduced the surface tension of water to 27.90 mN/m at a concentration of 0.005% (Arima et al., 1968). Other studies reported a reduction in surface tension of water from 70 to 36 mN/m at a CMC of 15.6 mg/L, when surfactin was used with a lower purity (Abdel-Mawgoud et al., 2008). In a study conducted by Deleu et al. (1999), the CMCs of surfactin, iturin A, and fengycin were reported to be 10, 20, and 11 mg/L, respectively. Other biosurfactants such as sophorolipids were described to have a CMC of 20 and 130 mg/L for pure and crude biosurfactant, respectively. At these concentrations, the surface tension of water was reduced to 36 and 39 mN/m (Otto et al., 1999). Further examples are summarized by Desai and Banat (1997). In general, biosurfactants have a lower CMC than chemical surfactants such as potassium oleate with 350 mg/L or fatty alcohol ether sulfate with 170 mg/L (Kosswig, 2012). The low CMCs of biosurfactants and especially for the lipopeptides surfactin, iturin A, and fengycin make them very interesting as smaller amounts of them are needed in comparison with petrochemically derived surfactants. Overall, even surfactin with a lower purity had a CMC lower than chemical counterparts, which is greatly beneficial for its application such as bioremediation or enhanced oil recovery. Here, the demand of high-purity products is less essential than, for example, in the pharmaceutical and food industry. Abdel-Mawgoud et al. (2008) characterized surfactin produced by a *B. subtilis* strain with respect to several chemical characteristics. The solubility in aqueous solution was given at pH >5, with the optimum at pH 8–8.5. Surfactin was stable in the pH range from 5 to 13. With respect to temperature and salinity, stability was not influenced after autoclaving and in salinities up to 6% NaCl (Abdel-Mawgoud et al., 2008).

Next to the outstanding surface-active and chemical properties, lipopeptides hold bioactive properties. With this feature, they stand out from many other surfactants. The bioactive properties of biosurfactants and their concomitant potential in being used in food safety and therapeutic applications are summarized by Meena and Kanwar (2015). Surfactin, for example, showed both antiviral, antibacterial, and antitumor activities, while iturin displayed mostly antifungal activity with limited antibacterial properties.

The manifold structures and concomitant attributes allow for several functions such as emulsifier, dispersant, foaming agent, thickening agent, detergent, viscosity-reducing agents, and antimicrobial ingredient. Therefore, several industrial sectors where an application is feasible will be presented in more detail.

6.5.2 Agricultural Applications

Every year, plant diseases caused by environmental conditions or by pathogens account for a great loss of plants in the agriculture sector. To prevent loss of crops by diseases, research investigated the applicability of biosurfactants as an alternative to chemical fertilizers due to their eco-friendly, less invasive, and more sustainable character. The bacteria, to which

both *Bacillus* spp. and *Pseudomonas* spp. belong, acting as an "active ingredient" are so-called plant growth-promoting rhizobacteria (PGPR). These PGBR can either protect plants against diseases or promote plant growth (Besset-Manzoni et al., 2018; Shameer and Prasad, 2018). Investigations have already demonstrated that these bacteria live in biofilms and their mode of action relies on a variety of physical triggers and microbial metabolites, such as the excretion of lipopeptides. In this field, Ongena and Jacques (2008) published a review highlighting the relevant facts regarding plant disease biocontrol employing *Bacillus* lipopeptides. *Bacillus* spp. is attributed to have antagonistic, inhibiting, spreading, and immuno-stimulating effects. Studies have already demonstrated that the individual lipopeptide families possess different characteristics and hence play different roles in the interaction with plants. Surfactin, for example, is attributed with many features such as being antibacterial and antifungal. Further influences are in the fields of biofilm formation, spreading and as signal for plant diseases. The actions of iturin are spreading and antifungal activities, and fengycin possesses antifungal activities and also acts as signal molecule for plant cells (Ongena and Jacques, 2008).

In a study conducted by Malfanova et al. (2012) to evaluate the antifungal activity of lipopeptides synthesized by *B. subtilis* HC8, fengycin showed the highest antifungal activity. Cawoy et al. (2015) indicated that strains producing all three families of lipopeptides were overall the most effective producers in terms of fungi inhibition. Strains lacking the production of iturin but coproducing both surfactin and fengycin and strains incapable of producing any lipopeptides showed lower inhibition capacities. Results indicated that iturin is the most active ingredient followed by fengycin. Surfactin was reported to have synergistic effects, for example, supports root tissue colonization and promotes nutrient supply by surface wetting and detergent properties. This synergistic effect of surfactin is based on its strong surface-active properties that trigger biofilm formation. Biofilm formation is essential for swarming on plant tissues. Also Paraszkiewicz et al. (2017) confirmed that the presence of surfactin in sufficient amounts is necessary for biofilm formation. However, although iturin and fengycin showed higher antimicrobial activities in this report, also surfactin is attributed with this effect due to so-called detergent-like effects on membranes (Heerklotz and Seelig, 2007).

Dimkić et al. (2017) used high-performance thin-layer chromatography (HPTLC) to separate individual lipopeptide mixtures and to test the antimicrobial activity against *P. syringae* pv. *aptata*, *Xanthomonas arboricola* pv. *juglandis*, and *L. monocytogenes* employing an agar overlay method. Iturin was determined to be the most effective antifungal family. Furthermore, the authors concluded that the antimicrobial activity of iturin was dependent on the homologues within the family. The inhibition zones for surfactin homologues were rather low, supporting the hypothesis that surfactin has synergistic effects and supports plant colonization. Due to the importance of synergistic effects causing plant benefits, lipopeptide family coproducers are very attractive in the agricultural sector to protect plants by diseases.

In a recent study conducted by Le Mire et al. (2018), the effectiveness of surfactin to protect wheat by up to 70% against the fungi *Zymoseptoria tritici* was shown. Interestingly, surfactin itself did not show any antifungal activity, but was reported to stimulate salicylic acid- and jasmonic acid-depending signaling pathways. These acids are important regulators for plant defenses against biotic stresses.

Also in a study conducted by Paraszkiewicz et al. (2017), growth of fungi was reduced for all strains tested, which were surfactin single producers; surfactin and iturin producers; and surfactin, iturin, and fengycin coproducers. However, *B. cinerea* and *A. flavus* were only

growth-inhibited by *Bacillus* coproducer strains. Likewise, Cawoy et al. (2015) explored that the production of individual congeners and the respective amounts within lipopeptide families vary depending on the target pathogen.

Bais (2004) demonstrated that *B. subtilis* strain 6051, able to synthesize surfactin, forms a biofilm on Arabidopsis roots and shows antibacterial effect against *P. syringae*. While the wild-type strain *B. subtilis* 6051 was effective against the pathogens, the derivative containing a *ΔsrfAA* mutation was less effective.

6.5.3 Detergents

Industrial processes are already implemented for sophorolipids (Soliance SA, Pomacle, France; Evonik Industries AG, Essen, Germany; and Saraya Co., Ltd., Osaka, Japan) and for rhamnolipids (AGAE Technologies, Corvallis, Oregon, the United States, and Biotensidon GmbH, Karlsruhe, Germany), and also Evonik Industries AG announced the commercialization of rhamnolipids in a press release from 2016 (Evonik Commercializes Biosurfactants, 2016). Lipopeptides produced by *B. subtilis* are also of great interest to this industrial sector, and currently, no products in the houseware sector are available containing these biosurfactants. Mukherjee (2007) investigated the compatibility and stability of cyclic lipopeptides in locally available laundry detergents. Results revealed that a mixture of detergent and crude biosurfactant led to an overall improved washing performance of up to 26% in removing sunflower oil or blood from cotton fabrics in comparison with the detergents itself. However, the crude biosurfactants alone had a lower efficiency than the detergents alone. Taira et al. (2017) further evaluated the effect of surfactin on subtilisin, a *Bacillus* protease often used in laundry formulations. While surfactants such as sodium dodecyl sulfate decreased the proteolytic activity, the presence of surfactin at low concentrations did not inhibit the subtilisin activity.

6.5.4 Nanoemulsions and Emulsions

(Nano-)emulsions are important structures in different fields such as the pharmaceutical, cosmetic, and food industry. Such structures allow the incorporation of, for example, health beneficial molecules such as vitamins that are poorly soluble in water and instable at certain conditions. The surface-active molecules incorporated to stabilize these emulsions have to provide both physical and chemical stability. With respect to physical stability, fundamental research on surface-active, interfacial, and emulsifying properties of lipopeptides was performed by several research groups (Deleu et al., 1999; Iglesias-Fernández et al., 2015; Onaizi et al., 2016). Among the three main lipopeptide families, surfactin was reported to be the most effective regarding the time to reach the equilibrium at the oil/water interface and the final interfacial tension, whereas iturin A possessed the best resistance to creaming/flocculation, and fengycin was most stable against coalescence (Deleu et al., 1999). Hence, as each food emulsion features its own character and is accompanied by different destabilization mechanisms, a matrix-specific lipopeptide stabilizer has to be characterized. Onaizi et al. (2016) and Iglesias-Fernández et al. (2015) also investigated the ability of surfactin to adsorb at solid-liquid and especially air-liquid interfaces. The latter investigations further increase the field of applications in, for example, aerated food products such as mousse.

With respect to chemical stability, He et al. (2017) investigated the physical and oxidative stability of microemulsions with docosahexaenoic acid, one of the most important omega-3 polyunsaturated fatty acids. Results revealed that the microemulsion stabilized with surfactin showed an enhanced physical and antioxidative stability than a microemulsion with Tween 80. Furthermore, the experiments indicated that surfactin at a concentration of 0.2 mmol/L had a better emulsifying capability in o/w emulsions than Tween 80, lecithin, and sucrose fatty acid ester at equal concentrations.

The incorporation of surfactin as stabilizer can have a further benefit over synthetic stabilizers depending on the application. As surfactin possesses antimicrobial properties, (nano-) emulsions stabilized by this biosurfactant have consequently antimicrobial effects. As a result, applications in the pharmaceutical industry, in cosmetics, or the food industry are conceivable. A nanoemulsion based on sunflower oil and stabilized by surfactin, for example, showed a high activity against *Salmonella typhi* and also against *L. monocytogenes* and *Staphylococcus aureus* (Joe et al., 2012). These findings give one example of application possibilities in the food safety area, as lipopeptides can be incorporated into the food matrix as protecting ingredient.

6.5.5 Food Industry

With respect to applications as ingredient or additive, the presence of surfactin in various fermented food products such as in natto, a Japanese soybean dish, is highly beneficial for approval. Juola et al. (2014) examined different natto samples with respect to their surfactin content. Interestingly, the highest amounts detected were up to 2.2 mg/g, which correlates to an amount of 80–100 mg surfactin per 50 g of natto. Further studies need to be conducted in order to determine the acceptable daily intake (ADI) for surfactin to declare it as nontoxic. But, regarding the fact that surfactin is already consumed daily by numerous humans and as *B. subtilis* is generally considered being a generally accepted as safe (GRAS) organism, an implementation of surfactin in the food industry is very conceivable.

One possible application of surfactin is in the reduction of biofilm formation on food processing equipment induced from food pathogens. Surfactin can adsorb on materials such as stainless steel, and the lipopeptide was able to reduce adhesion and biofilm formation of the pathogens *L. monocytogenes* and *P. fluorescens*. Further coeffects might be essential for the antimicrobial effects of surfactin (de Araujo et al., 2016).

Few studies were performed so far to incorporate surfactin directly into a food matrix to replace other ingredients. One of these attempts was carried out by Zouari et al. (2016). Sesame peel flour was used for the preparation of cookies as partial replacement for conventional white wheat flour. Parameters such as hardness, moisture content, and spread factor became worse when more sesame peel flour was used. However, when 0.1% *B. subtilis* SPB1 biosurfactant was added, the texture profile was highly improved, even in comparison with the conventional used emulsifier glycerol monostearate (Zouari et al., 2016).

Another possible application can be found during food processing, as investigated by Jiang et al. (2017). In this study, the potential of lipopeptides from *Bacillus* spp. to inhibit *A. carbonarius* and hence ochratoxin A contamination in the winemaking process was investigated. Ochratoxin A concentration should not exceed 2.0 µg/L in wine as it is a carcinogenic mycotoxin. Furthermore, this compound negatively affects the fermentation behavior of yeasts. The study revealed that the presence of lipopeptides during the winemaking process

reduced *A. carbonarius* and hence ochratoxin A concentrations. Also in comparison with SO_2, the lipopeptides showed better antifungal properties and also promoted the growth of yeasts and formation of esters and acids being involved in the aroma profile (Jiang et al., 2017).

6.5.6 Personal Care and Therapeutic Applications

Biosurfactants can also be used in personal care and therapeutic applications. One attempt was made by Bouassida et al. (2017). Here, *B. subtilis* SPB1 lipopeptide was used as alternative to the conventional used surfactant sodium dodecyl sulfate in toothpaste formulation. Next to good foaming abilities, the toothpaste containing lipopeptides furthermore was reported to be advantageous due to the antimicrobial activities the lipopeptides come along with.

6.6 BIOTECHNOLOGICAL PRODUCTION OF SURFACTIN

In the field of surfactin production, research often aims at increasing the yields to approach industrial implementation. The following chapter describes the recent progress regarding surfactin production and lipopeptide synthesis in general. Thereby, the chapter is divided into three categories. In the first part, strategies dealing with both medium and process parameter optimization will be presented. Secondly, techniques employing strain engineering are given, and the last part illustrates an overview of different cultivation strategies employed.

6.6.1 Optimization of Media Composition and Process Parameter

The optimization of both the medium composition and process parameter is often addressed in studies dealing with the increase of lipopeptide yields. In this field, the review paper by Rangarajan and Clarke (2015) and Shaligram and Singhal (2010) give a fundamental summary. With respect to medium optimization, either defined or complex media are used as basis, and novel approaches incorporate the utilization of cheap substrates labeled as agro-industrial waste products or by-products.

6.6.2 Carbon, Nitrogen and Trace Elements

With respect to media optimization, the well-established mineral salt medium used by Cooper et al. (1981) and the Landy medium (Landy et al., 1948) are often used as reference. By surveying literature, the complexity of surfactin and lipopeptide synthesis in general becomes obvious. In terms of growth, ammonium and glutamine are the preferred nitrogen sources for *Bacillus* spp. (Detsch, 2003; Fisher, 1999).

Among different nitrogen sources tested for surfactin production, ammonium nitrate was shown to be a very good nitrogen source for the strain *B. subtilis* YRE207 (Fonseca et al., 2007), whereas this source was less suited for the strain *B. subtilis* BBG208 (Yaseen et al., 2017). The same study also indicated that ammonium nitrate is a promising alternative for glutamic acid for the synthesis of fengycin (Yaseen et al., 2017). In both studies, also urea was an interesting nitrogen source that also benefits from its low prize. Alanine had a positive effect on surfactin synthesis when glutamic acid in the original Landy medium was replaced, whereas the

employment of other amino acids and complex media components significantly reduced the yield (Yaseen et al., 2017). Interestingly, while a replacement of glucose by different carbon sources did not enhance surfactin production, a significant increase in the concentration of fengycin was determined using mannitol (Yaseen et al., 2017). Depending on the strain, the C-N ratio is also critical. This ratio was addressed in a study conducted by Medeot et al. (2017) with the strain *B. subtilis* $MEP_2$18. The optimal ratio was reported as 10:1, and best carbon and nitrogen sources tested were glucose and ammonium nitrate, respectively. The original Cooper and Landy media have a C-N ratio of 13:1 and 27:1, respectively.

In general, medium optimization procedures come along with numerous experiments. Therefore, designs of experiment methods, such as response surface methodology or the Taguchi method, are useful tools to evaluate the effect of different components on the lipopeptide concentration.

Sen (1997) improved the original Cooper medium with respect to glucose, NH_4NO_3, $FeSO_4$, and $MnSO_4$ concentrations. The optimal concentrations determined for maximal surfactin production with the strain *B. subtilis* DSM 3256 were 36.5, 4.5, 4×10^{-3}, and 27.5×10^{-2} g/L, respectively. While this study confirmed a rather high glucose concentration being better suited, Willenbacher et al. (2015b) reported that a glucose concentration of 8 g/L gives better yields. Here, the surfactin concentration was increased from 0.7 to 1.1 g/L for the strain *B. subtilis* $DSM10^T$. In addition, this study also addressed environmental concerns and the original chelating agent Na_2EDTA of the Cooper medium was replaced by Na_3-citrate. In contrast, a study addressing the production of lichenysin by the strain *B. licheniformis* WX-02 identified 30 g/L glucose as optimal concentration when testing different concentrations in the range of 10–50 g/L (Qiu et al., 2014).

Employing design of experiment, the surfactin concentration obtained by *B. subtilis* ATCC 21332 was increased from 1.74 to 3.34 g/L with an optimized trace element composition (Wei et al., 2007). Here, optimal concentrations of Mg^{2+}, K^+, Mn^{2+}, Fe^{2+}, and Ca^{2+} were 2.4, 10, 0.01, and 0.008 mM and 7 μM, respectively, instead of the original 0.8, 30, 0.2, 0.3, and 7 μM. Hence, concentrations of Mg^{2+}, K^+, and Mn^{2+} were increased, and especially, Mg^{2+} and K^+ were found to have interactive effects. Mg^{2+} and K^+ are directly involved in surfactin synthesis, with Mg^{2+} needed in the PCP domain (Reuter et al., 1999) and K^+ stimulating surfactin secretion (Kinsinger et al., 2003). Other trace elements, such as Mn^{2+} and Fe^{2+}, had a lower impact on surfactin production itself, but cell growth was drastically reduced in the absence (Wei et al., 2007). However, a study conducted by Huang et al. (2015) revealed a positive effect of Mn^{2+} when using NH_4NO_3 as nitrogen source. *Bacillus* spp. show diauxic consumption and use first NH_4^+ followed by NO_3^- after depletion (Davis et al., 1999). An increase in Mn^{2+} to >0.05 mmol/L led to a shift toward nitrate utilization, and final concentrations of surfactin measured were 6.2-fold higher as well. Consequently, Mn^{2+} was shown to increase the activity of both nitrate reductase and glutamate synthetase. The latter one increases the ability of free amino acids (Huang et al., 2015).

Design of experiments was also conducted by Motta Dos Santos et al. (2016) in microplate bioreactor. The statistics revealed that higher levels of glutamic acid, glucose, yeast extract and $MgSO_4$, and L-tryptophan had a positive effect on surfactin production by the strain *B. subtilis* BBG131. Interestingly, higher amounts of trace elements were statistically not significant to enhance surfactin yields, when other chemicals with a higher effect were improved. After statistical analysis, the proposed medium for both optimal biomass production and optimal

$Y_{P/X}$ was compared with the original Landy medium in a bubbleless membrane bioreactor as shown in Coutte et al. (2013). Although no significant improvement in the surfactin concentration was obtained, the yield $Y_{P/X}$ increased from 135.34 mg/g cell dry weight to 305 mg/g. Also Gudiña et al. (2015) illustrated the complexity of the effects of added trace elements. For example, the reference medium containing 10% corn steep liquor achieved a crude biosurfactant concentration, which contained surfactin congeners, of 1.3 ± 0.1 g/L. In a next setup, individual optimal concentrations were determined for $FeSO_4$, $MnSO_4$, and $MgSO_4$. Here, for both 2.0 mM $FeSO_4$ and 0.8 mM $MgSO_4$, the optimal individual concentrations evaluated led to crude biosurfactant concentrations of 4.2 ± 0.1 and 3.5 ± 0.1 g/L. Interestingly, when combining these trace elements at their respective optimal concentrations, the concentration achieved was rather low with 2.7 ± 0.1 g/L. However, when these chemicals were added together with 0.2 mM $MnSO_4$, the highest crude biosurfactant concentration within this study was reported with 4.8 ± 0.2 g/L.

6.6.3 Alternative Substrates for Lipopeptide Production

Next to the optimization of single components in defined media, another approach to increase the yields is the utilization of waste streams or by-products such as cashew apple juice (Freitas de Oliveira et al., 2013), rapeseed meal (Jin et al., 2014), waste distiller's grain (Zhi et al., 2017), two-phase olive mill waste (alpeorujo) (Maass et al., 2016), orange peel (Kumar et al., 2016), or cassava-flour-processing effluent (Nitschke and Pastore, 2004). The utilization of such media components is especially interesting for high-volume and low-value applications (Rangarajan and Clarke, 2015). However, while investigating the effect of such supplements on the final lipopeptide concentration, the authors often do not compare their results with a reference medium. Such approaches have both advantages and disadvantages. For example, the overall production costs may be reduced when using alternative substrates that do not compete with the food industry and open new opportunities regarding lucrative waste management. On the other hand, for several waste streams, it is difficult to maintain a constant composition, and the purity plays an important role. For example, de Andrade et al. (2016b) replaced analytic-grade glycerol by concentrated glycerol from the biodiesel industry and studied the effect on surfactin production. The utilization of this waste might also come along with other nutritional elements such as phosphorus or nitrogen, depending on the initial source of triglycerides used. In contrast to prior expectations, the utilization of concentrated glycerol resulted in an overall delay in cell growth compared with analytic-grade glycerol. This indicated that impurities such as remaining salts and methanol, still present in the by-product at low concentrations, might have a strong, and in this study negative, influence on cell growth. In terms of biosurfactant production, higher foaming and hence higher biosurfactant synthesis were recorded for the medium containing analytic-grade glycerol with 325 mg/L in comparison with 71 mg/L.

6.6.4 Influence of Process Parameter

Experimental design methods were also employed to optimize process parameter. Jacques et al. (1999) investigated the effect of different temperatures (25–40°C), pH (5.5–8), shaking (100–200 rpm), and media components in shake flask cultures. The optimal process parameter

to increase the synthesis of surfactin, iturin A, and fengycin by the strain *B. subtilis* S499 was 30°C, pH 7, and 200 rpm. Fahim et al. (2012) investigated different rpm and concomitant different k_La values in shake flask experiments. They revealed that surfactin production is superior for higher k_La values, while fengycin synthesis was better under moderate oxygen supply. In terms of oxygen supply, Rangarajan et al. (2015) also reported a higher selectivity toward the synthesis of fengycin by the strain *B. megaterium* MTCC 8280 under oxygen-limiting conditions.

Yeh et al. (2006) investigated different k_La values in a 5 L jar fermenter with foam collector. The highest k_La, obtained by 300 rpm and 1.5 vvm, led to the highest surfactin concentration and overall production rate of 106 mg/(Lh). Monteiro et al. (2016) investigated the influence of different culture media, temperatures (15, 25, and 30°C), and initial pH (5–9) on the production of the three lipopeptides surfactin, iturin, and fengycin using the strain *B. amyloliquefaciens* 629. During their investigations, only one of the four media, namely, PDB, a medium containing plant-derived nutrient, allowed for a simultaneous production of all three lipopeptides at 25°C and 30°C. Nevertheless, the lowest concentrations of surfactin and fengycin were detected in this medium in comparison with the other media tested (medium optimal for lipopeptide production, MB1, and Luria-Bertani (LB)). For surfactin, overall higher concentrations were monitored at 15°C and for fengycin at 25°C. With respect to pH, no lipopeptides were detected after cultivations with an initial pH of 5, 8, or 9. An initial pH of 6 was furthermore more suitable than pH 7.

Slivinski et al. (2012) investigated the temperatures 25, 30, 37, and 45°C on the production of surfactin using a medium containing 50% sugarcane and 50% okara in a solid-state fermentation. Highest surfactin concentration was measured at 37°C after 60 h with 484 mg/L. At 45°C, the maximum concentration measured was 108 mg/L at 36 h. For both 25 and 30°C, the surfactin concentration continuously increased to a concentration of ~ 380 and 290 mg/L until 72 h.

6.6.5 Alternative Medium Optimization Approaches

Both de Andrade et al. (2016b) and Zhi et al. (2017) provided interesting approaches to optimize surfactin that will be described briefly.

To increase the yield of lipopeptides, de Andrade et al. (2016a) proposed a process that aimed to cosynthesize two different target products, namely, surfactin and 2,3-butanediol. The latter one shows already actual and potential applications in printing inks; rubbers; and cosmetic, food, and pharmaceutical products. A cooptimization may show an impact on the economic viability, as the overall value increases, especially when the main product of interest such as surfactin is just synthesized in small amounts. In order to achieve higher concentrations, they investigated different concentrations of the three media components cassava wastewater, whey, and activated carbon. Interestingly, the employment of central composite rotational design revealed an almost identical composition of the three media components to achieve the maximum concentrations for both surfactin and 2,3-butanediol.

Also Zhi et al. (2017) examined an interesting approach to synthesize surfactin using a waste by-product. In their study, waste distiller's grain was used as carbon source for culturing *B. amyloliquefaciens*. In the first step, strains were screened with respect to their ability to grow on distiller's grain and their surfactin concentrations achieved. As a result, the highest

concentration of surfactin was reached by the strain *B. amyloliquefaciens* MT45. However, this strain exhibited poor growth on the medium and a low carbon conversion. Therefore, the strains were also evaluated regarding their amylase, protease, and lipase activities. Strains exhibiting a high amylase activity in general reached higher cell counts when cultivating on waste distiller's grain. Hence, the surfactin-producing strain MT45 was cocultured with strains exhibiting a high hydrolase activity. This coculturing enhanced the surfactin concentration from 1.04 to almost 1.6 g/L. Further optimization was performed by evaluating the inoculation ratio of the two strains MT45 and X82. The overall surfactin concentration was the highest when using a ratio of 1:0.5 with 2.54 g/L.

6.6.6 Strain Engineering

As already illustrated in a previous chapter, the synthesis of surfactin is regulated by a complex quorum-sensing mechanism. Consequently, also strain engineering is often employed to enhance the surfactin yields by different approaches. A study by Coutte et al. (2010a) revealed that the disruption of the operon encoding for plipastatin in the strain *B. subtilis* 168, where a functional *sfp* gene was integrated, led to a fivefold increase in surfactin synthesis. The native promoter P_{srfA} was thereby replaced by a constitutive promoter, and activity measurements revealed an earlier expression. Interestingly, Willenbacher et al. (2016) demonstrated that an exchange of the native promoter P_{srfA} by a strong constitutive promoter was strain-dependent. An already relatively strong surfactin producer such as *B. subtilis* DSM10^T was negatively affected by the exchange, while a minor surfactin producer benefited from the replacement. Also, Yaseen et al. (2016) investigated the effect of different promoters on the synthesis of fengycin. The P_{fen} promoter of strain *B. subtilis* BBG21, a spontaneous mutant of the strain ATCC 21332, showed a very high efficiency compared with the promoter P_{pps}, which is located in the strains BBG111, a 168 derivative, and *B. amyloliquefaciens* FZB42. Interestingly, when the P_{pps} promoter in the strain BBG111 was replaced by the strong promoter P_{fen} of strain BBG21, a 10-fold increase in fengycin production was observed for BBG111, while the overall synthesis rate remained almost constant when integrating the P_{fen} promoter of strain ATCC 21332. Sequence analysis of P_{fen} in ATCC 21332 and its derivative BBG21 revealed that a point mutation in P_{fen} of BBG21 led to the overproduction of fengycin. Another study addressing promoter exchange was conducted by Qiu et al. (2014). The lichenysin synthesis of the strain *B. licheniformis* WX-02 was reported to be improved when the native promoter was replaced by the *srf* operon promoter P_{srf}.

Consumption exhibited a similar pattern (Zhao et al., 2012). Within the same group, a strain with a 12.77-fold increased fengycin expression was reported by genome shuffling (Zhao et al., 2016).

In terms of metabolic engineering, Coutte et al. (2015) generated a model predicting the knockout of respective genes to overproduce leucine, an important precursor for surfactin synthesis. The engineered strain based on this model held a 20.9-fold increased surfactin synthesis. Using the same syntax developed in this study, Dhali et al. (2017) engineered strains lacking either the gene *codY* or *lpdV*, and surfactin synthesis was increased by 5.8- and 1.4-fold, respectively. Furthermore, the results revealed that the *lpdV* mutant overproduced mainly the C_{14} isoform. Such approaches are very interesting as the synthesis can be headed toward a specific isoform.

6.6.7 Cultivation Strategies for Lipopeptide Production

In Section 6.1, findings regarding media composition and fundamental parameter such as temperature and pH were presented that provide a basis for bioreactor cultivation. However, the production of lipopeptides at a larger scale goes along with a main challenge, namely, the severe foaming, that is often not addressed in shake flask experiments. The occurrence of foam can be considered as both advantage and drawback, as it not only provides the first purification step but also goes along with the loss of culture media and surfactin-producing cells (Coutte et al., 2017; Rangarajan and Clarke, 2015). In this sense, the following sections focus on process strategies that either integrate or avoid foaming.

6.6.7.1 Batch, Fed-Batch, and Continuous Strategies

A batch process with the addition of antifoam was carried out by Davis et al. (1999). Under glucose-limited conditions, the strain *B. subtilis* ATCC 21332 was synthesized up to 31.2 mg/L surfactin, and the $Y_{P/X}$ obtained was 0.0068 g/g. Surfactin was thereby mainly produced when entering the stationary phase. Under nitrogen-limiting conditions, the maximum concentration of surfactin was 45.3 mg/L with an $Y_{P/X}$ of 0.021 g/g. Here, surfactin was mainly synthesized when the strain used nitrate as nitrogen source instead of ammonium. In a subsequent study, Davis et al. (2001) demonstrated that foaming is favorable. Highest surfactin enrichment was obtained when foaming was integrated in the cell culture stage instead of employing it as separate unit. In addition, the authors reported that lower stirrer speeds were more favorable than higher stirrer speeds. Employing 269 rpm, half of the media was lost due to foaming after 11 h, and hence, the time window for surfactin production was drastically reduced. Lower stirrer speeds consequently favored surfactin enrichment in the foam. This was confirmed by Yeh et al. (2006). In general, higher stirrer speed and aeration rates in a 5 L jar fermenter resulted in overall higher surfactin concentrations until 1.5 vvm and 300 rpm. A further increase in both parameters, on the contrary, decreased surfactin production rates. In another study employing the strain ATCC 21332, antifoam addition was avoided (Coutte et al., 2010b). Antifoams are often considered as undesired as they are costly and might have a negative effect on cell growth. Besides, they need to be addressed in product purification (Yeh et al., 2006). In a batch culture with the strain *B. subtilis* ATCC 21332 and Landy medium, foaming started after 6 h, and from the initial volume of 3 L, only 1.13 L was in the system at the end of cultivation (Coutte et al., 2010b). Both surfactin and fengycin were continuously extracted in the foam with total amounts produced of 714 and 43 mg, respectively. For surfactin, 60 mg were extracted from the broth, while fengycin was not determined in broth samples. From the total biomass of 12.4 g, 1.4 g was extracted from the foam. Consequently, the foaming yielded higher concentrations than the process employing antifoam. Next to the rpm, also the position of the impeller affects the performance. Chenikher et al. (2010) proposed to have one Rushton turbine impeller in the medium to allow for a proper mixing, and a second impeller was positioned slightly above the liquid level. When the volume increased due to feeding, the impeller promoted mixing at the air-liquid interface that favored foaming. The loss of both medium and cells was addressed in a feeding profile established by Guez et al. (2007) for the synthesis of mycosubtilin with the strain *B. subtilis* BBG100. Here, the feeding rate was adapted to the foam overflow rate. Jin et al. (2015) also increased the iturin A production

by employing a two-stage glucose feeding strategy. They addressed the cell-to-spore ratio and aimed at reducing the amount of spores, as spores are the final state in cell differentiation and consequently do not produce lipopeptides. A low feeding rate resulted in a relatively low iturin A concentration. Interestingly, by employing a rather high feeding rate, the ability of cells to consume glucose was found to change, and a glucose accumulation in the later stage of cultivation resulted in an increase of spores and thereby a reduction in the iturin A productivity. Hence, a combination of the feeding rates was tested. During the first stage, the high rate was employed, and in the late feeding stage, the feed rate was reduced. As a result, glucose accumulation did not occur, the cell-to-spore ratio was relatively constant, and the synthesis of iturin A was maintained during the whole time course of cultivation. Another approach was presented by Rangarajan et al. (2015) for the production of fengycin in the strain *B. megaterium* MTCC 8280. In their bioreactor batch cultivation, foaming was almost completely avoided due to a switch from submerged aeration to headspace aeration and applying a positive pressure. This enabled the reproduction of shake flask experiments where fengycin production was found to be the highest under oxygen-limiting conditions.

For lipopeptide production, the transition from fed-batch to continuous processes is very narrow. Especially when foaming is integrated and a feed is applied, the process can also be considered as a continuous setup. Such a process was also presented by Chen et al. (2006) in which foam fractionation was integrated and optimal conditions for surfactin production employing the strain *B. subtilis* BBK006 were obtained at a dilution rate of 0.2 L/h and a rather low glucose concentration in the feed with 0.25 g/L. Alonso and Martin (2016) also examined a continuous strategy in a small-scale experiment where the culture broth was actively pumped with a set flow rate into a foam trap. An air pump was mounted to the bottom of the foam column that allowed to produce foam. The overflow of the foam was set to a defined rate and hence allowed to maintain a constant volume in the foam column.

6.6.7.2 Alternative and Novel Process Set-Ups

SOLID CARRIER ASSISTED SUBMERGED CULTIVATION

Yeh et al. (2006) claimed that the addition of solid carriers to the cultivation process stimulates surfactin production using the strain *B. subtilis* ATCC 21332 as the additional surface might trigger cell growth and hence surfactin production as cells form biofilms at the surfaces. The optimized concentration of the activated carbon with 25 g/L as solid carrier led to a surfactin concentration of 3600 mg/L in comparison with the control without solid carriers of 100 mg/L. In a further study, Yeh et al. (2008) investigated a bioreactor design with the previously obtained experiences of increasing surfactin production by adding solid carriers. This enabled them to handle the severe foaming, and an addition of antifoam was not necessary. Their bioreactor is composed of a jar connected to a foam collector, a cell recycler, and a surfactin precipitation unit. In the medium, they added activated carbon. After 60 h of cultivation, a maximum surfactin concentration was measured with 6.45 g/L, and the overall volumetric production rate was calculated to be 0.106 g/(L h). The application of a cell-recycling device is furthermore advantageous, as a severe loss of volume and hence surfactin-producing cells is often an issue when processes involving foaming are created.

ROTATING DISC BIOREACTOR

The rotating disk system described by Chtioui et al. (2010) is a bubble-free process and takes advantage of the ability of *Bacillus* strains to form biofilms. The setup was further investigated in 2012 for the simultaneous synthesis of surfactin and fengycin employing the strain *B. subtilis* ATCC 21332 (Chtioui et al., 2012). The disks rotated at a speed of 30 L/min, and the disks were mounted to be half immersed in the medium. Air was supplied by an inlet in the medium-free area. In their study, they investigated two different air flow rates and used either 7 or 14 disks. At a lower air flow rate of 100 L/h, more planktonic cells and more biofilm formation were obtained at the end of cultivation with 14 disks. In general, the total biomass obtained after 72 h of cultivation was rather low in all experimental setups with 2.2–3 g cell dry weight per liter, indicating a low growth rate. During the experiment, oxygen limitation occurred in the medium especially during the exponential growth phase. This resulted in the production of acetoin. Interestingly, fengycin concentrations were about 2.5- and 4.5-fold higher than surfactin concentrations. In this experimental setup, surfactin production started at the beginning of the stationary phase, and fengycin concentrations were relatively significant at that time. The high production of fengycin might be correlated to the low oxygenation levels or to the formation of biofilm.

BUBBLELESS MEMBRANE BIOREACTOR

Coutte et al. (2010b) investigated the production of the lipopeptides surfactin and fengycin in a bubbleless membrane bioreactor using the strain *B. subtilis* ATCC 21332. In this way, foam formation was avoided by employing aeration by a hollow fiber membrane air-liquid contactor. Different aeration setups were tested with either external or submerged aeration modules. With respect to surfactin production, the concentrations in the different setups ranged between 188 and 242 mg/L. A higher difference was observed for fengycin production, where the concentrations ranged between 47 and 392 mg/L. Interestingly, for surfactin, the highest concentration was obtained in the setup using an external aeration with polyethersulfone, and for fengycin, this setup yielded the lowest concentration. The highest concentration was obtained with a submerged aeration. Interestingly, washing of the membranes in all three setups revealed that a high proportion of surfactin was adsorbed onto the membranes. On the contrary, only small amounts of fengycin adsorbed onto the membranes. However, the choice of a proper membrane is very important as results demonstrated the effect of adsorbed surfactin on the k_La. In addition, cells accumulated on the membranes. As similar overall amounts of both surfactin and fengycin were produced in comparison with a simple batch process with foam overflow, the usefulness of such a design was highlighted.

In 2013, an improved and continuous process with cell recycling using the developed membrane bioreactor was presented by Coutte et al. (2013). In detail, the medium was fed into the bioreactor at a constant flow rate of 0.3 L/h, and the same amount was allowed to leave the system. This overflow passed a microfiltration unit, and cells were recycled into the system. The permeate containing, among others, surfactin was collected in a second tank. A further purification step was integrated by mounting an ultrafiltration membrane unit to tank 2. Thereby, surfactin was separated from other residual substrates and metabolites. In comparison with the batch process, an improved surfactin productivity is using the strain *B. subtilis* BBG131, a 168 derivative, of 54.7 mg/(Lh) instead of 17.4 mg/(Lh) in a fed-batch process. Under integration of cell recycling, the productivity was further increased to 110 mg/(Lh), when a dilution rate of 0.2 L/h was set.

ANAEROBIC CULTIVATION

Bacillus subtilis was for long considered being a strict aerobic microorganism. However, *B. subtilis* is also able to grow under anaerobic conditions using nitrate or nitrite as final electron acceptor. In the absence of an alternative electron acceptor, *B. subtilis* undergoes fermentative growth with lactate, acetate, and ethanol as end products (Härtig and Jahn, 2012; Nakano et al., 1997). For surfactin synthesis, *B. subtilis* ATCC 21332 was shown to exhibit a better $Y_{P/X}$ under nitrate-limited oxygen-depleted (0.075 g/g) conditions, and the maximal concentration attained was 439.0 mg/L in comparison with ammonium-limited oxygen-depleted (0.012 g/g and 53.2 mg/L) and carbon-limited oxygen-depleted (0.0069 g/g and 41.3 mg/L) conditions (Davis et al., 1999). The high $Y_{P/X}$ also surpassed the values obtained during aerobic conditions. These findings were confirmed by Willenbacher et al. (2015a) employing a foam-free anaerobic cultivation with the strain *B. subtilis* DSM10^{T}. In comparison with a batch process employing foam fractionation with an $Y_{P/X}$ of 0.192 g/g, the anaerobic process gave a yield of 0.278 g/g.

6.6.8 Downstream Processing of Lipopeptides

The choice of an appropriate downstream process is dependent on the target product and the concomitant purity needed. In general, the number of downstream processing steps increases with increasing purity, and consequently, also production costs rise. Mnif and Ghribi (2015a) and Rangarajan and Clarke (2016) give a broad overview of the downstream techniques applied and the respective advantages and disadvantages.

Acid precipitation is the most frequently used procedure to obtain crude biosurfactant (Alvarez et al., 2012; Dunlap et al., 2011; Grover et al., 2010; Vater et al., 2002) and is greatly advantageous as it is both easy to conduct and a cheap technique. Direct solvent extraction is also often applied. Thereby, the proper selection of a solvent can target at a specific lipopeptide family (Cazorla et al., 2007; Dimkić et al., 2017; Geissler et al., 2017). As previously indicated, foam fractionation is often integrated into the process in order to handle the severe foaming (Davis et al., 2001; Willenbacher et al., 2014). When the foam fractionation is directly incorporated into the process, this process step is called in situ product removal (ISPR). ISPR was shown to be very promising as a first enrichment step can be obtained and the subsequent number of downstream processing steps can be reduced. Other techniques such as thin-layer chromatography (TLC) (Cazorla et al., 2007), high-performance liquid chromatography (HPLC) (Dunlap et al., 2011), adsorption on resins or charcoal, and membrane ultrafiltration are used among others. Ultrafiltration is a promising approach for the environmental friendly and less time-consuming recovery of biosurfactants with high yields, but equipment costs are high (Isa et al., 2007). Reversed phase HPLC, so far, is probably the most appropriate technique to obtain lipopeptides of high purity.

6.7 CHALLENGES, NEEDS, AND FUTURE TRENDS IN LIPOPEPTIDE PRODUCTION

As highlighted in the previous sections, many studies were successfully employed to increase the yields of lipopeptides. Nevertheless, the complex regulatory system of *Bacillus* spp. still poses a main challenge that results in concentrations and yields to a level far below an industrial-scale application.

Another obstacle often observed is the degradation of surfactin. While iturin and fengycin are mainly produced during the stationary phase, surfactin is synthesized during the late exponential phase (Ongena and Jacques, 2008). Consequently, when the surfactin concentration is monitored during the time course of cultivation, surfactin synthesis mostly strongly correlates with cell growth. As reported, in the transition to the stationary phase, a decrease in surfactin concentration was often reported (Dhanarajan et al., 2014; Maass et al., 2016; Yeh et al., 2006). Consequently, putative mechanisms must be present that degrade lipopeptides. Nitschke and Pastore (2004) proposed that proteolytic activities might lead to a degradation of surfactin. However, surfactin degradation in *B. subtilis* ATCC 21332 exhibiting a high proteolytic activity was not observed. Interestingly, when the crude biosurfactant obtained by this strain was inoculated in culture broth of a strain where a degradation was detected, the recent stable biosurfactant was degraded as well. Maass et al. (2016) named three mechanisms that might be responsible for surfactin degradation. Additional supplements need to be supplied, proteases are present in the culture medium hydrolyzing the peptide moiety of the lipopeptide, or the bacteria itself consume the lipopeptide as these might possess an inhibitory effect at a certain concentration. As they did not observe a complete degradation, Maass et al. (2016) concluded that the last option is the most reasonable. Yeh et al. (2006) assumed that cells assimilate surfactin as carbon source after glucose depletion to maintain cell growth for a certain time. Nevertheless, these observations must be examined in further studies to figure out if the yields might be increased when surfactin is not degraded.

Another aspect that may be addressed in future studies is the complex regulatory mechanism and the lifestyle of *Bacillus* spp. As reported in the study by Jin et al. (2015), a reduced number of spores led to an increase in iturin concentration. However, spore formation is only one out of several differentiation states that *Bacillus* spp. can undergo (Romero, 2013). As a matter of fact, once entered a differentiation state other than surfactin production, cells are no longer able to change their physiological state for surfactin production (Lopez et al., 2009). Further studies addressing this obstacle are necessary especially when aiming at high cell density cultivations. If cells differentiate, the ability to produce surfactin in sufficient amounts is highly limited.

In addition to strain development and process improvement, more studies must be targeted at further real applications and at promoting toward an approval as ingredient in food, cosmetic, and other applications. It was shown that surfactin has a low toxicity (Juola et al., 2014), but although often claimed as being a highly promising biosurfactant, only a few concrete comparisons were made. In addition, more research must be performed exploring the properties of individual lipopeptide congeners, and hence, also appropriate purification techniques need to be established.

6.8 CONCLUSION AND OUTLOOK

This chapter provided information regarding both the synthesis, chemical properties, possible application fields, and an overview of approaches to increase yields.

In summary, it can be concluded that results obtained within a study addressing, for example, media optimization cannot be transferred directly to any other strain. In addition, an issue often facing is the presentation of results. Often, lipopeptide concentrations were determined at a few or just one time point during cultivation. With respect to different growth behavior, it

might be reasonable to assume that the point of maximum productivity was missed. This fact makes it complicated to compare research.

Nevertheless, surfactin is a promising microbial-derived surfactant, and although a large number of potential applications in different industrial sectors have been published, the widespread use of surfactin and other lipopeptides is not conceivable in the foreseeable future. Much research still has to be performed coupling the presented methods and addressing the issues illustrated in this chapter.

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1.3 Research proposal

For a long time, *B. subtilis* was considered being a strict aerobic organism. However, research in the 1990s demonstrated that *B. subtilis* can also grow anaerobically by fermentation or by nitrate respiration and involved genes and regulatory mechanisms were explored (Cruz-Ramos et al. 1995, Hoffmann et al. 1995, Nakano et al. 1996, Sun et al. 1996, Hoffmann et al. 1998, Nakano et al. 1998a). Indeed, the ability to grow both in the presence and absence of oxygen can be attributed to the natural habitat of *B. subtilis* in the soil where the strain faces changes in oxygen availability.

However, studies on the production of *Bacillus* lipopeptides under anaerobic conditions is up to date a little research field but was already shown in 1985 in a culture employing *B. licheniformis* (Jahaveri et al. 1985). Subsequently, Davis et al. (1999) reported on maximal product per biomass yields $Y_{P/X}$ for surfactin under oxygen-depleted nitrate limited conditions culturing strain *B. subtilis* ATCC 21332. A further recent study has demonstrated that the anaerobic cultivation of *B. subtilis* DSM 10^T allows to synthesize surfactin (Willenbacher et al. 2015a) and the $Y_{P/X}$ values surpassed those obtained in aerobic cultures with foam fractionation (Willenbacher et al. 2014). Additionally, Willenbacher et al. (2015a) highlighted the potential of foam-free environments associated with anaerobic cultivations, as conventional aerobic processes are accompanied by severe foaming. In aerobic cultures, indeed, foaming can be reduced or even inhibited completely by mechanical foam breakers or by the addition of antifoam agents. However, mechanical foam breakers increase the energetical input, and antifoam agents can cause difficulties in subsequent downstream processes (St-Pierre Lemieux et al. 2019). Additionally, both techniques can cause cellular stress and consequently reduced productivities (St-Pierre Lemieux et al. 2019). In this sense, the current study aimed at further improving and characterizing the anaerobic growth of *B. subtilis* by nitrate respiration to synthesize surfactin in a foam-free environment. Different strategies were employed to approach a process that is advantageously over conventional aerobic processes.

First, the suitability of a genome reduced *B. subtilis* strain to synthesize surfactin was investigated. It was hypothesized that the employed genome reduction saves metabolic resources and hence surfactin titers were expected to increase. Different temperatures were

tested under both aerobic and anaerobic conditions. The product yields ($Y_{P/X}$, $Y_{P/S}$), biomass yield ($Y_{X/S}$), specific productivity (q) and specific growth rate (μ) were recorded. Cultivation performances of reference strain *B. subtilis* JABs24, as well as the established surfactin-forming wild-type strain *B. subtilis* DSM 10^T were evaluated for comparison.

Second, anaerobic growth of *B. subtilis* JABs24 was furthermore characterized including reporter strains. These reporter strains allow to monitor the expression levels of the promoters P_{narG} and P_{nasD}, as both the nitrate reductase NarGHI and nitrite reductase NasDE are crucial for anaerobic growth by nitrate respiration. The impact of nitrite as metabolic intermediate as well as ammonium as metabolic end product of nitrate respiration were evaluated. In addition, different glucose levels as well as bioreactor cultivations performed as batch and fed-batch processes were run to investigate the impact of varying glucose levels on anaerobic nitrate respiration and surfactin synthesis.

In a third approach, an anaerobically self-inducible surfactin synthesis process was presented. Therefore, the native promoter of the *srfA* operon P_{srfA} was replaced by both P_{narG} and P_{nasD}, which allowed the induction of surfactin synthesis when cells enter oxygen limited conditions. It was hypothesized that a switch from aerobic growth to anaerobic growth is beneficial for surfactin production as advantages of both aerobic and anaerobic growth are combined. Shake flask cultivations with varying oxygen availabilities were performed to demonstrate the proof of concept of the proposed self-inducible surfactin synthesis system. In addition, different bioreactor process strategies targeting at aerobic-anaerobic self-inducible switch processes were presented that aimed at transferring and optimizing the results of shake flask cultivations to bioreactor cultivations.

Chapter 2 Publications

2.1 Evaluation of surfactin synthesis in a genome reduced *Bacillus subtilis* strain

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Evaluation of surfactin synthesis in a genome reduced *Bacillus subtilis* strain

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Abstract

Strain engineering is often a method of choice towards increasing the yields of the biosurfactant surfactin which is naturally synthesized by many *Bacillus* spp., most notably *Bacillus subtilis*. In the current study, a genome reduced *B. subtilis* 168 strain lacking 10% of the genome was established and tested for its suitability to synthesize surfactin under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C. This genome reduced strain was named IIG-Bs20-5-1 and lacks, amongst others, genes synthesizing the lipopeptide plipastatin, the antibiotic bacilysin, toxins and prophages, as well as genes involved in sporulation. Amongst all temperatures tested, 37 °C was overall superior. In comparison to the reference strain JABs24, a surfactin synthesizing variant of *B. subtilis* 168, strain IIG-Bs20-5-1 was both aerobically and anaerobically superior with respect to specific growth rates μ and yields $Y_{X/S}$. However, in terms of surfactin production, strain JABs24 reached higher absolute concentrations with up to 1147.03 mg/L and 296.37 mg/L under aerobic and anaerobic conditions, respectively. Concomitant, strain JABs24 reached higher $Y_{P/S}$ and $Y_{P/X}$. Here, an outstanding $Y_{P/X}$ of 1.541 g/g was obtained under anaerobic conditions at 37 °C. The current study indicates that the employed genome reduced strain IIG-Bs20-5-1 has several advantages over the strain JABs24 such as better conversion from glucose into biomass and higher growth rates. However, regarding surfactin synthesis and yields, the strain was overall inferior at the investigated temperatures and oxygen conditions. Further studies addressing process development and strain engineering should be performed combining the current observed advantages of the genome reduced strain to increase the surfactin yields and to construct a tailor-made genome reduced strain to realize the theoretically expected advantages of such genome reduced strains.

Keywords: Lipopeptide, Biosurfactant, Strain development, Genome reduction, Anaerobic

Introduction

Bacillus subtilis is commonly denoted the model Gram-positive bacterium. Due to its inherent characteristics, such as the natural secretion of proteases, high titers and low toxic by-product formation, this microorganism is an established bacterial platform for a variety of industrial applications (van Dijl and Hecker 2013). Amongst others, processes with *B. subtilis* as industrial host for the synthesis of proteases and riboflavin are implemented (Singh et al. 2017). Another promising metabolite is the biosurfactant surfactin, which is a cyclic lipopeptide synthesized by *Bacillus* spp. Surfactin is attributed with a variety of characteristics. These properties, ranging from exceptional surface-activity and broad spectrum physico-chemical properties, as well as antimicrobial effects, make surfactin an interesting candidate for a variety of applications such as in the agricultural, detergent and food industry (Geissler et al. 2019). However, up to date, neither a strain nor a process was described having the potential of large-scale high-titer production of surfactin as the main target product. Past research in order to increase the yields can be divided in three groups, namely (i) optimization of medium and process parameter (Freitas de Oliveira et al. 2013; Gudiña et al. 2015), (ii) applying diverse process strategies (Coutte et al. 2013; Alonso and Martin 2016) and (iii) performing strain engineering (Coutte et al. 2015; Willenbacher et al. 2016).

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The complete sequence of the *B. subtilis* strain 168 genome, which comprises 4100 protein-encoding genes, was revealed by Kunst et al. (1997) and Barbe et al. (2009) resequenced this genome. Kobayashi et al. (2003) expanded the first sequence by determining essential genes in *B. subtilis* needed to sustain bacterial life. This laid the foundation of creating minimal genome cells (MGC) for *Bacillus*. MGC are defined as cells with a minimal gene set able to sustain life in the unlimited presence of nutrients and in the absence of environmental stress (Koonin 2000). Ideally, a genome reduced strain is expected to have a similar growth behavior and to yield higher product titers as the cells need less energy for genome replication, and dispensable proteins do not have to be transcribed and translated (Choe et al. 2016).

A *B. subtilis* strain derived from strain 168 with a genome reduction of 7.7% was engineered by Westers (2003). The strain lacked genes encoding for prophages as well as AT-rich islands. Under laboratory conditions, the reduction did not affect growth and viability. With respect to the heterologous production of a model protein, a positive effect of the genome reduction was not observed, and authors assumed that a redirection of energy resources into product formation did not occur. Also Ara et al. (2007) applied genome reduction to *B. subtilis* 168 in order to create a strain for the effective production of alkaline cellulase. The genome of the final strain MG1M was 0.99 Mb smaller than its parental strain and the strain showed a similar growth behavior. However, cellulase and protease activity were similar to the parental strain 168 and consequently the product titer was not increased by genome reduction. Within the same group, another genome reduced strain missing 0.87 Mb was created (Morimoto et al. 2008) and this strain was reported to have an improved extracellular and protease productivity from transformed plasmids carrying the respective genes.

Strain 168 is well-suited to employ strain engineering and was hence also used in this study as initial strain. However, strain 168 is only able to synthesize surfactin after the correction of the frameshift mutation in *sfp* (Julkowska et al. 2005; Coutte et al. 2010). To allow for better evaluation of the performed genome reduction, strain 168 with a functional *sfp*$^+$ was used as reference. The employed genome reduced strain within this study also carries a functional *sfp* gene and the genome is ~ 10% smaller. Amongst others, genes encoding for plipastatin, bacylisin, toxins and prophages, as well as genes involved in sporulation, were deleted markerless by Wenzel and Altenbuchner (2015). The type-strain *B. subtilis* DSM 10^T was furthermore used as additional reference strain. Strain DSM 10^T is reported to be a natural high surfactin producing strain yielding up to 1.1 g/L surfactin in shake flask cultivation (Willenbacher et al. 2015b). In addition, for this strain, literature dealing with both the aerobic and anaerobic synthesis of surfactin was reported (Willenbacher et al. 2014, 2015a) giving a reliable comparison to results obtained within this study.

The current study aimed at evaluating the effects of genome reduction on the synthesis of surfactin in *B. subtilis* 168 with a functional *sfp* gene. To get a more fundamental background, the cultivations were performed at different temperatures and under both aerobic and anaerobic conditions. The constructed genome reduced strain was evaluated with respect to biotechnological efficiency parameters including growth rates and yields and compared to the type-strain DSM 10^T as well as the laboratory strain 168 carrying a functional *sfp*.

Materials and methods

Chemicals and materials

All chemicals used were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and were of analytical grade. The reference material for the lipopeptide surfactin (≥ 98%) was obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany).

DNA manipulation, plasmid construction and propagation

Molecular techniques were carried out according to (Green and Sambrook 2012). The plasmids used or constructed in this study are listed in Additional file 1: Table S1 together with their construction procedure. To amplify the desired DNA fragments, polymerase chain reactions (PCRs) were performed using a polymerase (Q5® High-Fidelity DNA Polymerase #M0491S, New England BioLabs®, Frankfurt am Main, Germany). The PCRs were run on a PCR thermal cycler (LifeECO BTC42096, Hangzhou Bioer Technology Co. Ltd., China). As a template for PCR, the chromosomal DNA (cDNA) of *B. subtilis* 168 was used unless otherwise specified. The cDNA of each strain was extracted with a DNA extraction kit (DNeasy® Blood & Tissue Kit from Qiagen (Hilden, Germany) as instructed by the manufacturer. All oligonucleotides used for PCR were synthesized by Eurofins MWG Operons (Ebersberg, Germany) (Additional file 1: Table S2). DNA fragments were digested with restriction enzymes purchased from New England BioLabs® (Frankfurt am Main, Germany). To purify PCR products or DNA fragments cut from agarose gel were purified employing respective kits (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel GmbH, Düren, Germany). The purified DNA fragments were ligated by T4 DNA ligase (Thermo Fisher Scientific, Karlsruhe, Germany). The constructed plasmid DNAs were finally extracted (innuPREP Plasmid Mini Kit, Analytik Jena AG, Jena, Germany) and sequenced

(GATC Biotech AG, Konstanz, Germany). To propagate the desired plasmids, *Escherichia coli* JM109 (Yanisch-Perron et al. 1985) was employed. The transformants of *E. coli* were selected on LB agar supplemented with 100 µg/mL ampicillin or spectinomycin according to the plasmid selection marker.

Construction of the *B. subtilis* strains

All final strains used in this study are given in Table 1 and their construction procedure are thoroughly described in Additional file 1: Table S3. The parental strains of *B. subtilis*, namely the wild-type surfactin producing strain DSM 10^T and the laboratory model strain 168, were purchased from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Transformation of *B. subtilis* strains were performed according to "Paris method" (Harwood and Cutting 1990) and the transformants were selected on LB with 100 µg/mL spectinomycin. As a parental strain for genome manipulation, the genome reduced strain IIG-Bs20-3, a derivative of strain 168 with tryptophan prototrophy and deletion of prophages and antibiotic biosynthesis genes, was used in this study (Wenzel and Altenbuchner 2015) To induce the competence in IIG-Bs20-3, a cassette containing *comK* and *comS* under control of the mannitol-inducible promoter (*mtlA*) was inserted at the 3′-end of the histidine biosynthesis operon as reported by Rahmer et al. (2015) to generate strain IIG-Bs20-5. Markerless integration of the P_{mtlA}-*comKS* cassette was performed based on a histidine auxotrophy system developed by Motejadded and Altenbuchner (2007). To enable the production of surfactin in the desired strains, the frameshift mutation in *sfp* (shown as *sfp**) was removed by transformation of the cells with pJOE8949.1. Plasmid pJOE8949.1 was an integrative plasmid carrying the functional copy of *sfp*. The gene integration was performed based on selection with spectinomycin and anti-selection on LB medium with mannose according to the method of the mannose deletion system (Wenzel and Altenbuchner 2015).

Cultivation conditions and preparation of culture samples

Mineral salt medium and flask preparation

The mineral salt medium investigated by Willenbacher et al. (2014) was used for aerobic cultivations. The final medium contained 1% glucose (*m*/*v*), 4.0×10^{-6} M Na_2 EDTA × 2 H_2O, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4 \times 7$ H_2O, 1.0×10^{-6} M $MnSO_4 \times H_2O$, 0.1 M NH_4Cl, 0.03 M KH_2PO_4, 0.04 M $Na_2HPO_4 \times 2$ H_2O and 8.0×10^{-4} M $MgSO_4 \times 7$ H_2O. For 1 L medium, KH_2PO_4, $Na_2HPO_4 \times 2$ H_2O and NH_4Cl were diluted in a total volume of 969 mL demineralized H_2O and this solution was autoclaved after adjusting the pH to 7 with 10 M NaOH. The remaining components were added afterwards from individual stock solutions: 20 mL of an autoclaved 50% (*m*/*v*) glucose solution, 1 mL of a filter-sterilized trace element solution containing 4×10^{-3} M Na_2 EDTA × 2 H_2O, 7.0×10^{-3} M $CaCl_2$, 4×10^{-3} M $FeSO_4 \times 7$ H_2O, 1×10^{-3} M $MnSO_4 \times H_2O$, and 10 mL of a $MgSO_4$ solution with 8×10^{-2} M $MgSO_4 \times 7$ H_2O. Aerobic cultivations were performed in 1 L baffled shake flasks with 100 mL medium. For anaerobic cultivations, the nitrogen source was replaced by 0.025 M NH_4Cl and 0.1 M $NaNO_3$. 96.9 mL of this solution were filled in 100 mL serum flasks. The flasks were sealed using crimp seals with a septum. Prior to autoclaving, all serum flasks were equipped with a filter in order to allow air outflow. Afterwards, 2 mL of the 50% (*m*/*v*) glucose solution, 0.1 mL of the filter-sterilized trace element stock solution and 1 mL of the $MgSO_4$ stock solution were added using a sterile syringe and canula. To remove residual oxygen in the serum flasks, sterile N_2-gas was purged through the medium for 5 min via filters.

Table 1 Overview of strains used in the current study

Strain	Genotype or description
B. subtilis	
$DSM10^T$	Wild-type strain[a]
JABs24	*trp*$^+$ *sfp*$^+$ Δ*manPA*, (Morabbi Heravi and Altenbuchner 2018) and (Reuß et al. 2017), see electronic supplementary material for detailed strain history
IIG-Bs-20-5-1	*sfp*$^+$ P_{mtlA}-*comKS trp*$^+$ Δ[SPβ] Δ[*skin*] Δ[PBSX] Δ[proΦ1] Δ[proΦ2] Δ[proΦ3] Δ[proΦ4] Δ[proΦ5] Δ[proΦ6] Δ[proΦ7] Δ[*pks*] Δ[*manPA-yjdF-yjdGHI-yjzHJ*] Δ[*sboAX-albABCDEFG*] Δ*ppsABCDE* Δ*bacABCDEF* Δ[*ytpAB-ytoA*] Δ[*sdpABCIR*] Δ[*bpr-spollGA-sigEG*] Δ[*ntdABC-glcP*], (Wenzel and Altenbuchner 2015), see electronic supplementary material for detailed strain history

[a] Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German collection of microorganisms and cell cultures)

Preparation of inoculum cultures

The first pre-culture was prepared by inoculating 20 mL LB medium (5 g/L tryptone, 10 g/L NaCl, 10 g/L yeast extract) with 100 µL of the respective glycerol stock in a 100 mL baffled shake flask. The shake flasks were incubated at 120 rpm and 30 °C for 24 h in an incubator shaker (Newbrunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany). The second pre-culture was prepared in 250 mL baffled shake flasks by diluting the respective LB-pre-culture each 1:10 and 1:20 in a final volume of 50 mL aerobic mineral salt medium. These pre-cultures were incubated for another 12 h and 24 h for the 1:10 and 1:20-dilutions, respectively.

Main culture

All aerobic and anaerobic cultivations were performed at 25, 30, 37 and 40 °C and 120 rpm in an incubator shaker (Newbrunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany). Serum flasks were incubated in a horizontal manner. For each temperature, four shake flasks and four serum flasks were prepared. Each two flasks were inoculated with a time difference of 12 h using the 1:10-dilution pre-cultures for the first set of duplicates, and the 1:20-dilution pre-cultures for the second set of duplicates. All shake flasks and serum flasks were inoculated with a resulting OD_{600} of 0.1.

Sampling and sample analysis

Samples were taken regularly from the individual four flasks to cover every other hour of the growth phase and samples were analyzed regarding the OD_{600}, glucose and surfactin concentrations. The OD_{600} was determined using a spectrophotometer (Biochrom WPA CO8000, Biochrom Ltd., Cambridge, UK). Prior to further analysis, cells were removed by centrifuging for 10 min at 4700 rpm at 4 °C (Heraeus X3R, Thermo Fisher Scientific GmbH, Braunschweig, Germany).

Surfactin was analyzed using a HPTLC system (CAMAG, Muttenz, Switzerland) with a validated method as described previously (Geissler et al. 2017). In brief, a threefold extraction of 2 mL cell-free broth with each 2 mL chloroform/methanol 2:1 (*v*/*v*) was conducted. The pooled solvent layers obtained after each extraction were evaporated to dryness in a rotary evaporator (RVC2-25 Cdplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 10 mbar and 40 °C. For HPTLC analysis, samples were resuspended in 2 mL methanol and applied as 6 mm bands on HPTLC silica gel 60 plates from Merck (Darmstadt, Germany). A surfactin standard curve was applied in the range of 30–600 ng/band. The development was conducted using chloroform/methanol/water (65:25:4, *v*/*v*/*v*) over a migration distance of 60 mm. After the development, the plate was scanned at 195 nm to quantify surfactin.

Glucose concentrations were determined using a HPTLC method as well. Proper diluted cell-free supernatants were applied as 6 mm bands and the plate was developed with acetonitrile/H_2O (85:15, *v*/*v*) over a migration distance of 70 mm. After development, the plate was dipped in the derivatization solution diphenylamine (DPA) for 3 s and the plate was heated for 20 min at 120 °C using the TLC plate heater. DPA reagent was prepared by first dissolving 2.4 g diphenylamine and 2.4 g aniline in 200 mL methanol and then adding 20 mL 85% phosphoric acid.

For further data analysis, the OD/cell dry weight (CDW) conversion factor was determined in a pre-liminary test. Therefore, the strains were cultivated as triplicates as described above for aerobic conditions until reaching the range of maximum OD_{600}. 40 mL culture were filled in dried and pre-weighted falcons and centrifuged for 10 min at 4700 rpm and 4 °C. The supernatant was discarded, and the cell pellet was washed with saline solution prior to a second round of centrifugation. After discarding the supernatant, the weight of the cell pellets were determined after drying the loaded falcons at 110 °C for 24 h and the conversion factor was calculated. In this sense, the OD/CDW conversion factor for all strains used was determined as 3.76 ± 0.17 with a %RSD of 4.47%.

Data analysis

Biomass concentrations (g/L), glucose concentrations (g/L) and surfactin concentrations (g/L) for aerobic and anaerobic cultivations during the time course of cultivation were plotted for the individual strains and temperatures. Several process parameter were calculated for all cultivations conducted. Here, the mean values obtained for two time points were used, labelled m_1 and m_2. The first time point used for calculation was at 0 h of cultivation with all corresponding data. The second time point corresponded to CDW_{max}. Again, all data measured at this time point were used for calculation. In addition, calculations were based on absolute values, as this compensated for the different amounts of samples taken, especially for the long lasting anaerobic cultivations. Using absolute values furthermore allowed for better comparison with literature and future planned bioreactor cultivations.

The biomass yield on substrate $Y_{X/S}$ (g/g), product yield on substrate $Y_{P/S}$ (g/g) and the product yield on biomass $Y_{P/X}$ (g/g) were calculated using Eqs. 1, 2 and 3, respectively.

$$Y_{X/S} = \frac{\Delta m_{CDW}}{\Delta m_{glucose}} \tag{1}$$

$$Y_{P/X} = \frac{\Delta m_{surfactin}}{\left(\frac{(m_{CDW_1} + m_{CDW_2})}{2}\right)} \tag{2}$$

$$Y_{P/S} = \frac{\Delta m_{surfactin}}{\Delta m_{glucose}} \quad (3)$$

The growth rate was calculated using Eq. 4.

$$\mu = \frac{\ln \frac{m_{CDW_2}}{m_{CDW_1}}}{t_2 - t_1} \quad (4)$$

The specific productivity $q_{spec.,surfactin}$ ($g_{surfactin}/g_{CDW}$ h) was calculated using Eq. 5.

$$q_{spec.,surfactin} = \frac{\Delta m_{surfactin}}{\left(\frac{(m_{CDW_1}+m_{CDW_2})}{2}\right) \cdot \Delta t} \quad (5)$$

In order to evaluate the reliability of the obtained data, an overall maximum relative standard deviation (RSD_{max}) was determined for CDW, surfactin concentration and glucose concentration individually. Therefore, the relative standard deviations of the flasks at t_{CDWmax} were used and the mean value of the corresponding duplicates at $t_{0\ h}$. Both the mean RSD (RSD_{mean}) as well as the corresponding standard deviation ($SD_{RSD\ mean}$) were determined for these individual RSD. The RSD_{max} was then calculated as $RSD_{mean} + SD_{RSD\ mean}$, and was determined as 12.23%, 8.53% and 0.10% for CDW, surfactin concentration and glucose concentration, respectively.

Results

We hypothesized that the genome reduced strain *B. subtilis* IIG-Bs20-5-1 shows a similar growth behavior as compared to the reference strain *B. subtilis* JABs24 and yields higher surfactin concentrations. To get a more fundamental background on the effects of genome reduction, the cultivations were performed at four different temperatures and under both aerobic and anaerobic conditions. In addition, the high surfactin producing strain *B. subtilis* DSM 10^T was used as further reference strain to evaluate the overall performance of the two main strains deriving from the non-surfactin producer *B. subtilis* 168.

Aerobic cultivations are superior to anaerobic cultivations with respect to CDW_{max} and surfactin concentration

Figure 1 displays the CDW, glucose consumption and surfactin concentration during the time course of aerobic (A) and anaerobic (B) cultivation employing the strain *B. subtilis* JABs24 at 37 °C. The exemplary graphs depict the main differences for aerobic and anaerobic conditions, which are valid for all strains at all temperatures tested. The main data, including $CDW_{max.}$, $surfactin_{CDW\ max.}$, $glucose_{CDW\ max.}$, $t_{CDW\ max.}$, as well as the overall $surfactin_{max.}$ with the corresponding $t_{overall\ surfactin\ max.}$ are further summarized in Table 2 (aerobic) and Table 3 (anaerobic). Briefly, aerobic cultivations yielded 4.6- to 10-fold higher CDW, reached two to tenfold higher surfactin concentrations and the time of complete glucose consumption and concomitant the time to reach CDW_{max} were much shorter by factors 2 to 6 for all cultivations employed.

Under aerobic conditions, except for strain JABs24 at 25 °C, where a glucose concentration of 0.81 g/L was measured, glucose was completely consumed under aerobic conditions when CDW_{max} was reached. For all strains, the highest CDW was reached at 37 °C with 1.95 g/L, 2.17 g/L and 2.10 g/L for strains JABs24, IIG-Bs20-5-1 and DSM 10^T, respectively. For both IIG-Bs20-5-1 and DSM 10^T, the lowest CDW was obtained at 25 °C with 1.54 g/L and 1.58 g/L, respectively. JABs24 yielded the lowest CDW at 30 °C with 1.27 g/L. Considering the mean CDW_{max} of the three strains at the temperatures tested, the overall %RSD of the CDW_{max} obtained was 15.94%, 12.77% and 10.71% for strains JABs24, IIG-Bs20-5-1 and DSM 10^T, respectively. The time to reach the highest CDW was shorter the higher the temperature was. In comparison, at 25 °C the cultivation time of CDW_{max} varied between 54 and 72 h, whereas at 40 °C glucose consumption was obtained after 15 to 18 h of cultivation. Amongst the three strains tested, strain JABs24 yielded the highest surfactin concentration at 37 °C with 1147.03 mg/L. For strain IIG-Bs20-5-1, the highest concentration was also detected at 37 °C with 993.03 mg/L. Strain DSM 10^T, however, synthesized the highest concentration of 446.12 mg/L at 25 °C, followed by 37 °C with 353.93 mg/L. During the stationary phase, surfactin concentrations further increased by a factor of 1.09 to 2.24. In general, lowest increase was monitored for strain IIG-Bs20-5-1, followed by strain JABs24 and highest increase was obtained for strain DSM 10^T. The final overall highest concentration for the latter strain was monitored at 37 °C with an increase from 353.93 to 793 mg/L, which is lower than the highest concentrations obtained for strains JABs24 and IIG-Bs20-5-1.

As shown in Table 3, summarizing the data for anaerobic cultivations, glucose in general was not completely consumed under anaerobic conditions when CDW_{max} was reached. A trend regarding glucose consumption was not observed amongst the strains and temperatures tested. For example, for strain IIG-Bs20-5-1 with increasing temperature, 4.63 g/L, 0.00 g/L, 0.00 g/L and 3.25 g/L were measured prior to an observed decline in CDW. Similar to aerobic cultivations, the time to reach CDW_{max} was shorter the higher the temperature was set. Excluding strain DSM 10^T, the time varied between 144 and 176 h at 25 °C, and 24 to 42 h at 40 °C. Strain DSM 10^T, however, reached

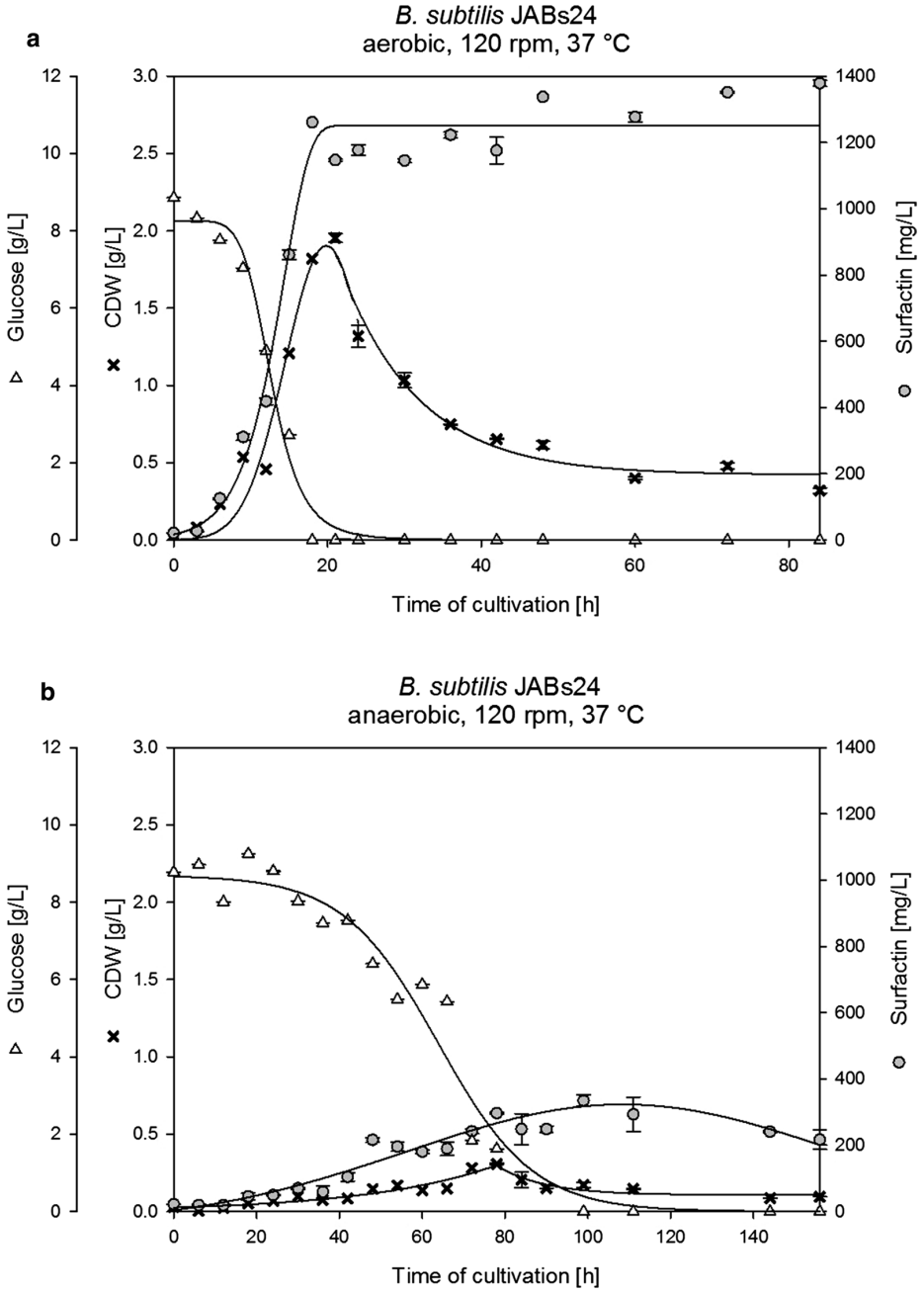

Fig. 1 Exemplary time course of CDW (black cross) and surfactin (grey circle) in comparison to glucose (open triangle) during the cultivation of *B. subtilis* JABs24 under **a** aerobic and **b** anaerobic conditions at 37 °C

the CDW_{max} after 90 h of cultivation at 30 °C, 37 °C and 40 °C. With respect to CDW_{max}, all strains reached the lowest value at 25 °C. Amongst the other temperatures tested, no trend was observed, and data varied between 0.26 and 0.45 g/L. With respect to surfactin, anaerobic cultivations showed a similar pattern as compared to aerobic

Table 2 Summary of the results obtained for aerobic cultivations at various temperatures

Aerobic shake flask cultivation							
T (°C)	*B. subtilis* strain	CDW_{max} (g/L)	$Surfactin_{CDW\ max}$ (mg/L)	$Glucose_{CDW\ max}$ (g/L)	$t_{CDW\ max}$ (h)	Overall $surfactin_{max}$ (mg/L)	$t_{overall\ surfactin\ max}$ (h)
25	JABs24	1.63	766.15	0.81	54	1189.15	156
	IIG-Bs20-5-1	1.54	654.29	0.00	72	970.80	156
	DSM 10^T	1.58	446.12	0.00	60	740.32	84
30	JABs24	1.27	602.98	0.00	36	836.23	42
	IIG-Bs20-5-1	2.12	676.46	0.00	36	740.34	144
	DSM 10^T	1.84	209.06	0.00	30	415.79	156
37	JABs24	1.95	1147.03	0.00	21	1378.50	84
	IIG-Bs20-5-1	2.17	993.03	0.00	18	1083.92	48
	DSM 10^T	2.10	353.93	0.00	18	793.61	60
40	JABs24	1.88	670.58	0.00	18	1079.58	24
	IIG-Bs20-5-1	2.09	681.63	0.00	18	766.72	72
	DSM 10^T	2.04	203.45	0.00	15	406.16	84

Table 3 Summary of the results obtained for anaerobic cultivations at various temperatures

Anaerobic serum flask cultivation							
T (°C)	*B. subtilis* strain	CDW_{max} (g/L)	$Surfactin_{CDW\ max}$ (mg/L)	$Glucose_{CDW\ max}$ (g/L)	$t_{CDW\ max}$ (h)	Overall $surfactin_{max}$ (mg/L)	$t_{overall\ surfactin\ max}$ (h)
25	JABs24	0.26	87.67	0.41	176	87.67	176
	IIG-Bs20-5-1	0.19	79.46	4.63	144	79.46	144
	DSM 10^T	0.16	96.90	5.70	156	96.90	156
30	JABs24	0.26	209.53	5.03	90	209.53	90
	IIG-Bs20-5-1	0.41	189.64	0.00	90	189.64	90
	DSM 10^T	0.33	76.14	6.17	90	146.64	144
37	JABs24	0.31	296.37	1.62	78	333.92	99
	IIG-Bs20-5-1	0.39	215.31	0.00	60	273.38	111
	DSM 10T	0.45	180.65	0.00	90	181.86	111
40	JABs24	0.39	75.80	2.35	42	211.24	90
	IIG-Bs20-5-1	0.26	75.96	3.25	24	158.34	90
	DSM 10^T	0.39	110.03	1.71	90	111.56	84

cultivations. At 37 °C, all strains synthesized the highest amounts of surfactin with 296.37 g/L, 215.31 g/L and 180.65 g/L at CDW_{max}. At 25 °C, the overall $surfactin_{max}$ matched the concentration at CDW_{max}. A similar result was obtained for strains JABs24 and IIG-Bs20-5-1 at 30 °C. The highest increase of surfactin was detected for strain DSM 10^T at 30 °C, as well as for strains JABs24 and IIG-Bs20-5-1 at 40 °C where the concentration reached values of 1.93- to 2.79-fold higher. Considering the overall highest surfactin concentration, strain DSM 10^T was, similar to aerobic cultivations, inferior to strain JABs24 yielding the highest values.

Strain IIG-Bs20-5-1 yields higher growth rates μ and $Y_{X/S}$ than strain JABs24

As illustrated in Fig. 2, the growth rates μ (1/h) increased with increasing temperature for all strains tested under both aerobic and anaerobic conditions. In accordance to the higher CDW_{max} reached and the reduced time to reach CDW_{max}, aerobic cultivations possessed higher growth rates. For example, at 25 °C and 40 °C, strain JABs24 reached a growth rate of 0.070 1/h and 0.236 1/h under aerobic, and of 0.012 1/h and 0.061 1/h under anaerobic conditions. Strain DSM 10^T yielded higher growth rates under aerobic conditions than the other two strains except at 25 °C. The genome reduced strain

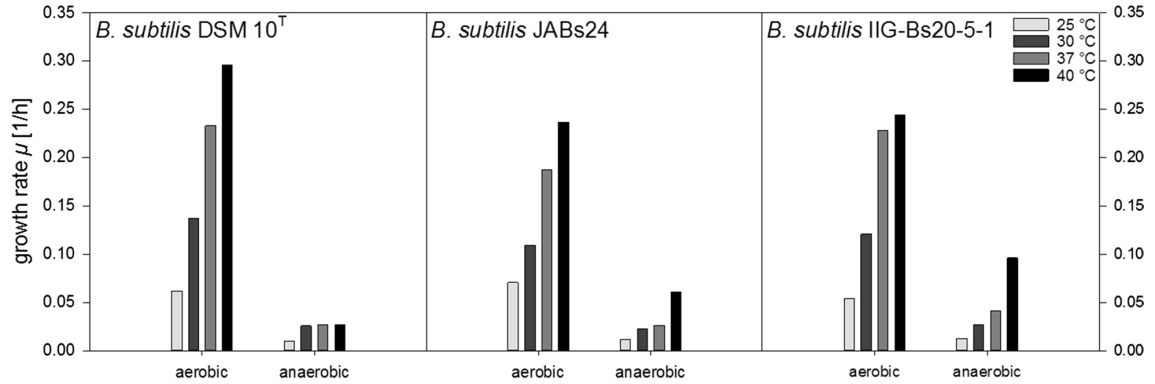

Fig. 2 Overview of the calculated growth rates μ (1/h) for strains *B. subtilis* DSM 10^T, *B. subtilis* JABs24 and *B. subtilis* IIG-Bs20-5-1 under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C

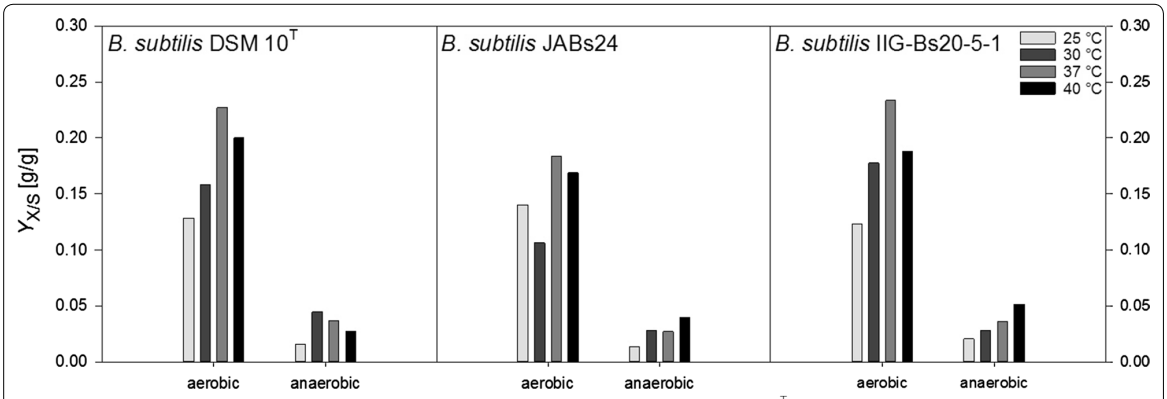

Fig. 3 Overview of the calculated biomass yields on substrate $Y_{X/S}$ (g/g) for strains *B. subtilis* DSM 10^T, *B. subtilis* JABs24 and *B. subtilis* IIG-Bs20-5-1 under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C

furthermore performed better than strain JABs24, albeit the exception of 25 °C. At this condition, JABs24 was superior to both other strains. Under anaerobic conditions, strain IIG-Bs20-5-1 surpassed the growth rates for JABs24 and DSM 10^T at all temperatures tested, with the highest growth rate obtained at 40 °C with 0.096 1/h and the lowest at 25 °C with 0.013 1/h.

Figure 3 depicts the results obtained for the biomass yield on substrate $Y_{X/S}$ (g/g). Under aerobic conditions, the highest conversion from glucose into biomass was obtained at 37 °C with yields of 0.184 g/g, 0.234 g/g and 0.227 g/g for strains JABs24, IIG-Bs20-5-1 and DSM 10^T, respectively. Except for strain JABs24 at 25 °C, values increased with increasing temperature and after reaching a maximum at 37 °C, yields declined at 40 °C. Though, values at 40 °C were still higher than at 30 °C. Under anaerobic conditions, $Y_{X/S}$-values were much lower and did not surpass 0.051 g/g, which was obtained for strain IIG-Bs20-5-1 at 40 °C. In comparison to the data determined for the aerobic cultivations, a similar trend or strain possessing generally the highest conversion at the temperatures tested cannot be emphasized.

Strain JABs24 yields outstanding values for $Y_{P/X}$ with the genome reduced strain being slightly inferior

Figure 4 displays the product yield per substrate $Y_{P/S}$ (g/g) for the different temperatures tested. 37 °C was superior under both aerobic and anaerobic conditions. Similar to the growth rate μ and the $Y_{X/S}$, aerobic calculated $Y_{P/S}$ were much higher than anaerobic values. The highest $Y_{P/S}$ were obtained under aerobic conditions at 37 °C for strain JABs24 with 0.108 g/g and for strain IIG-Bs20-5-1 with 0.105 g/g. These values were about twofold higher than at the other temperatures tested. Under anaerobic cultivations, the $Y_{P/S}$ at 37 °C reached 0.027 g/g and 0.018 g/g, respectively for these two strains. The variation of the

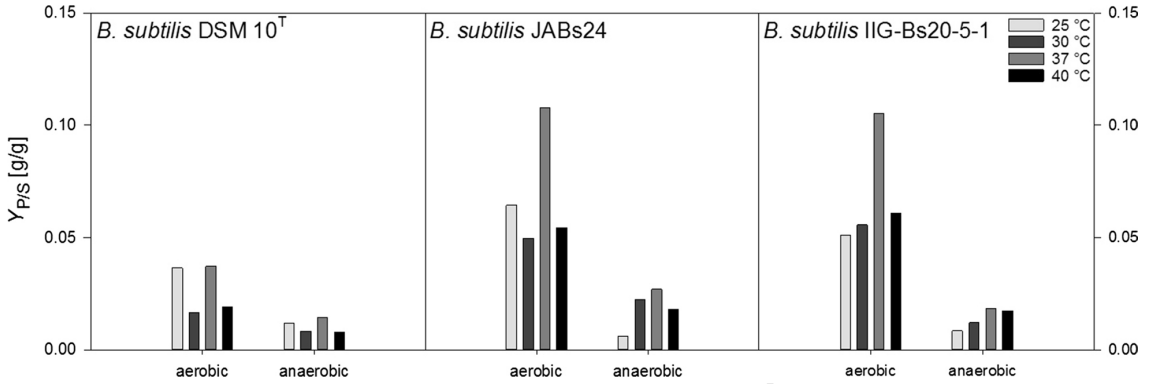

Fig. 4 Overview of the calculated product yields on substrate $Y_{P/S}$ (g/g) for strains *B. subtilis* DSM 10^T, *B. subtilis* JABs24 and *B. subtilis* IIG-Bs20-5-1 under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C

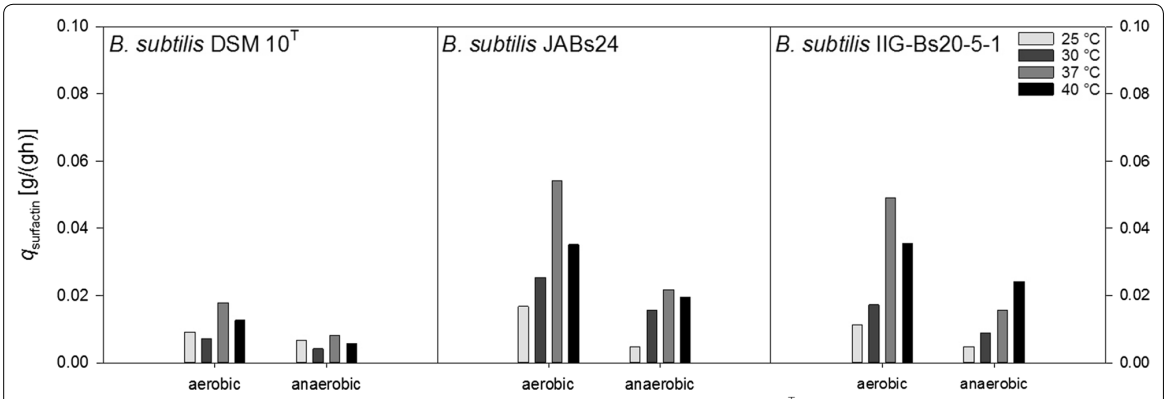

Fig. 5 Overview of the calculated surfactin productivities $q_{surfactin}$ (g/g h) for strains *B. subtilis* DSM 10^T, *B. subtilis* JABs24 and *B. subtilis* IIG-Bs20-5-1 under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C

$Y_{P/S}$ was overall lower than under aerobic conditions. The $Y_{P/S}$ for strain DSM 10^T did not surpass 0.037 g/g under aerobic, and 0.015 g/g under anaerobic conditions, both obtained at 37 °C, which correlates to the overall lower obtained surfactin concentrations.

Figure 5 illustrates the specific productivity $q_{surfactin}$ (g/g h). With respect to the long cultivation times under anaerobic conditions, the values calculated were rather low. Here, a trend could be observed for strain IIG-Bs20-5-1, and the overall productivity increased from 0.005 to 0.024 g/g h with increasing temperature. For strain JABs24, highest productivity was obtained at 37 °C with 0.022 g/g h. Strain DSM 10^T showed the overall lowest productivity and values did not exceed 0.008 g/g h. Under aerobic conditions, 37 °C was superior for all strains tested and productivities of 0.054 g/g h, 0.049 g/g h and 0.018 g/g h were recorded for strains JABs24, IIG-Bs20-5-1 and DSM 10^T, respectively. 40 °C was furthermore superior to 30 °C, similar to the observations for the $Y_{X/S}$.

Figure 6 shows the product yields per biomass $Y_{P/X}$ (g/g). The $Y_{P/X}$ for strain DSM 10^T obtained at 25 °C surpassed the values at the other three temperatures tested and reached 0.541 g/g and 0.942 g/g under aerobic and anaerobic conditions, respectively. 30 °C yielded the lowest values, and 37 °C was furthermore superior to 40 °C. For strain DSM 10^T the $Y_{P/X}$ was 1.48- to 2.58-fold higher under anaerobic conditions at all temperatures run. For strain JABs24, yields obtained anaerobically were 1.02- to 1.32-fold higher at 30 °C to 40 °C, but 0.73-fold lower at 25 °C. The highest yield was determined anaerobically at 37 °C with 1.541 g/g. The overall lowest yield was determined aerobically at 25 °C with 0.627 g/g. Yields determined for the genome reduced strain IIG-Bs20-5-1 were overall slightly lower than for strain JABs24, but in general surpassed the $Y_{P/X}$ of strain DSM 10^T. However,

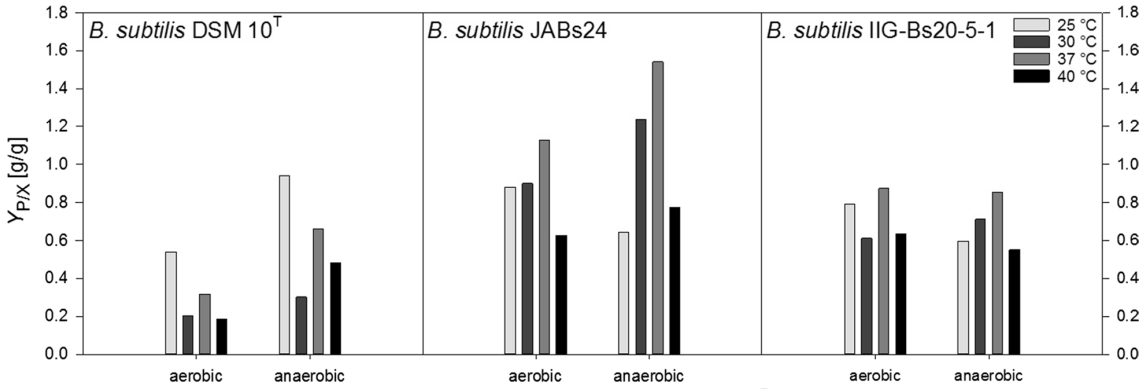

Fig. 6 Overview of the calculated product yields on biomass $Y_{P/X}$ (g/g) for strains *B. subtilis* DSM 10^T, *B. subtilis* JABs24 and *B. subtilis* IIG-Bs20-5-1 under aerobic and anaerobic conditions at 25 °C, 30 °C, 37 °C and 40 °C

other than for the two reference strains, differences in aerobic and anaerobic yields were not as significant and the $Y_{P/X}$ was mostly inferior under anaerobic conditions. For example, the $Y_{P/X}$ were 0.75- to 0.97-fold lower at 25 °C, 37 °C and at 40 °C. At 30 °C, the $Y_{P/X}$ under aerobic and anaerobic conditions were 0.611 g/g and 0.712 g/g, respectively. The overall highest $Y_{P/X}$ were obtained at 37 °C with 0.873 g/g and 0.854 g/g for aerobic and anaerobic cultivations.

Discussion

The current research aimed at investigating the suitability of a genome reduced strain to produce surfactin. We hypothesized that the surfactin yields in strain *B. subtilis* IIG-Bs20-5-1 benefit from the employed genome reduction, and that the strain shows a similar growth pattern than the primary reference strain JABs24.

Evaluation of growth, surfactin synthesis and yields obtained under the conditions tested

The growth behavior of strains 168 *sfp*^{0} and DSM 10^T was reported to be similar by Kabisch et al. (2013) and Julkowska et al. (2005). For specific growth rates, both strains yielded ~0.450 1/h (Kabisch et al. 2013), and strain BBG258, a 168 *sfp*$^+$ derivative, yielded 0.499 1/h in a study conducted by Dhali et al. (2017). However, although the aerobic growth rates obtained within this study were much lower with < 0.234 1/h, it must be noted that strain 168 varies between laboratories due to domestication, and DSM 10^T is reported to have different variants as well, such as ATCC 6051 (Kabisch et al. 2013) and NCBI 3610 (Julkowska et al. 2005). Hence, the assignment remains a challenge and comparisons are hindered (Zeigler et al. 2008). Contrariwise to literature, considering the overall growth rates obtained within this study at 30 °C, 37 °C and 40 °C, strain DSM 10^T was superior to strain JABs24 under aerobic conditions. It might be that the restoration of *sfp* led to a reduced growth rate, as secondary metabolites such as lipopeptides are thought to be metabolically demanding as they are synthesized by large non-ribosomal peptide synthetases (Fischbach and Walsh 2006). In addition, *B. subtilis* undergoes a complex machinery of cell differentiation with surfactin being an important signal molecule (López and Kolter 2010). Reestablishing of surfactin synthesis is expected to change the overall cellular differentiation process, as reported by Julkowska et al. (2005) where surfactin induced swarming of cells. Interestingly, under anaerobic conditions strain DSM 10^T was inferior to JABs24 and the genome of JABs24 hence might be able to better adapt to anaerobic conditions.

Next to the growth rate, data obtained for the surfactin concentrations for strains JABs24 (Ongena et al. 2007; Coutte et al. 2010) and DSM 10^T (Willenbacher et al. 2015b), as well as for the anaerobic yields calculated for strain DSM 10^T are well in accordance to literature (Willenbacher et al. 2015a). However, next to the genetic alterations of strains used in different studies, comparisons are also difficult when different media or process set-ups and parameter are used as in Willenbacher et al. (2014). In this study, strain DSM 10^T was cultivated in a 2.5 L benchtop bioreactor under aerobic conditions with surfactin recovery employing foam fractionation. $Y_{P/X}$, $Y_{X/S}$ and $Y_{P/S}$ obtained were 0.192 g/g, 0.268 g/g and 0.052 g/g. In the current study, at 30 °C, the $Y_{P/X}$ was in a similar range with 0.204 g/g, and both $Y_{X/S}$ and $Y_{P/S}$ were much lower with 0.158 g/g and 0.017 g/g, respectively.

Also with respect to the temperature, different strains, media and parameter used make it difficult to draw meaningful conclusions. Nevertheless, within this study,

37 °C was overall superior for all parameter tested and amongst the strains cultivated, which is well in accordance to different literature (Sen and Swaminathan 1997; Rahman and Ano 2009; Ghribi et al. 2012; Meena et al. 2018).

Davis et al. (1999) examined different batch cultures and reported that the highest $Y_{P/X}$ was obtained under nitrate-limited oxygen-depleted conditions with 0.075 g/g. In comparison, aerobic nitrogen-limited conditions yielded only 0.021 g/g. However, although the current results also indicated that, regardless nitrogen-limitation, anaerobic cultivations yielded higher $Y_{P/X}$, literature often reports that surfactin production is favored at good oxygenation (Yeh et al. 2006; Abdel-Mawgoud et al. 2008; Fahim et al. 2012; Ha et al. 2018). Although the $Y_{P/X}$ is often not calculated, data presented by Abdel-Mawgoud et al. (2008) also indicated that the $Y_{P/X}$ is increased at higher aeration in a medium containing only nitrate as sole nitrogen source. In the current study the $Y_{P/X}$ was generally superior under anaerobic conditions, but it has to be further elucidated which mechanisms are causing this effect. For example, the presence of nitrate itself and the concomitant nitrate respiration might result in the overall higher $Y_{P/X}$. As *B. subtilis* is also able to grow aerobically on nitrate as sole nitrogen source, further studies are desirable to evaluate the effect of the nitrogen source nitrate, especially as *B. subtilis* contains two nitrate reductases, with NasBC being active both aerobically and anaerobically in the assimilatory pathway, and NarGHJ being only induced anaerobically in the presence of the alternative electron acceptor nitrate (Nakano et al. 1998; Ye et al. 2000).

To sum up, the results obtained within the current study are comparably reliable and especially the high $Y_{P/X}$ of 1.127 g/g and 1.541 g/g obtained for strain JABs24 under both aerobic and anaerobic conditions at 37 °C must be emphasized which surpasses many reported $Y_{P/X}$ in literature for different process set-ups (Davis et al. 1999; Chtioui et al. 2012; Willenbacher et al. 2014, 2015a; Coutte et al. 2015; Dhali et al. 2017). Coutte et al. (2010) also reached a high $Y_{P/X}$ of 1.08 g/g in a 168 sfp^+ derivative strain, but it has to be pointed out that a functional *sfp* from *B. subtilis* ATCC 21332 was integrated instead of restoration of the original *sfp* as performed in this study. In this sense, with the $Y_{P/X}$ being superior for strain JABs24, the most crucial question is now, if the genome reduction led to a benefit as hypothesized.

Strains JABs24 vs. IIG-Bs20-5-1—evaluation of the effect of genome reduction

Constructing genome reduced strains is an interesting technique. The designed strains ideally show a similar or even better growth behavior than the parental strain (Choe et al. 2016). In addition, when a strain shall be used as catalyst for the synthesis of a specific product, genome reduced strains are expected to yield higher titers as the deletion of non-essential genes saves metabolic resources (Choe et al. 2016). In 2008, the suitability of genome reduced *B. subtilis* with the aim of producing a target product was reported by Morimoto et al. (2008). However, the product used within that study was synthesized from a plasmid carrying the respective gene. In the current study, the target product surfactin is naturally produced by *B. subtilis* and the synthesis is based on a complex quorum sensing system (Hamoen 2003). Dhali et al. (2017) reported an increase in surfactin yield from 527 ± 80 to 1556 ± 123 mg/g CDW by deleting the gene encoding for CodY in a 168 sfp^+ derivative. In our study, we expected an increase in surfactin synthesis as, for example, (i) the gene *pps*, which encodes for the large non-ribosomal peptide synthetase of the lipopeptide plipastatin was deleted and the cell can hence save resources, (ii) sporulation factors were deleted which influence cell differentiation and a higher ratio of cells might become surfactin producers, (iii) prophages were deleted which is expected to, amongst others, reduce the formation of autolysins and hence cell lysis.

A strain with several identical deletions was constructed by Westers (2003) and they reported that the strain possessing a 7.7% smaller genome exhibited a comparable growth behavior and biomass yield on substrate under laboratory conditions as the reference strain. However, they also pointed out that the suitability of their constructed genome reduced strain as bacterial cell factory remains open. In our study, the genome reduced strain IIG-Bs20-5-1 yielded either similar or even higher CDW_{max}, but especially under anaerobic conditions, the time of cultivation to reach CDW_{max} was shorter than for strain JABs24. This observation is further on reflected in the determined growth rate μ, which is overall both aerobically and anaerobically higher for strain IIG-Bs20-5-1. The $Y_{X/S}$ of strain IIG-Bs20-5-1 also surpassed the respective yields of strain JABs24 at almost all conditions tested, indicating a better conversion efficiency from glucose into biomass. Comparing the data for the growth rate μ, $Y_{X/S}$ and the time to reach CDW_{max} of strain JABs24 and IIG-Bs20-5-1, it gives the idea that the employed genome reduction has a higher positive impact especially under anaerobic conditions and at 37 °C. However, the better growth and the obtained higher biomass did not lead to higher surfactin concentrations and product yields per biomass $Y_{P/X}$. In both parameter, strain JABs24 was superior. With some exceptions, aerobically at 30 °C and 40 °C, and anaerobically at 40 °C, strain IIG-Bs20-5-1 yielded higher surfactin concentrations at CDW_{max}. However, apart from the surfactin concentration at

CDW_{max}, the overall $surfactin_{max}$ was higher for JABs24 throughout all experiments. The $Y_{P/X}$ for strain JABs24 was beyond that generally higher under anaerobic conditions, which is well in accordance to the observations from Willenbacher et al. (2015a). Interestingly, the $Y_{P/X}$ was in a similar range for strain IIG-Bs20-5-1 under aerobic and anaerobic conditions at the respective temperatures, indicating that the genome reduction has a rather negative effect on the surfactin synthesis under anaerobic conditions. In this context, the employed genome reduction led to improved growth rates and $Y_{X/S}$, but did neither positively influence the surfactin synthesis nor productivity, nor the yields $Y_{P/S}$ and $Y_{P/X}$.

To sum up, aerobic cultivations were superior with respect to μ, $Y_{X/S}$ and $Y_{P/S}$, while anaerobic cultivations overall yielded better $Y_{P/X}$, especially for strain JABs24. In comparison to the reported high surfactin producing wild-type DSM 10^T, both strain JABs24 as well as the genome reduced strain IIG-Bs20-5-1 reached excellent values for surfactin concentration and $Y_{P/X}$. In direct comparison, the genome reduced strain was superior to its main reference strain with respect to both the growth rate μ and $Y_{X/S}$. Anaerobic conditions furthermore favored the growth and conversion from glucose into biomass for IIG-Bs20-5-1 compared to strain JABs24, however, the surfactin synthesis was negatively affected anaerobically. Although hypothesized that the genome reduced strain leads to better surfactin yields, strain JABs24 reached outstanding surfactin concentrations aerobically and $Y_{P/X}$ values anaerobically.

Additional file

Additional file 1: Table S1. Plasmids used in this study. **Table S2.** Oligonucleotides used in this study. **Table S3.** Strains used in this study.

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Authors' contributions
MG planned and executed the experiments, collected data, created the graphs and drafted the manuscript. IK performed part of the experiments and collected and evaluated corresponding data. KMH and JA constructed the strains and contributed to interpretation of the experiments. MH significantly contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

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Availability of data and materials
Data relevant to this study which is not included in the manuscript is available as additional file. Please turn to the corresponding author for all other requests.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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2.2 Towards the anaerobic production of surfactin using *Bacillus subtilis*

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Towards the Anaerobic Production of Surfactin Using *Bacillus subtilis*

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The anaerobic growth of *B. subtilis* to synthesize surfactin poses an alternative strategy to conventional aerobic cultivations. In general, the strong foam formation observed during aerobic processes represents a major obstacle. Anaerobic processes have, amongst others, the distinct advantage that the total bioreactor volume can be exploited as foaming does not occur. Recent studies also reported on promising product per biomass yields. However, anaerobic growth in comparison to aerobic processes has several disadvantages. For example, the overall titers are comparably low and cultivations are time-consuming due to low growth rates. *B. subtilis* JABs24, a derivate of strain 168 with the ability to synthesize surfactin, was used as model strain in this study. Ammonium and nitrite were hypothesized to negatively influence anaerobic growth. Ammonium with initial concentrations up to 0.2 mol/L was shown to have no significant impact on growth, but increasing concentrations resulted in decreased surfactin titers and reduced nitrate reductase expression. Anaerobic cultivations spiked with increasing nitrite concentrations resulted in prolonged lag-phases. Indeed, growth rates were in a similar range after the lag-phase indicating that nitrite has a neglectable effect on the observed decreasing growth rates. In bioreactor cultivations, the specific growth rate decreased with increasing glucose concentrations during the time course of both batch and fed-batch processes to less than 0.05 1/h. In addition, surfactin titers, overall $Y_{P/X}$ and $Y_{P/S}$ were 53%, ∼42%, and ∼57% lower than in serum flask with 0.190 g/L, 0.344 g/g and 0.015 g/g. The $Y_{X/S}$, on the contrary, was 30% lower in the serum flask with 0.044 g/g. The productivities q were similar with ∼0.005 g/(g·h). However, acetate strongly accumulated during cultivation and was posed as further metabolite that might negatively influence anaerobic growth. Acetate added to anaerobic cultivations in a range from 0 g/L up to 10 g/L resulted in a reduced maximum and overall growth rate μ by 44% and 30%, respectively. To conclude, acetate was identified as a promising target for future process enhancement and strain engineering. Though, the current study demonstrates that the anaerobic cultivation to synthesize surfactin represents a reasonable perspective and feasible alternative to conventional processes.

Keywords: *Bacillus subtilis*, anaerobic cultivation, lipopeptide, surfactin, process control strategy, nitrate respiration, acetate, foam-free

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INTRODUCTION

The cyclic lipopeptide surfactin synthesized by *Bacillus subtilis* is often described as a promising alternative to surfactants of petrochemical and oleochemical origin (Henkel et al., 2017) with additional antimicrobial properties (Ongena and Jacques, 2008; Li et al., 2019). However, conventional aerobic processes targeting at surfactin production share one major bottleneck, namely excessive foaming. The presence of foam in biotechnological processes often results, amongst others, in lower productivity (St-Pierre Lemieux et al., 2019). Excessive foaming may hinder probes to measure correctly, blocks exhaust air filters and hence pressure increases, and leads to heterogeneity in the cultivation broth (St-Pierre Lemieux et al., 2019). Different processes targeting surfactin production were reported that either integrate or avoid foaming. Integration of foaming is mostly performed as *in situ* product removal (Cooper et al., 1981; Davis et al., 2001; Chen et al., 2006; Willenbacher et al., 2014). Here, the ability of surfactin to accumulate at air-liquid interfaces is used as advantage and can be regarded as a first enrichment and purification step. Nevertheless, the uncontrolled foaming is reported to hinder process control and next to surfactin, also producer cells and the medium is lost for further cultivation (Willenbacher et al., 2014; Rangarajan and Clarke, 2015; Coutte et al., 2017). The membrane-bioreactor presented by Coutte et al. (2010) is an alternative foam-free cultivation strategy. This set-up yielded concentrations of 0.242 g/L surfactin. However, productivity was reduced during the time course of cultivation due to the adsorbance of surfactin onto the membranes which further reduced oxygen transfer. Chtioui et al. (2012) designed a rotating disk bioreactor where surfactin was produced by *B. subtilis* ATCC 21332 both in free cells and cells immobilized as a biofilm on the rotating disks. Aeration was performed above the liquid level and was reported to not be sufficient and surfactin concentrations did not surpass 0.212 g/L. Another strategy to synthesize surfactin was illustrated by Davis et al. (1999). Different batch cultivations with e.g., nitrate-limitation, carbon-limitation or oxygen-limitation demonstrated that highest specific product yield per biomass ($Y_{P/X}$) was achieved in nitrate-limited oxygen-depleted cultures. The authors reported that anaerobic growth occurred in oxygen-depleted conditions. However, aeration was still maintained at 0.5 vvm indicating the presence of microaerophilic conditions. This strategy was further adapted by Willenbacher et al. (2015a) employing strain *B. subtilis* DSM 10^T. Anaerobic conditions were obtained as aeration was completely avoided, which also resulted in a foam-free environment without the need of adding antifoam. This process reached high values for the product yield per biomass $Y_{P/X}$ with 0.278 g/g employing 2.5 g/L glucose. The anaerobic cultivation takes advantage of the ability of *B. subtilis* to use nitrate as alternative electron acceptor in the absence of oxygen. During nitrate respiration, nitrate is reduced to ammonium via nitrite using the enzymes nitrate reductase NarGHI and nitrite reductase NasDE (Nakano et al., 1998a). A recent study demonstrated that anaerobic serum flask cultivations employing strain *B. subtilis* JABs24, which is the well-established laboratory strain 168 with the ability to synthesize surfactin due to integration of a functional *sfp* gene, reached excellent values for $Y_{P/X}$ with 1.541 g/g and these values surpassed aerobic results (Geissler et al., 2019b). Next to the foam-free environment that can be achieved employing anaerobic cultivations, another advantage is the more than hundred thousand times higher solubility of nitrate compared to oxygen in the medium. This allows for more flexibility in the design of batch and fed-batch processes. Furthermore, the development of nitrate respiration processes might generally be beneficial for products sensitive against oxidation. However, recent studies of both Willenbacher et al. (2015a) and Geissler et al. (2019b) have reported a much lower cell dry weight accompanied by overall inferior surfactin titers compared to aerobic counterparts. As a consequence, this study aimed at further evaluating the relevance of nitrite and ammonium as well as the impact of glucose concentrations for an envisioned foam-free anaerobic surfactin bioproduction process.

We hypothesized that the presence of both nitrite and ammonium has a negative impact on anaerobic nitrate respiration, while different initial glucose concentrations play a minor role. To further substantiate this hypothesis, reporter strains carrying P_{narG}-*lacZ* and P_{nasD}-*lacZ* fusion were included to evaluate effects on the most important enzymes during anaerobic nitrate respiration.

MATERIALS AND METHODS

Chemicals and Materials

All chemicals used were of analytical grade and were purchased from Carl Roth GmbH & Co., KG (Karlsruhe, Germany). The surfactin reference standard (≥98% purity) and glucose standard were obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany).

Microorganisms, Genetic Engineering and Strain Maintenance

All strains used within this study are listed in **Table 1**. Strain *B. subtilis* JABs24, constructed as described in Geissler et al. (2019b), is derived from the laboratory strain 168 with functional *sfp* and was used as initial strain for the construction of the reporter strains MG1 and MG5. The oligonucleotides (Eurofins Genomics Germany GmbH, Ebersberg, Germany) and plasmids used are listed in **Supplementary Table S1** and **Table 2**, respectively. The promoter regions of *narG* and *nasD* were amplified through PCR (peqSTAR 96X, VWR GmbH, Darmstadt, Germany) using primers s1001/s1002, and s1011/s1012, respectively. The PCR products were purified (GeneMATRIX basic DNA purification Kit, EURx Sp. Z o.o, Gdańsk, Poland) and ligated into plasmid pJOE4786.1 (Altenbuchner et al., 1992) with T4 DNA ligase (New England BioLabs GmbH, Frankfurt am Main, Germany) after digestion with *Sma* I (New England BioLabs GmbH, Frankfurt am Main, Germany). The obtained plasmids, pKAM0182 for P_{narG} and pSHX1 for P_{nasD}, were transformed into chemical competent

TABLE 1 | Overview of strains used in the current study.

Strain	Genotype or description	References
B. subtilis		
JABs24	*trp*$^+$ *sfp*$^+$ Δ*manPA*	Geissler et al. (2019b)
MG1	*trp*$^+$ *sfp*$^+$ Δ*manPA amyE::*[P_{narG}-*lacZ, spcR]*	This study
MG5	*trp*$^+$ *sfp*$^+$ Δ*manPA amyE*::[P_{nasD}-*lacZ, spcR*]	This study
E. coli		
JM109	*mcrA recA1 supE44 endA1 hsdR17* ($r_K^- m_K^+$) *gyrA96 relA1 thi* Δ*(lac-proAB) F'* [*traD36 proAB*$^+$ *lacI*^{q} *lacZ* Δ*M15*]	Yanisch-Perron et al. (1985)

TABLE 2 | Plasmids used in this study.

Plasmid	Properties or insert	References
pJOE4786.1	ori_{pUC18}, *bla*, *ter-'lacI-lacZα-ter*	Altenbuchner et al. (1992)
pKAM0182	pJOE4786.1 + PCR s1001 – s1002 (*Sma* I)	This study
pSHX1	pJOE4786.1 + PCR s1011 – s1012 (*Sma* I)	This study
pKAM312	ori_{pBR322}, *rop*, *ermC*, *bla*, *amyE'-*[*ter-*P_{glcR}-*lacZ-spcR*]*-'amyE*	Morabbi Heravi and Altenbuchner (2018)
pKAM452	pKAM312 containing promoter region of *narG* (pKAM0182), integrated by *Age* I and *Nde* I	This study
pSHX2	pKAM312 containing promoter region of *nasD* (pSHX1), integrated by *Age* I and *Nde* I	This study

E. coli JM109. Strains carrying the plasmid were selected on LB plates supplemented with ampicillin (100 μg/mL). Isolated plasmids pKAM0182 and pSHX1 (QIAamp DNA Mini Kit (50), QIAGEN GmbH, Hilden, Germany) were digested with *Nde* I and *Age* I (New England BioLabs GmbH, Frankfurt am Main, Germany) and fragments of interest were isolated (MinElute Gel Extraction Kit (50), QIAGEN GmbH, Hilden, Germany). Afterwards, digestion products were ligated into pKAM312 (Morabbi Heravi and Altenbuchner, 2018) resulting in pKAM452 and pSHX2 and transformed in competent *E. coli* JM109. Plasmids pKAM452 (P_{narG}-*lacZ*) and pSHX2 (P_{nasD}-*lacZ*) were isolated and were transformed into natural competent *B. subtilis* JABs24. By double cross-over into the *amyE* gene, reconstructed strains were selected by agar plates supplemented with either spectinomycin (100 μg/mL) or erythromycin (10 μg/mL), and by starch plates dyed with Lugol's iodine solution. Positive colonies were further checked by PCR using primers s7406 and s7409 to confirm insertion of P_{narG}-*lacZ* and P_{nasD}-*lacZ* into *amyE* gene.

Media Composition

The LB medium used for the first pre-culture composed of 5 g/L tryptone, 10 g/L NaCl and 10 g/L yeast extract. An adapted medium based on the medium described by Willenbacher et al. (2015b) was used for all further pre-cultures and main cultures. The glucose concentration was 20 g/L for the second mineral salt pre-culture, 10 g/L and 2.5 g/L glucose for the batch and fed-batch bioreactor process, as well as either 2.5, 5, 7.5, or 10 g/L glucose for the serum flask cultivations as indicated in the respective results. The buffer composed of 0.03 mol/L KH_2PO_4 and 0.04 mol/L $Na_2HPO_4 \cdot 2\ H_2O$ in the mineral salt pre-culture and serum flask cultivations, and $5.71 \cdot 10^{-3}$ mol/L KH_2PO_4 and $4.29 \cdot 10^{-3}$ mol/L $Na_2HPO_4 \cdot 2\ H_2O$ in the bioreactor cultivations. The nitrogen source used for the mineral salt pre-culture was 0.1 mol/L $NaNO_3$ and for the bioreactor and serum flask cultivation 0.1 mol/L $NaNO_3$ and $5.0 \cdot 10^{-4}$ mol/L $(NH_4)_2SO_4$. In case of $MgSO_4$ and trace element solution, which were prepared separately as autoclaved, respectively, filter sterilized stock solutions, all cultivations had the same final concentrations with $8.0 \cdot 10^{-6}$ mol/L Na_3-citrate, $7.0 \cdot 10^{-6}$ mol/L $CaCl_2$, $4.0 \cdot 10^{-6}$ mol/L $FeSO_4 \cdot 7\ H_2O$, $1.0 \cdot 10^{-6}$ mol/L $MnSO_4 \cdot H_2O$, $4.0 \cdot 10^{-6}$ mol/L $Na_2MoO_4 \cdot 2\ H_2O$ and $8.0 \cdot 10^{-4}$ mol/L $MgSO_4 \cdot 7\ H_2O$. A 25% (*w*/*w*) autoclaved (121°C, 20 min) glucose solution was used for the bioreactor feed. For the cultivations investigating the effect of ammonium, the concentration of $(NH_4)_2SO_4$ was adjusted. In case of nitrite and acetate experiments, the targeted concentrations were added from an autoclaved 0.1 mol/L $NaNO_2$ or 277.88 g/L Na-acetate stock solution, respectively.

Cultivation Conditions

Pre-cultures were run at 120 rpm and 37°C in an incubator shaker (NewbrunswickTM/Innova 44, Eppendorf AG, Hamburg, Germany). A first overnight pre-culture was performed in a 100 mL baffled shake flask by inoculating 20 mL LB medium with 100 μL of the respective glycerol stock. This pre-culture was diluted 1:100 in mineral salt medium for a second pre-culture and incubated for 36 h. The shake flask size was adjusted to the amount of inoculation material needed and flasks were filled with 10–13% of the mineral salt medium.

Anaerobic Serum Flask Cultivation

Anaerobic serum flasks were prepared as described in Geissler et al. (2019b). Briefly, the buffer and nitrogen sources were autoclaved inside the flasks and all other solutions were added afterwards through a disinfected septum using a syringe. Anaerobic conditions were set by flushing the flasks with nitrogen and degassing through a filter element.

Anaerobic Bioreactor Cultivation

Bioreactor cultivations were performed in 42 L custom-built bioreactors (ZETA GmbH, Graz/Lieboch, Switzerland). The bioreactors are mounted on a scale and are equipped with pH (EasyFerm Bio HB Arc 120, Hamilton Bonaduz AG, Bonaduz, Switzerland) and pO_2 (VisiFerm DO ARC 120 H0, Hamilton Bonaduz AG, Bonaduz, Switzerland) probes. Acid, base and feed solutions were on individual scales and added via peristaltic pumps. Bioreactors were equipped with three Rushton turbines and four baffle plates. The buffer and nitrogen source were autoclaved inside the bioreactor and the other components were added sterile through a septum. Prior to inoculation, the medium was flushed with N_2 to ensure anaerobic conditions and pO_2 measurement throughout cultivation confirmed absence of oxygen. Stirrer speed was kept at 120 rpm and pH 7 was maintained by adding either 1 mol/L NaOH or 1 mol/L H_3PO_4. Temperature was set to 37°C.

Sampling and Sample Analysis

For all cultivations performed, samples were taken in regular intervals. The OD_{600} was measured with a spectrophotometer (Biochrom WPA CO8000, Biochrom Ltd., Cambridge, United Kingdom). The cell dry weight was calculated by dividing the OD_{600} by the factor 3.762 which was determined previously (Geissler et al., 2019b). Prior to further analyses, samples were centrifuged for 10 min at 4°C and 4816 *g* (Heraeus X3R, Thermo Fisher Scientific GmbH, Braunschweig, Germany) and stored at −20°C until further processing.

Glucose was measured with a HPTLC system (CAMAG AG, Muttenz, Switzerland) as described in Geissler et al. (2019b). Briefly, the mobile phase used was acetonitrile/H_2O (85:15, *v*/*v*) and plates were developed over a migration distance of 70 mm. After development, plates were derivatized with diphenylamine (DPA) reagent. DPA was prepared by diluting 2.4 g diphenylamine and 2.4 g aniline in 200 mL methanol and then adding 20 mL 85% phosphoric acid. After derivatization, plates were scanned at 620 nm and the glucose concentration was calculated in dependence of the standard curve.

Surfactin analysis was performed as described in Geissler et al. (2017) using a HPTLC method. Briefly, samples were extracted three times with an equal volume of chloroform:methanol 2:1 (*v*/*v*). The pooled solvent layers were evaporated and the crude surfactin was resuspended in methanol to match the initial sample volume. Plates were developed using the mobile phase chloroform:methanol:water 65:25:4 (*v*/*v*/*v*) over a migration distance of 60 mm. After development, the plates were scanned at 195 nm and evaluation was performed by peak area in correspondence to a standard curve.

Spectrophotometric assays (Merck KGaA, Darmstadt, Germany) were used to measure nitrate (Cat. No. 1.09713.0001), nitrite (Cat. No. 1.14776.0001) and ammonium (Cat. No. 1.14752.0001) concentrations.

Acetate concentration was determined with an enzymatic kit (Cat. No. 10148261035, r-biopharm AG, Pfungstadt, Germany).

For ß-galactosidase assay, a volume of 100 µL of cell suspension from strain MG1 or MG5 was mixed with 900 µL Z-Buffer (0.06 mol/L Na_2HPO_4, 0.04 mol/L NaH_2PO_4, 0.01 mol/L KCl, 1 mmol/L $MgSO_4 \cdot 7\ H_2O$, 0.04 mol/L mercaptoethanol). After addition of 10 µL toluol, the mixture was incubated for 30 min at 37°C and 750 rpm. 200 µL of 20 mmol/L *ortho*-nitrophenylgalactopyranoside was added and the reaction was stopped when the solution turned yellow by adding 500 µL of 1 mol/L Na_2CO_3. Samples were centrifuged for 2 min at 19283 *g* and 250 µL were transferred to a microtiter plate. Absorbance was measured at both 420 nm and 550 nm and the Miller Units (MU) were calculated according to the following equation:

$$MU = 1000 \cdot \frac{(OD_{420\,nm} - (1.75 \cdot OD_{550\,nm}))}{t \cdot \upsilon \cdot OD_{600\,nm}}$$

Data Analysis

For the bioreactor cultivations performed, as well as for serum flask cultivations if required, the biomass yield per substrate $Y_{X/S}$ [g/g], product yield per substrate $Y_{P/S}$ [g/g], the product yield per biomass $Y_{P/X}$ [g/g], the specific surfactin productivity q [g/(g·h)] and the specific growth rate μ [1/h] were determined. The respective equations are listed in the **Supplementary Material (S2)**. Depending on the evaluation, either surfactin or acetate was considered as product P. These parameters were calculated in two distinct approaches. Maximum yields were determined by calculating the respective parameter in between sampling points and overall yields were calculated based on the data of inoculation and at CDW_{max} of the process. For the bioreactor cultivation employing a feed, the glucose fed at sampling was added to the respective time point.

RESULTS AND DISCUSSION

Influence of Different Ammonium Concentrations on Anaerobic Growth and Effect on Promoter Activity of P_{narG} and P_{nasD}

Under aerobic conditions, ammonium is the preferred nitrogen source and in the presence of both ammonium and nitrate, nitrate consumption is induced after depletion of ammonium (Davis et al., 1999). During anaerobic nitrate respiration, however, nitrate is used as alternative electron acceptor and is thereby reduced to nitrite which is further reduced to ammonium by the enzymes nitrate reductase NarGHI and nitrite reductase NasDE, respectively (Hoffmann et al., 1998; Nakano et al., 1998a; Härtig and Jahn, 2012). As these enzymes are crucial for anaerobic nitrate respiration, the corresponding gene expressions were monitored by the respective promoters P_{narG} and P_{nasD}. As the concentration of ammonium is expected to increase during cultivation due to nitrate reduction, it was hypothesized that the increase in ammonium might have a negative impact on both enzyme activity as well as gene expression, the latter one being studied using the reporter strains. In addition, a low initial ammonium concentration was hypothesized to be sufficient as ample pool for the incorporation of ammonium into biomass until ammonium is provided by nitrate respiration. In this sense, the influence of different ammonium concentrations

on the growth of *B. subtilis* under anaerobic conditions was examined. In a first screening, *B. subtilis* JABs24 was cultivated in duplicate employing 2.5 g/L glucose with five various ammonium concentrations ranging from 0.001 mol/L NH_4^+ up to 0.2 mol/L NH_4^+. The overall growth rates μ were in the range of 0.068 ± 0.006 1/h. Also with respect to the final CDW, which was in the range of 0.194 ± 0.015 g/L, and the time to reach CDW_{max}, an influence of the ammonium concentration was not observed indicating that a high initial ammonium concentration as well as the increase in ammonium due to nitrate respiration did not negatively influence anaerobic growth when employing 2.5 g/L glucose. However, as reported by Willenbacher et al. (2015a), an increase in the initial glucose concentration from 7.5 g/L to 10 g/L resulted in a lower CDW and $Y_{X/S}$ employing strain *B. subtilis* DSM 10^T. The initial NH_4^+ concentration in this study was 0.1 mol/L. Assuming a complete conversion from nitrate to ammonium, final concentrations were about 0.16 and 0.14 mol/L NH_4^+ for 7.5 and 10 g/L glucose, respectively. In this sense, further cultivations employing the reporter strains *B. subtilis* MG1 (P_{narG}-*lacZ*) and MG5 (P_{nasD}-*lacZ*) with both 7.5 and 10 g/L glucose, as well as 0.001 mol/L, 0.1 mol/L, and 0.2 mol/L NH_4^+ were further used to examine the combinative effect of different ammonium and glucose concentrations on anaerobic growth by nitrate respiration.

Figure 1 displays an exemplary cultivation plot of strain MG1 employing 10 g/L glucose and 0.2 mol/L NH_4^+. The biomass increased up to 0.5 g/L after 70.5 h of cultivation. Glucose was almost depleted when CDW_{max} was reached and hence the drop in CDW_{max} was caused by glucose depletion. About 70 mmol/L NO_3^- was reduced and ammonium increased by ∼60 mmol/L. Nitrite peaked at the beginning of cultivation to 0.558 mmol/L and was further reduced to 0.007 mmol/L at CDW_{max}. Another increase was measured when CDW decreased. This nitrite pattern was observed in almost all cultivations tested. The activity of P_{narG} increased up to 119 MU after 32.5 h of cultivation and slightly decreased to 76 MU until CDW_{max}. For a better evaluation, the results of the different ammonium and glucose concentrations tested are summarized in **Table 3**.

A higher glucose concentration and especially the presence of 0.2 mol/L NH_4^+ within the same glucose level resulted in a higher CDW_{max}. Discrepancies, however, must be considered as the determined CDW_{max} in several cultivations was prior to glucose depletion or even slightly after glucose depletion due to the time intervals of sampling. A CDW_{max} of 0.388 g/L was reported by Geissler et al. (2019b) employing strain *B. subtilis* JABs24 using 10 g/L glucose, 0.1 mol/L NO_3^- and 0.025 mol/L NH_4^+. This value is in a similar range in comparison to this study. For strain MG1 with 7.5 g/L, both the $Y_{X/S}$ and the overall growth rate μ increased when more ammonium was added to the medium. For all other cultivations, this trend for both the overall growth rate μ and the $Y_{X/S}$ was not observed. Hence, no generally admitted influence of ammonium could be observed at these experimental conditions, which is also in agreement to the results employing 2.5 g/L glucose. Under aerobic conditions, for example, Leejeerajumnean et al. (2000) reported that 26 tested *Bacillus* strains, among them strain *B. subtilis* NCIMB 3610, grew in the presence of 931 mmol/L NH_4^+ at pH 7. Müller et al. (2006)

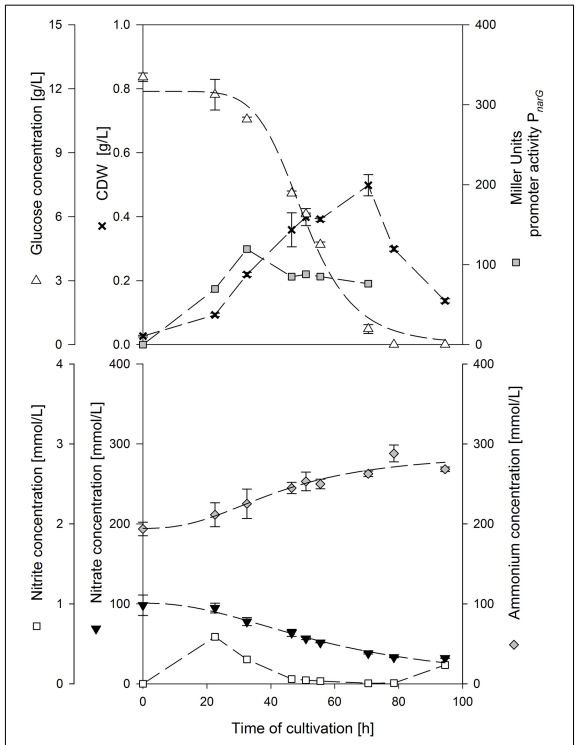

FIGURE 1 | Exemplary anaerobic serum flask cultivation employing strain *B. subtilis* MG1 in a mineral salt medium containing 10 g/L glucose, 0.1 mol/L NO_3^- and 0.2 mol/L NH_4^+.

described that 500 mmol/L did not cause growth inhibition using strain *B. subtilis* 168. A defect was observed with more than 750 mmol/L NH_4^+, but the authors stated that this is due to osmotic or ionic stress and not due to the presence of ammonium itself. However, no reports on the effect of ammonium under anaerobic conditions were found.

Interestingly, the surfactin concentration was lower the more ammonium was present at the beginning of cultivation, with a more drastic change in between 0.1 mol/L and 0.2 mol/L NH_4^+. Exemplary, employing 10 g/L glucose and 0.001 mol/L NH_4^+ resulted in a surfactin concentration of 156.20 mg/L, while the titer obtained with 0.2 mol/L NH_4^+ was only 87.35 mg/L for strain MG1. On the contrary, when the ratio of NO_3^-:NH_4^+ was shifted towards higher nitrate concentrations in studies on the surfactin synthesis under aerobic conditions, a decrease in biomass and surfactin concentration, but an increase in the $Y_{P/X}$ was observed. For example, media containing only NH_4^+ or NO_3^- resulted in a surfactin titer of ∼1 g/L and ∼0.35 g/L at an CDW_{max} of 2 g/L after 21 h and of 0.5 g/L after 36 h of cultivation with strain *B. subtilis* JABs24, respectively. This results in $Y_{P/X}$ values of 0.466 g/g and 0.668 g/g (data not shown). On the contrary, Davis et al. (1999) reported on an improvement in the $Y_{P/X}$ when cultivating *B. subtilis* ATCC 21332 in a

TABLE 3 | Summary of the results and calculated yields for the serum flask cultivations of strains *B. subtilis* MG1 (P_{narG}-*lacZ*) and MG5 (P_{nasD}-*lacZ*) employing 7.5 g/L and 10 g/L glucose and different ammonium concentrations.

	Strain	7.5 g/L glucose			10 g/L glucose		
		0.001 mol/L NH_4^+	0.1 mol/L NH_4^+	0.2 mol/L NH_4^+	0.001 mol/L NH_4^+	0.1 mol/L NH_4^+	0.2 mol/L NH_4^+
CDW_{max} [g/L]	MG1	0.299 ± 0.033	0.319 ± 0.013	0.485 ± 0.033	0.459 ± 0.007	0.379 ± 0.007	0.498 ± 0.033
	MG5	0.399 ± 0.040	0.392 ± 0.007	0.465 ± 0.027	0.332 ± 0.093	0.405 ± 0.020	0.425 ± 0.040
Overall μ [1/h]	MG1	0.050 ± 0.004	0.061 ± 0.001	0.076 ± 0.004	0.044 ± 0.008	0.034 ± 0.002	0.042 ± 0.002
	MG5	0.059 ± 0.002	0.067 ± 0.002	0.062 ± 0.003	0.055 ± 0.002	0.045 ± 0.001	0.051 ± 0.009
$Y_{X/S}$ [g/g]	MG1	0.040 ± 0.001	0.044 ± 0.004	0.078 ± 0.012	0.037 ± 0.003	0.029 ± 0.001	0.040 ± 0.003
	MG5	0.056 ± 0.004	0.081 ± 0.001	0.058 ± 0.003	0.048 ± 0.004	0.036 ± 0.002	0.052 ± 0.008
Surfactin [mg/L]	MG1	109.97 ± 3.23	94.55 ± 2.00	83.19 ± 7.37	156.20 ± 3.46	136.40 ± 4.40	87.35 ± 3.99
	MG5	85.53 ± 13.41	85.61 ± 3.97	48.49 ± 2.02	74.90 ± 17.95	66.42 ± 15.35	32.63 ± 7.82
maximum Miller Units	MG1	238.37 ± 3.08	156.51 ± 8.88	115.90 ± 2.90	231.70 ± 9.16	152.07 ± 25.33	119.45 ± 8.24
	MG5	669.29 ± 35.62	692.48 ± 44.51	666.46 ± 16.74	779.24 ± 20.70	982.14 ± 196.30	708.56 ± 78.74
Miller Units at CDW_{max}	MG1	176.57 ± 30.27	145.76 ± 8.51	77.12 ± 0.45	127.07 ± 0.95	116.23 ± 1.85	76.21 ± 4.02
	MG5	605.28 ± 13.14	543.52 ± 38.63	569.58 ± 47.52	740.50 ± 173.45	746.76 ± 136.21	559.63 ± 70.19

nitrate-limited oxygen-depleted process. Consequently, further medium optimization studies on the impact of different nitrate concentrations at constant ammonium levels on surfactin synthesis are a crucial approach also to investigate the impact on both the dissimilatory and assimilatory nitrogen metabolism.

With respect to the promoter activities, averaged maximum Miller Units for P_{narG} were overall lower with ∼170 MU than for P_{nasD} with ∼750 MU. During the time course of cultivation, as illustrated in **Supplementary Figure S3** and summarized in **Table 3**, the promoter activity decreased after reaching the maximum until CDW_{max}. For P_{narG}, both the maximum promoter activity as well as the activity at CDW_{max} was lower the more ammonium was present. Differences amongst the glucose concentrations tested were less distinct. Hence, the impact of glucose was less significant than the ammonium concentration and lower ammonium concentrations yielded a higher P_{narG} activity. The activity of P_{nasD} was overall much higher with Miller Units up to 1000, but the pattern was similar to P_{narG} and activity reached a maximum and further declined until CDW_{max}. For the P_{nasD} activity, values at 10 g/L glucose were overall higher in comparison to the respective data at 7.5 g/L glucose. However, highest values were obtained for 0.1 mol/L NH_4^+. Hence, a trend was not observed regarding the influence of increasing ammonium concentrations. This is in agreement to the results of Nakano et al. (1998a) reporting that the presence of ammonium did not alter the anaerobic expression levels of a transcriptional fusion *nasD-lacZ* strain.

Generally, in the current experimental set-up, the increase of ammonium showed a tendency to an overall improvement with respect to CDW_{max} while a distinct trend for growth rate μ, $Y_{X/S}$ and promoter activity P_{nasD} was not observed. However, a decrease in both P_{narG} activity and surfactin synthesis was noticed. Due to this ambiguous effect of ammonium on growth but the negative effect on surfactin synthesis and P_{narG} activity, an initial ammonium concentration of 0.001 mol/L was used for all further experiments, as surfactin is the product of interest. In addition, as a further increase in CDW during anaerobic growth beyond 10 g/L glucose will result in a further accumulation of ammonium, the reduction to a minimum from the beginning is expected to be more profitable at long-term view.

Influence of Different Nitrite Concentrations on Anaerobic Growth

As previously described and illustrated in **Figure 1**, nitrite concentrations peaked shortly after inoculation and decreased during the time course of further cultivation. On the one hand, nitrite is often stated to be toxic and hence cells need to detoxify accumulated nitrite, on the other hand, the reduction to ammonium by nitrite reductase is an electron sink which allows the reoxidation of NADH to NAD^+ (Cruz Ramos et al., 1995; Nakano et al., 1998a; Reents et al., 2006). NAD^+ itself is needed for glycolysis, oxidative decarboxylation and the citric acid cycle. Inversely, in the absence of nitrate or nitrite as electron acceptor, *B. subtilis* growth by fermentation and NAD^+ would be regenerated through end product phosphorylation, with the main fermentative metabolites produced being lactate, acetate and 2,3-butandiol (Cruz Ramos et al., 2000).

To further elucidate the impact of nitrite on anaerobic growth, the influence of various concentrations in the range from 0 mmol/L up to 20 mmol/L on the growth behavior of strain JABs24 employing 2.5 g/L glucose was investigated. In the time frame cultivated, the overall growth rate was reduced which was basically due to an increase in lag-phase. For 8 mmol/L and 6 mmol/L NO_2^-, strains restored growth after a lag-phase of around 30 h. With lower nitrite concentrations of 4, 2.5, and 1 mmol/L, growth was detected after a lag-phase of 26, 24, and 12 h of cultivation, respectively. These results are further affirmed by the overall growth rate and maximum growth rate, illustrated in **Figure 2**. A decrease in the overall growth rate μ from 0.068 1/h to 0.027 1/h was observed for 0 mmol/L and 10 mmol/L NO_2^- added in the time window tested. For the maximum growth rates, mean values varied in between 0.141 1/h and 0.208 1/h. However, higher nitrite concentrations did not result in lower growth rates. This is also in agreement to literature, where anaerobic growth of different *B. subtilis*

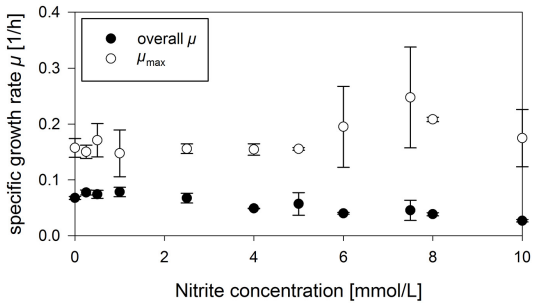

FIGURE 2 | Influence of different nitrite concentrations on the overall and maximum specific growth rate μ in anaerobic serum flask cultivations employing strain *B. subtilis* JABs24.

strains employing either 10 mmol/L nitrate or nitrite resulted in the same OD-values indicating the suitability of nitrite as alternative electron acceptor (Hoffmann et al., 1998; Cruz Ramos et al., 2000; Marino et al., 2001). Interestingly, authors did not report on an increased lag-phase as observed in this study. For 20 mmol/L NO_2^- and 2.5 g/L glucose, growth was not detected during the current experiment. To further investigate the effect of high nitrite concentrations and a longer time frame, a further cultivation employing 10 g/L glucose with both 10 and 20 mmol/L NO_2^- was performed. For 20 mmol/L, growth was not detected even after 90 h of cultivation. For 10 mmol/L NO_2^-, CDW values up to 0.5 g/L were measured after ∼115 h. Compared to the cultivations employing different ammonium concentrations, the time to reach CDW_{max} was hence almost twice as long. However, the final CDW was comparably high and the concentration of nitrite was also drastically reduced until CDW_{max} was reached (data not shown).

To sum up the effect of nitrite, only at very high levels, nitrite is indeed growth limiting or even inhibiting. Concentrations up to 10 mmol/L did significantly increase the time of cultivation employing strain *B. subtilis* JABs24, but the maximum growth rates μ as well as CDW_{max} prior to glucose depletion were similar to the references without further additives and nitrite was also reduced to ammonium.

Impact of Various Glucose Concentrations on Anaerobic Growth and Effect on Promoter Activity of P_{narG} and P_{nasD}

During the study of Willenbacher et al. (2015a) using strain *B. subtilis* DSM 10^T, a benefit of employing 10 g/L glucose with respect to higher CDW and surfactin concentrations was not given. Willenbacher et al. (2015a) stated that a concentration of 10 g/L glucose leads to overflow metabolism in *B. subtilis*. However, no data regarding this hypothesis, such as acetate concentrations, were shown. The previous results of cultivations with strain *B. subtilis* JABs24 and various ammonium concentrations indicated that the effect of 7.5 g/L and 10 g/L is less severe than reported by Willenbacher et al. (2015a) considering the CDW_{max}, but an increase in glucose indeed decreased overall growth rates μ and resulted in lower $Y_{X/S}$ values (**Table 3**). As the glucose concentration plays a major role in the design of batch and fed-batch processes, cultivations with 2.5, 5, 7.5, and 10 g/L glucose were performed in serum flasks employing the strains *B. subtilis* JABs24, MG1 and MG5 as one triplicate set-up.

Figure 3A illustrates the change in CDW_{max}, $Y_{X/S}$ as well as μ_{max} and overall μ of the serum flask cultivations employing different glucose concentrations. CDW_{max} increased by employing higher glucose concentrations from 0.239 ± 0.011 g/L up to 0.492 ± 0.011 g/L. In addition, the time to reach CDW_{max} increased from 30 h to 69 h the higher the glucose concentration was. The observation from Willenbacher et al. (2015a) could consequently not be confirmed and higher cell densities were actually reached the more glucose was added. However, it must be emphasized that different strains were used and in addition, even a frequent sampling does not assure to measure the CDW when glucose is about to be depleted, which was also reported by Geissler et al. (2019b). For the overall growth rate μ, a decrease from 0.072 ± 0.002 1/h to 0.041 ± 0.000 1/h was observed with increasing glucose concentration. In contrast, the differences in μ_{max} and the $Y_{X/S}$ were less distinct. For μ_{max}, values ranged from 0.098 ± 0.004 1/h to 0.133 ± 0.001 1/h, and for the overall $Y_{X/S}$ between 0.044 ± 0.002 g/g and 0.070 ± 0.005 g/g.

Figure 3B displays the surfactin and acetate concentrations at CDW_{max} as well as the corresponding overall $Y_{P/S}$. The surfactin concentration employing 10 g/L glucose was ∼2-fold higher with 189.72 ± 10.50 mg/L than at the other concentrations tested. On the contrary, maximum surfactin titers at the other glucose levels tested were in a similar range and only a slight trend towards an increase in surfactin at higher glucose levels was monitored. The $Y_{surfactin/S}$ showed a similar trend than the $Y_{X/S}$ and decreased with increasing glucose concentrations from 0.023 ± 0.008 g/g to 0.016 ± 0.001 g/g. As reported by Willenbacher et al. (2015a), authors assumed that overflow metabolism led to their results employing 10 g/L glucose. In the current study, acetate concentrations were measured for the samples at CDW_{max}. Acetate is reported to be the most abundant end product during anaerobic growth of *B. subtilis* (Cruz Ramos et al., 2000). The acetate concentrations produced during anaerobic growth increased from 1.15 ± 0.12 g/L to 2.17 ± 0.24 g/L with increasing glucose concentrations. The $Y_{acetate/S}$ was more than 10-fold higher than the $Y_{surfactin/S}$ and decreased as well with increasing glucose concentration from 0.382 ± 0.051 g/g to 0.202 ± 0.020 g/g. This result is, however, rather counter intuitive, as higher glucose concentrations actually do not result in more acetate per glucose. Although further fermentative end products were not expected as nitrate respiration is reported to suppress fermentative growth (Cruz Ramos et al., 2000), lactate was measured as well. As assumed, at CDW_{max} employing 10 g/L glucose the lactate concentration reached 0.24 ± 0.01 g/L, and 0.16 ± 0.01 g/L employing 7.5 g/L glucose. This indicates that the glucose flux, in contrast to acetate, into lactate is comparably low, but that a high amount of glucose converts into acetate and not to the target product. Nakano et al. (1998b) reported that several enzymes of the citric acid cycle show a reduced activity

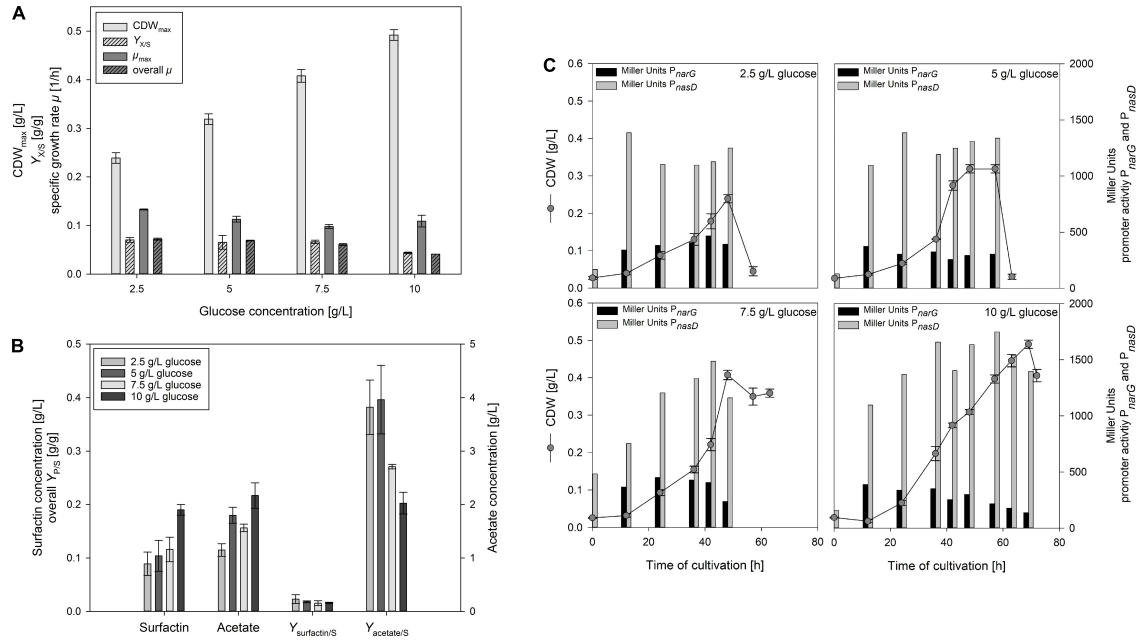

FIGURE 3 | Influence of different glucose concentrations in anaerobic *B. subtilis* JABs24, MG1 and MG5 serum flask cultivations on **(A)** CDW_{max}, biomass per substrate yield $Y_{X/S}$, specific maximum and overall growth rate μ and **(B)** surfactin and acetate concentrations at CDW_{max}, surfactin per substrate yield $Y_{surfactin/S}$ and acetate per substrate yield $Y_{acetate/S}$. In addition, **(C)** CDW and corresponding Miller Units measured for expression of promoters P_{narG} and P_{nasD} during the time course of cultivation.

anaerobically and authors assumed that citrate deficiency might cause *citZ* repression. In this sense, carbon flux studies to investigate glucose degradation are another interesting option for future studies.

Similarly to the previous cultivations, the effect of glucose on anaerobic nitrate respiration was also investigated by the inclusion of the reporter strains MG1 and MG5. **Figure 3C** illustrates the growth curves as well as the corresponding measured promoter activities for both P_{narG} and P_{nasD}. For P_{narG}, the activity at CDW_{max} was lower with increasing glucose concentrations and decreased from 392 MU to 137 MU. However, MU values in the early stages of cultivation also reached highest MU values in between 370 – 440 MU. Hence, the promoter activity of the nitrate reductase was lower the longer the cultivation lasted. In combination with the previous results (**Table 3**), both high glucose as well as high ammonium concentrations decreased the P_{narG} activity, but further studies have to address the question if actually the decreasing concentration of nitrate has an impact on P_{narG} activities. In accordance to the previous results, the activity of P_{nasD} was much higher with MU values up to ∼1700 MU in comparison to P_{narG}. The promoter activity for P_{nasD} also reached a maximum prior to CDW_{max} and a slight trend was observed with an increase in activity at higher glucose levels. In addition, the decrease in activity for P_{narG} was observed earlier during cultivation, while the maximum activity of P_{nasD} was achieved later. This would also explain the nitrite peak observed and is in agreement to the results of Nakano et al. (1998a), who reported that the presence of nitrite along with the global regulator ResDE stimulates the expression of *nasD*. In this sense, nitrate first has to be reduced, and the nitrite produced stimulates *nasD* expression.

To sum up, the serum flask cultivations illustrated that the growth rate was reduced with increasing glucose concentration and that the promoter activity of P_{narG} declined as well during the time course of cultivation. However, the experiments have also demonstrated that the cell density increased the more glucose was added. In addition, results revealed that a high amount of glucose is converted into acetate, while lactate can be considered as a neglectable metabolite in this experimental set-up. Furthermore, acetate production was not lower with less glucose present in the medium. The synthesis of acetate might also be needed to generate ATP, although nitrate respiration is the most efficient alternative respiratory mechanism compared to aerobic cultivations with oxygen as electron acceptor (Strohm et al., 2007).

Batch vs. Fed-Batch Bioreactor Cultivation

To further evaluate the impact of various glucose concentrations, the aim was to perform a batch bioreactor cultivation with

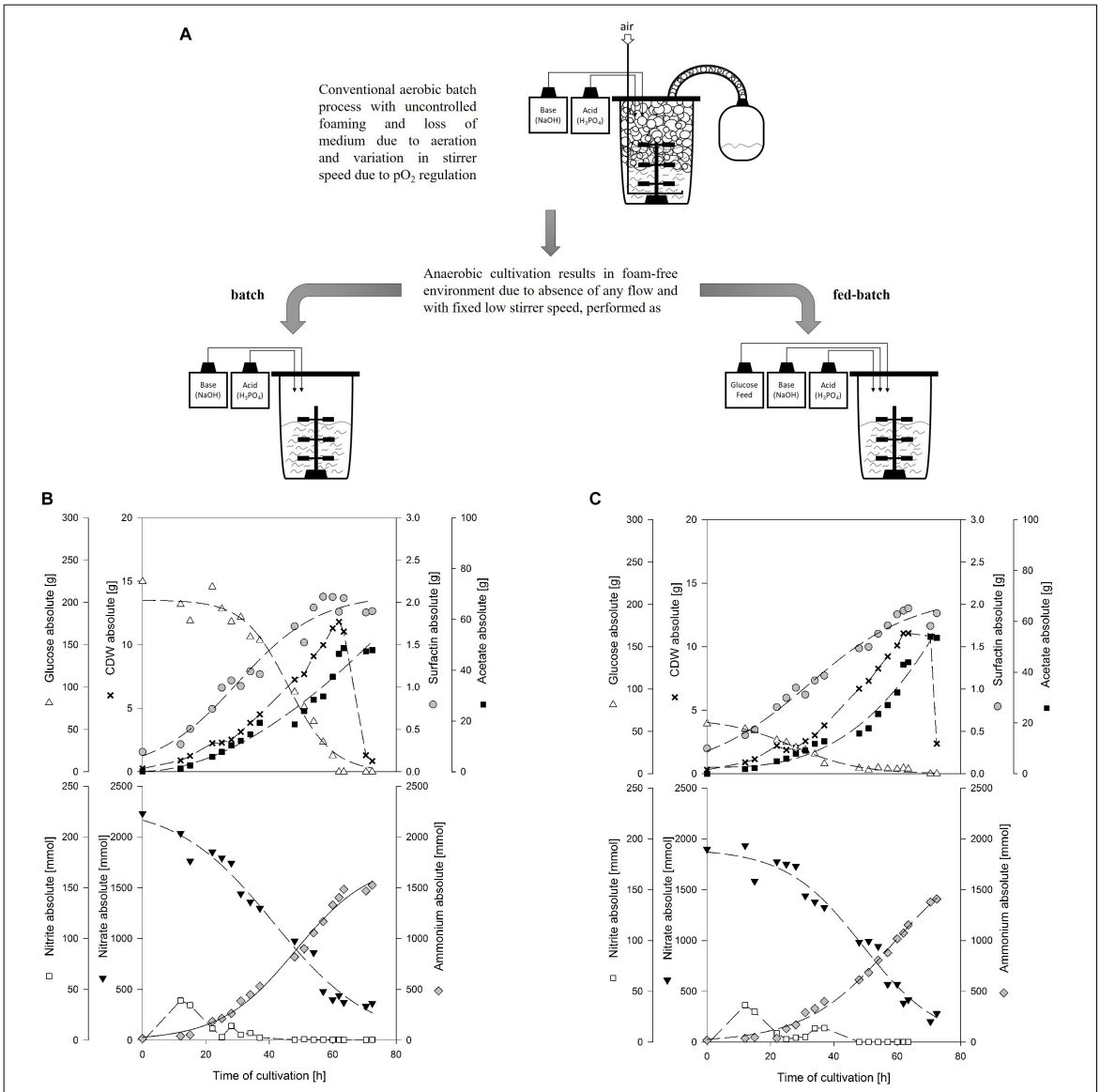

FIGURE 4 | Diagrammatic representation of conventional aerobic batch process with foaming and anaerobic foam-free batch and fed-batch process **(A)**. Time course of 20 kg anaerobic bioreactor cultivation with strain *B. subtilis* JABs24 in a medium containing 0.1 mol/L NO_3^- and 0.001 mol/L NH_4^+ performed as **(B)** batch with 10 g/L glucose and **(C)** fed-batch with 2.5 g/L glucose employing a feed with 600 g of a 25% glucose solution.

strain *B. subtilis* JABs24 employing 10 g/L glucose and a fed-batch cultivation with an initial glucose concentration of 2.5 g/L glucose. A diagrammatic representation of the processes performed as well as the advantage over aerobic batch cultivations is given in **Figure 4A**. For the fed-batch process, an exponential feed phase which matches the glucose added in the batch cultivation was performed to evaluate the impact of a constantly low glucose concentration on growth, surfactin and acetate production.

Figure 4B (batch) and **Figure 4C** (fed-batch) illustrate the absolute values for cell dry weight, glucose, surfactin and acetate, as well as the absolute values of the anaerobic respiration metabolites nitrate, nitrite and ammonium for the two process strategies applied. All important process results as well as

TABLE 4 | Summary of the results and calculated yields for the batch and fed-batch bioreactor process in comparison to a reference serum flask cultivation, as well as comparison to process parameters and yields obtained in different cultivation strategies reported.

	This study	**This study**	**This study**	**Willenbacher et al. (2015a)**	**Chtioui et al. (2012)**	**Coutte et al. (2010)**	**Davis et al. (1999)**
B. subtilis strain	JABs24	JABs24	JABs24	DSM 10^T	ATCC 21332	ATCC 21332	ATCC 21332
Cultivation strategy	Batch bioreactor anaerobic	Fed-batch bioreactor anaerobic	Serum flask 10 g/L glucose anaerobic	Batch bioreactor 2.5/10 g/L glucose anaerobic	Rotating disk bioreactor aerobic	Membrane bioreactor aerobic	Batch bioreactor Oxygen-depleted nitrate-limited
Medium	20 kg	20 kg	0.1 L	1.0 L	1.2 L	3 L	1 L
CDW_{max} [g]	11.81	11.07	x	0.320*/0.586*	3.75	9.3	6.0*
CDW_{max} [g/L]	0.62	0.57	0.492	0.320/0.586	3.125*	3.1*	6.0*
CDW_{max} [h]	62	63.5	69	48*/140*	72	30*	26*
Surfactin at CDW_{max} [g]	1.89	1.95	x	0.06*/0.17*	0.254	0.727	0.350*
Surfactin at CDW_{max} [g/L]	0.100	0.101	0.190	0.06*/0.17*	0.212*	0.242*	0.350*
μ max [1/h]	0.112 (15 h)	0.093 (22 h)	0.109 (12 h)	0.105/0.074	x	x	x
$Y_{P/X}$ max [g/g]	0.170 (15 h)	0.261 (12 h)	n.d.	x	x	x	x
$Y_{X/S}$ max [g/g]	0.164 (37 h)	0.193 (31 h)	n.d.	x	x	x	x
$Y_{P/S}$ max [g/g]	0.024 (54 h)	0.040 (25 h)	n.d.	x	x	x	x
$q_{surfactin}$ max [g/(g·h)]	0.057 (15 h)	0.023 (22 h)	n.d.	x	x	x	x
overall μ [1/h]	0.062	0.056	0.041	0.049*/0.022*	0.064*	0.154*	0.203*
overall $Y_{P/X}$ [g/g]	0.140	0.150	0.344	0.278/0.259	0.068	0.078*	0.075
overall $Y_{X/S}$ [g/g]	0.051	0.064	0.044	0.120/0.049	0.189	0.164*	0.316*
overall $Y_{P/S}$ [g/g]	0.007	0.010	0.015	0.033/0.011	0.013	0.013	0.018*
overall $q_{surfactin}$ [g/(g·h)]	0.004	0.005	0.005	0.005/0.002	0.001*	0.002*	0.003*
Acetate [g/L] at CDW_{max}	2.46	2.27	2.17	x	x	x	x
L-Lactate [g/L] at CDW_{max}	0.16	0.19	0.24	x	x	x	x

**calculated or estimated within this study or previous published works of Willenbacher et al. (2015a) based on the data or figures given in the respective study.*

calculated yields are furthermore summarized in **Table 4**. For comparison, results of the reference serum flask cultivation employing 10 g/L glucose and yields of further non-conventional cultivation strategies to produce surfactin reported in literature are given as well.

The glucose concentration in the batch process was depleted after 62 h of cultivation and the CDW_{max} was reached at this time with 11.81 g (OD_{600} of 2.35). For the fed-batch process, the feed was started after 37 h of cultivation with a set growth rate of 0.04 1/h and an initial feed rate of 0.01 kg/h. This growth rate was chosen based on the results of the serum flask cultivations and was expected to not cause glucose accumulation during the feed phase. The glucose concentration at feed start was 0.61 g/L and maintained in a range of 0.31 ± 0.04 g/L during the feed phase. The CDW_{max} before glucose consumption was 11.07 g (OD_{600} of 2.15). For both cultivations and similar to the serum flask cultivations, cell density dropped after glucose depletion. According to Espinosa-de-los-Monteros et al. (2001) cell lysis occurred after the growth phase instead of sporulation. The surfactin concentration in both cultivations increased throughout cultivation and at CDW_{max}, absolute values of 1.89 g and 1.95 g were reached. Consequently, both process strategies resulted in comparable surfactin titers and glucose limitation neither improved nor impaired surfactin productivity.

Analysis of nitrate revealed that there was no limitation and the reduction of nitrate as well as the increase in ammonium until the end of cultivation indicated that nitrate respiration occurred until growth stopped. At CDW_{max}, 439.41 mmol and 417.30 mmol NO_3^- were present in the medium for the batch and fed-batch process, respectively. Considering the results of both bioreactor and the serum flask cultivations, the nitrate demand for anaerobic nitrate respiration can be calculated as ∼150 mmol (NO_3^-)/g (CDW). Ammonium increased constantly up to 1403.49 and 1153.00 mmol until CDW_{max} was reached. For both cultivations, nitrite peaked at the beginning of cultivation and a second low nitrite peak was observed which actually occurred in a phase with reduced growth. After 48 h of cultivation nitrite was below 1 mmol. The highest concentration of nitrite was measured for both experiments after 12 h of cultivation with 39.13 mmol for the batch and 36.41 mmol for the fed-batch process. These observations match the data obtained from the serum flask cultivations where a nitrite peak was observed as well.

With respect to acetate, both processes showed a drastic increase in acetate with more than 40 g at CDW_{max}. The acetate concentration increased almost parallel to the biomass and a significant difference in between the two process strategies was not observed. The overall $Y_{acetate/X}$ and the $Y_{acetate/S}$ for both batch and fed-batch process were in a similar range at CDW_{max} and reached 3.920 g/g and 0.206 g/g for the batch, and 3.961 g/g and 0.260 g/g for the fed-batch cultivation, respectively. The results obtained are also in agreement with the serum flask cultivations and illustrate that a lower glucose concentration even

below 0.31 g/L throughout cultivation did not result in lower acetate production.

In comparison to acetate, lactate was produced as minor by-product in both cultivations with less than 4 g at CDW_{max}. In a study performed by Espinosa-de-los-Monteros et al. (2001), authors reported that acetic acid and acetoin accumulated in cultivations with excess nitrate, whereas lactate and butanediol were produced when nitrate became limiting due to the presence of excess reduced cofactors. This would be in accordance to the current study, as nitrate decreased from 0.1 mol/L to ∼0.02 mol/L, but further investigations regarding these findings are necessary. Contrariwise, Cruz Ramos et al. (2000) reported a production of 23.3 mmol/L lactate and 16.4 mmol/L acetate cultivating strain *B. subtilis* 168 with nitrate as electron acceptor. However, they reported that the presence of nitrate reduced the formation of lactate and increased the production of acetate. In comparison to the current study, 50 mmol/L glucose and 50 mmol/L pyruvate were used as carbon source, which makes a comparison difficult, as the influence of pyruvate is not known. In addition, only 10 mmol/L NO_3^- were used. This, based on the results of the current study, is not sufficient to ensure nitrate respiration throughout the cultivation in the presence of these amounts of carbon source. Consequently, it might be that the cultivation switched from nitrate respiration to fermentative growth and by that lactate was produced. This would also be in agreement with another statement made by Cruz Ramos et al. (2000) namely that the presence of nitrate actually represses the transcription of lactate dehydrogenase *ldh* and acetolactate synthase *alsS* genes.

Regarding the yields and process parameters, the fed-batch process reached higher maximum and overall yields $Y_{P/X}$, $Y_{X/S}$ and $Y_{P/S}$. This is in agreement with the results of different glucose concentrations tested, as lower glucose levels led to an improved $Y_{X/S}$ and $Y_{P/S}$ and this is hence also valid for a feed phase. However, the employment of a fed-batch process did result neither in a better biomass or surfactin production, nor in a significantly lower acetate or lactate formation. Interestingly, in comparison to the serum flask cultivation, the surfactin concentration in both bioreactor cultivations reached only 0.100 g/L and 0.101 g/L, while 0.190 g/L surfactin was produced in the serum flask with less biomass. This is also illustrated by the $Y_{P/X}$, which is more than double as high with 0.344 g/g in the serum flask. This observation is also in agreement with the $Y_{X/S}$, indicating a better glucose conversion into biomass in the bioreactor cultivation in comparison to the serum flask, while the $Y_{P/S}$ is superior in the serum flask cultivations.

To conclude, the fed-batch and batch cultivations showed that cell growth was observed as long as glucose was present in the medium, illustrating the general feasibility of anaerobic cultivations with strain *B. subtilis* JABs24 for the production of surfactin. Obviously, the next step in bioreactor process development would be to elongate the feed phase. In addition, neither process showed a significant superiority indicating that the initial glucose does not influence the overall performance of the cultivation. However, acetate production was also not reduced at lower glucose levels which makes this metabolite another interesting candidate for further investigations regarding its impact on anaerobic growth.

Impact of Various Acetate Concentrations on Anaerobic Growth and Effect on Promoter Activity of P_{narG} and P_{nasD}

During the previous cultivations, nitrite was shown to be reduced during the time course of cultivation even at an initial concentration up to 10 mmol/L, while acetate was detected in high amounts and the concentration curve was almost parallel to the growth curve with final acetate concentrations up to 2.5 g/L. Under aerobic conditions, the production of acetate is often correlated to overflow metabolism (Presecan-Siedel et al., 1999; Kabisch et al., 2013). In addition, *B. subtilis* is not able to grow on acetate aerobically due to the absence of genes of the glyoxylate shunt (Kabisch et al., 2013). However, under anaerobic fermentative conditions, acetate synthesis is important for generation of energy because acetate synthesis goes along with the AckA dependent formation of ATP (Cruz Ramos et al., 2000). Under anaerobic respiratory conditions, no distinct hypothesis was found that explains the acetate formation under nitrate respiratory conditions. The utilization of nitrate as alternative electron acceptor is reported to be the most favorable in view of ATP yield (Marino et al., 2001). Nevertheless, acetate is also often declared as growth inhibiting substance, but the effect varies in between different bacterial species as demonstrated by Lasko et al. (2000) testing *E. coli*, *Acetobacter aceti*, *Staphylococcus capitis*, *Gluconobacter suboxydans*, *Lactobacillus acetotolerans,* and *L. bulgaricus* in aerobic cultures. In another study, addition of 128 mmol/L acetate resulted in a reduced growth rate from 0.75 1/h to 0.4 1/h in an *E. coli* culture at pH 7.4 (Pinhal et al., 2019). However, little information is available on the effect of acetate on *B. subtilis*. Studies dealing with acetate and declaring growth inhibiting effect mostly refer to experiments with *E. coli*.

In contrast to nitrite, the concentration of acetate was increasing steadily. Hence, serum flask cultivations were performed with various initial acetate concentrations. Other than for nitrite, cultivations were performed directly with 10 g/L glucose to monitor effects in longer cultivations. The cultivation results with strains JABs24, MG1 and MG5 as triplicate are given in **Table 5**. The maximum CDWs were in a similar range and varied in between 0.465 and 0.532 g/L and no correlation was found between initial acetate concentration and CDW_{max}. The time to reach CDW_{max} was ∼69 h for the reference and ∼105 h for 10 g/L acetate added. In this sense, acetate has an influence comparable to that of nitrite with respect to the time of cultivation to reach CDW_{max}. However, while μ_{max} for nitrite was in an overall similar range (**Figure 2**), μ_{max} for acetate was lower the more acetate was added. This indicates that acetate did not prolong the lag-phase such as demonstrated for nitrite but has an overall negative effect on cellular growth. Furthermore, the overall growth rate μ was reduced from 0.041 ± 0.000 1/h to 0.029 ± 0.001 1/h applying 0 g/L and 10 g/L acetate, respectively. Due to the similar final CDW values obtained, the $Y_{X/S}$ for all cultivations was as expected in the same range and

TABLE 5 | Summary of effect of different acetate concentrations on anaerobic growth.

$c_{acetate}$ [g/L]	0	0.5	1.5	2.5	5	10
CDW_{max} [g/L]	0.492 ± 0.010	0.470 ± 0.049	0.470 ± 0.033	0.474 ± 0.025	0.532 ± 0.011	0.465 ± 0.011
μ_{max} [1/h]	0.123 ± 0.020	0.095 ± 0.005	0.083 ± 0.013	0.072 ± 0.009	0.076 ± 0.002	0.069 ± 0.006
Overall μ [1/h]	0.041 ± 0.000	0.042 ± 0.004	0.037 ± 0.002	0.033 ± 0.003	0.039 ± 0.001	0.029 ± 0.001
$\Delta c_{acetate}$ [g/L]	2.152 ± 0.249	2.689 ± 0.222	2.850 ± 0.108	3.014 ± 0.226	3.143 ± 0.372	2.987 ± 0.438
$Y_{acetate/X}$ [g/g]	4.386 ± 0.557	5.742 ± 0.251	6.084 ± 0.234	6.386 ± 0.686	5.906 ± 0.621	6.405 ± 0.813
$Y_{acetate/S}$ [g/g]	0.202 ± 0.020	0.262 ± 0.018	0.244 ± 0.020	0.271 ± 0.014	0.276 ± 0.047	0.265 ± 0.037

the mean yield was determined as 0.042 ± 0.004 g/g. Hence, added acetate did not reduce biomass formation. With respect to the acetate produced on top of the initial concentration, the reference cultivation reached the lowest values, while for the other cultivations a slight overall increase was detected. In accordance to this slight increase, also the yield $Y_{acetate/X}$ showed this effect with the same significant change in between 0 g/L and 0.5 g/L acetate added. To further illustrate the trend of the activities of both P_{narG} and P_{nasD}, the data after 36 h and 67 h of cultivation, as well as at CDW_{max}, are illustrated in **Figure 5**. In general, the activity decreased during the time course of cultivation within the different acetate concentrations tested. In regard to the promoter activity at CDW_{max}, a reduction in activity was observed for P_{narG} and Miller Units decreased from 137 MU for 0 g/L acetate to 35 MU in the cultivation with 10 g/L acetate added. Considering the activity of P_{nasD}, values were as previously reported much higher, and except for 10 g/L the activity at CDW_{max} was also reduced from 1397 MU for the reference and 815 MU employing 5 g/L. Interestingly, considering the percentage change, the decrease in activity was overall more severe for the nitrate reductase and further studies regarding the negative effect of either acetate directly or for example the onset of nitrate limitation as mentioned before on the reduced activity must be performed.

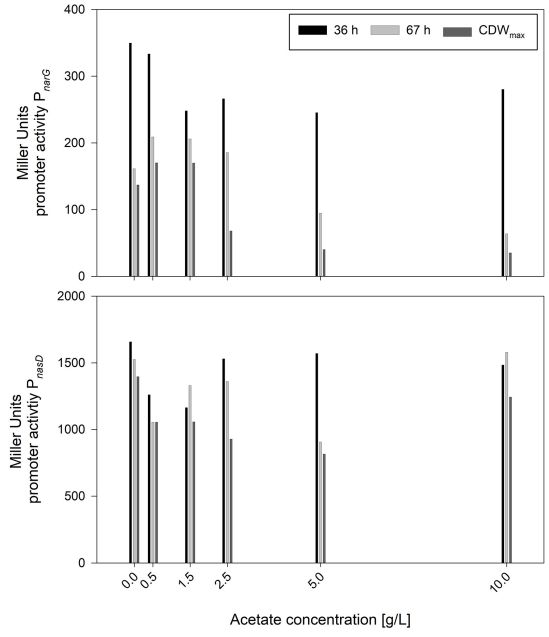

FIGURE 5 | Influence of different acetate concentrations on the expression of P_{narG} and P_{nasD} after 36 and 67 h of cultivation and at CDW_{max} employing strains *B. subtilis* MG1 and MG5.

To sum up, the inhibitory effect of acetate was more pronounced than for ammonium and especially nitrite, as acetate synthesis occurred throughout growth and concentrations did not decrease as shown for nitrite. High acetate concentrations reduced both the overall growth rate as well as the maximum growth rate and also the expression of *narG* and *nasD* were shown to be affected. Indeed, it is of upmost interest to further study this effect. These observations are counterintuitive, as acetate formation results in ATP supply, but in the same time its accumulation led to notable growth inhibition. Further studies regarding the role of acetate, e.g., by deleting the genes involved in acetate synthesis, namely acetate kinase *ackA* and phosphate acetyltransferase *pta*, displays one option. Regarding this approach, Cruz Ramos et al. (2000) already reported that a *B. subtilis* Δ*pta* mutant strain produced less acetate in the presence of nitrate, but growth was also drastically reduced, indicating the importance of this metabolic pathway for anaerobic growth by nitrate respiration. Still, strains unable to produce acetate or able to utilize acetate anaerobically might be an interesting alternative for surfactin production processes. For example, a *B. subtilis* strain carrying the glyoxylate shunt genes from *B. licheniformis* DSM 13 was reported to be more robust and showed a better growth aerobically (Kabisch et al., 2013). Longing for more robust strains able to grow anaerobically and synthesize surfactin without acetate accumulation clearly presents an opportunity for further strain engineering. Even if growth rates are lower, but productivity is maintained throughout a prolonged time window, a significantly increased product titer could be achieved in a foam-free environment.

SUMMARIZING REMARKS AND FURTHER CONSIDERATIONS FOR FUTURE RESEARCH

The results presented illustrate the feasibility of an anaerobic nitrate respiration process which also lays the basis for the

TABLE 6 | Comparison of anaerobic cultivations with *B. subtilis* in literature with the medium used and OD-values reached.

Strain	Medium	Condition	Carbon source [g/L]	Nitrogen source [mmol/L]	OD_{max}	References
B. subtilis JABs24 (derived from 168)	Defined (modified Cooper's mineral salt medium)	anaerobic	Glucose: 10	NO_3^-: 100 NH_4^+: 1	1.85 (serum flask) 2.35 (bioreactor)	This study
B. subtilis DSM 10^T	Defined (modified Cooper's mineral salt medium)	anaerobic	Glucose: 10	NO_3^-: 117.7 NH_4^+: 100	1.76	Willenbacher et al. (2015a)
B. subtilis DSM 10^T	Defined (modified Cooper's mineral salt medium)	anaerobic	Glucose: 7.5	NO_3^-: 117.7 NH_4^+: 100	2.568	Willenbacher et al. (2015a)
B. subtilis LCB6 (derived from I168)	Defined (Spizizen's minimal medium)	aerobic anaerobic	Glycerol: 10 mL/L	NO_3^-: 24 NH_4^+: 30	∼2 ∼0.1	Clements et al. (2002a)
B. subtilis LCB6 (derived from I168)	Defined (Spizizen's minimal medium)	aerobic anaerobic	Glucose: 10	NO_3^-: 24 NH_4^+: 30	3 0.12	Clements et al. (2002b)
B. subtilis 168	Defined (minimal medium)	anaerobic	Glucose: 9 Pyruvate: 4.4	NO_3^-: 10 NO_2^-: 10 x	1 0.7 1	Cruz Ramos et al. (2000)
B. subtilis JH642	Complex (LB with supplements)	anaerobic	Glucose: 0.18	NO_3^-: 10 NH_4^+: 4 NO_2^-: 10 NH_4^+: 4 x	1.1 1 0.7	Hoffmann et al. (1998)
B. subtilis JH642	Defined (minimal medium)	aerobic anaerobic	Glucose: 9	x NO_3^-: 10 NO_2^-: 10 x	10 1.1 1.05 1	Marino et al. (2001)

establishment of other processes where either foaming is a major issue, or the target product is sensitive towards oxygen.

The ability of *B. subtilis* to grow anaerobically was reported in previous studies. **Table 6** summarizes different studies with the respective medium, carbon and nitrogen sources used and OD-values achieved.

Next to fundamental research on anaerobic growth, several studies used this approach to synthesize lipopeptides, and in this case surfactin (Javaheri et al., 1985; Davis et al., 1999; Willenbacher et al., 2015a; Geissler et al., 2019b). This process strategy results in promising $Y_{P/X}$ values, meaning less biomass waste is produced per gram surfactin, and, which is an important aspect for operating and process control, foaming is completely avoided. This also allows using the full capacity of a bioreactor which leads to an improvement in the volumetric productivity, while the volume in foaming processes is often reduced so that the foam can accumulate in the headspace (St-Pierre Lemieux et al., 2019). Foam-free strategies also make the addition of antifoam agents and the implementation of foam breakers, which results in high energetical input, needless. Furthermore, both techniques to degrade foam can result in cellular stress and consequently reduced productivity (St-Pierre Lemieux et al., 2019). Another advantage of anaerobic cultivations is that the stirrer speed can be kept throughout the process, while pO_2 regulation generally goes along with increasing aeration rates and stirrer speeds. Both parameters significantly influence foaming and as the process values increase with increasing biomass, also foam formation is enhanced (St-Pierre Lemieux et al., 2019).

However, several concluding remarks should be mentioned that need to be addressed in further studies, especially as the target product surfactin reached much lower concentrations in the bioreactor cultivations. One issue faced was the pH value. Ideally, also as demonstrated by Willenbacher et al. (2015a), the anaerobic cultivation leads to a basic pH shift. During that cultivation, nitrogen airflow was adjusted above the liquid level, with the aim to reduce the backflow of oxygen from the air. In the current study, the pH shifted to acidic conditions and hence base needed to be added to maintain the pH. In preliminary bioreactor cultivations, different nitrogen flows were tested. Indeed, employing a nitrogen flow through the medium resulted in a decrease in pH, but also resulted in foaming. Employing a nitrogen flow above the liquid level was also not sufficient, which illustrates the difficulties in transferring results from a 1 L bioreactor cultivation as in Willenbacher et al. (2015a) to a 20 kg cultivation as in this study. The decrease in pH was assumed to be both due to the production of acidic products such as acetate, and due to the accumulation of CO_2 in the medium which converts to H_2CO_3 (Killam et al., 2003). Although acetate was not measured

by Willenbacher et al. (2015a), it is presumably to assume that acetate was produced due to similarities in e.g. CDW, and hence the effect of the acidic pH shift in the current study is most likely due to accumulated CO_2. Nevertheless, as the data for surfactin and CDW reported by Willenbacher et al. (2015a) and Geissler et al. (2019b) are well in accordance to the results obtained in this study, it can be hypothesized that there is no pivotal negative effect when CO_2 is not stripped.

The anaerobic growth of *B. subtilis* is considered an interesting research field and many studies deal with fundamental research on sequencing, cloning or regulatory mechanisms (Cruz Ramos et al., 2000; Clements et al., 2002a,b). Consequently, many efforts are needed to improve the production process to further improve the yields. As summarized by Geissler et al. (2019a), there are three methods to improve the titers, namely medium and process parameter optimization, strain engineering and establishing process strategies. For example, addition of further carbon sources, amino acids or vitamins was reported to improve anaerobic growth (Carvalho et al., 2010; Javed and Baghaei-Yazdi, 2016). In terms of process strategies, appropriate feed profiles must be established to, for example, maintain a certain glucose/nitrate-ratio which influences the synthesis of metabolic by-products such as acetate and lactate (Espinosa-de-los-Monteros et al., 2001). The incorporation of a short aerobic phase displays an interesting option as this resulted in faster growth as reported by Cruz Ramos et al. (2000) and might be beneficial when aerobic pre-cultures are used as performed in this and many other studies. However, on the contrary, Willenbacher et al. (2015a) employed anaerobic pre-cultures and in comparison to the results of Geissler et al. (2019b) no significant differences were observed that could be attributed to the pre-cultures. The third strategy, strain engineering, poses another option to improve the anaerobic growth and the surfactin synthesis of *B. subtilis*. In this field, promoter exchange or gene knockout studies display a promising approach. As illustrated in the previous results, the synthesis and accumulation of acetate was pointed out as bottleneck and is hence a starting point for future studies.

CONCLUSION

The present study demonstrated the applicability of anaerobic foam-free processes by nitrate respiration for the synthesis of surfactin in a *B. subtilis* cultivation. Nevertheless, even at low substrate concentrations, significant production of acetate could be observed. As such, acetate was identified as a target metabolite for ongoing research and strain development. Furthermore, future studies should investigate the reported decrease in the promoter activity P_{narG} during the time course of cultivation as well as its decrease in the presence of high ammonium and acetate concentrations. Concluding, this study constitutes an important step towards the development of longer, more robust and more productive processes with *B. subtilis* using anaerobic nitrate respiration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the manuscript/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MHo planned and executed the experiments, collected data, created the graphs, and drafted the whole manuscript. DF, LS, and KR performed part of the experiments and collected and evaluated corresponding data. SX under supervision of KH constructed the reporter strains. LL supported in interpretation of results. MHe contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.554903/full#supplementary-material

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2.3 Evaluation of an oxygen-dependent self-inducible surfactin synthesis in *B. subtilis* by substitution of native promoter P_{srfA} by anaerobically active P_{narG} and P_{nasD}

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Evaluation of an oxygen-dependent self-inducible surfactin synthesis in *B. subtilis* by substitution of native promoter P_{srfA} by anaerobically active P_{narG} and P_{nasD}

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Abstract

A novel approach targeting self-inducible surfactin synthesis under oxygen-limited conditions is presented. Because both the nitrate (NarGHI) and nitrite (NasDE) reductase are highly expressed during anaerobic growth of *B. subtilis*, the native promoter P_{srfA} of the surfactin operon in strain *B. subtilis* JABs24 was replaced by promoters P_{narG} and P_{nasD} to induce surfactin synthesis anaerobically. Shake flask cultivations with varying oxygen availabilities indicated no significant differences in native P_{srfA} expression. As hypothesized, activity of P_{narG} and P_{nasD} increased with lower oxygen levels and surfactin was not produced by P_{srfA}::P_{narG} as well as P_{srfA}::P_{nasD} mutant strains under conditions with highest oxygen availability. P_{narG} showed expressions similar to P_{srfA} at lowest oxygen availability, while maximum value of P_{nasD} was more than 5.5-fold higher. Although the promoter exchange P_{srfA}::P_{narG} resulted in a decreased surfactin titer at lowest oxygen availability, the strain carrying P_{srfA}::P_{nasD} reached a 1.4-fold increased surfactin concentration with 696 mg/L and revealed an exceptional high overall $Y_{P/X}$ of 1.007 g/g. This value also surpassed the $Y_{P/X}$ of the reference strain JABs24 at highest and moderate oxygen availability. Bioreactor cultivations illustrated that significant cell lysis occurred when the process of "anaerobization" was performed too fast. However, processes with a constantly low agitation and aeration rate showed promising potential for process improvement, especially by employing the strain carrying P_{srfA}::P_{nasD} promoter exchange. Additionally, replacement of other native promoters by nitrite reductase promoter P_{nasD} represents a promising tool for anaerobic-inducible bioprocesses in *Bacillus*.

Keywords: *Bacillus subtilis*, Lipopeptide biosurfactants, Surfactin, Microaerobic, Promoter exchange, Oxygen

Introduction

The cyclic lipopeptide surfactin synthesized by *Bacillus subtilis* displays promising characteristics in a variety of industrial sectors (Geissler et al. 2019a) due to its excellent surface-active properties and antimicrobial activities (Falardeau et al. 2013; Li et al. 2019). In addition, surfactin is a promising alternative to surfactants of petrochemical and oleochemical origin (Henkel et al. 2017). However, two bottlenecks regarding surfactin production must be mentioned that are often addressed in research. First, the excessive foaming during conventional aerobic processes, and second, the overall low titers of wild-type strains that are insufficient for industrial production.

Studies reported both on difficulties in operation due to uncontrolled foaming and on a great loss of cultivation medium at high agitation and aeration rates (Davis et al. 2001; Yao et al. 2015; Yeh et al. 2006). If product separation by foaming is integrated into the process as a first surfactin enrichment, cells are also lost for further

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surfactin production (Willenbacher et al. 2014). Issues with blocked exhaust air filters and hence increase in pressure can also occur. However, high agitation and aeration rates are indispensable when defined oxygen levels shall be maintained in commonly employed aerobic cultivations. Indeed, Yeh et al. (2006) reported that up to a certain high level of aeration and stirrer speed surfactin synthesis was improved in carrier-assisted cultivation due to improved oxygen transfer rate and mass transfer efficiency. In contrast to these circumstances, other studies described that an enhanced surfactin production rate was reached in oxygen-limited conditions (Davis et al. 1999; Kim et al. 1997). In this sense, the setpoints for both aeration and agitation rate are crucial to have the optimal performance in a bioreactor cultivation with a defined strain. Interestingly, an anaerobic cultivation of *B. subtilis* as demonstrated by Willenbacher et al. (2015) resulted in a completely foam-free approach for surfactin formation. Nevertheless, anaerobic nitrate respiration reveals some restrictions. Both the overall low growth rates of *B. subtilis* producer strains and comparatively low surfactin titers make these processes inferior to aerobic counterparts. Still, promising and high values with regard to product per biomass yields were obtained (Geissler et al. 2019b; Willenbacher et al. 2015). Another aspect is the negative impact of nitrite as well as acetate on anaerobic cell growth. Especially, the latter one increased drastically throughout nitrate respiration of *B. subtilis* and as such is an interesting candidate for strain engineering (Hoffmann et al. 2020).

With respect to the overall low surfactin titers of wild-type strains, rational strain engineering is also often employed, as heterologous production of surfactin in other host strains is difficult to realize (Hu et al. 2019). In this field, substitution of the native promoter P_{srfA}, whose regulation is dependent on a complex quorum-sensing mechanism (Geissler et al. 2019a), constitutes one promising approach. The replacement of P_{srfA} by constitutively promoters was reported in several studies (Coutte et al. 2010a; Willenbacher et al. 2016). Results demonstrated that both gene expression during the time course of cultivation as well as the ability of the wild-type strain to produce surfactin influence final surfactin concentrations. In contrast to constitutive promoters, several studies reported on improved surfactin titers up to 17-fold employing IPTG-inducible promoters (Jiao et al. 2017; Sun et al. 2009; Wang et al. 2018). However, IPTG is rather expensive, which poses a bottleneck for large-scale production.

The current study aimed to address the afore mentioned aspects and examined an oxygen-dependent, self-inducible expression system employing the promoters of the nitrate reductase P_{narG} and the nitrite reductase P_{nasD}. Hence, the presented method takes advantage of the ability of *B. subtilis* to grow anaerobically by nitrate respiration, the most effective life style to generate ATP after aerobic growth with oxygen as electron acceptor (Härtig and Jahn 2012). The adaptation of aerobic growing cells to anaerobic conditions is dependent on the interplay of three major regulators, which are ResDE, Fnr and Rex (a detailed overview is given by Härtig and Jahn (2012)). In a first step, nitrate is reduced to nitrite by the catabolic nitrate reductase NarGHI (Nakano et al. 1998). In a second step, nitrite is further reduced to ammonium by the nitrite reductase NasDE. Indeed, NasDE is also involved in aerobic assimilatory reduction of nitrate to nitrite. However, the expression of NasDE was reported to be significantly induced in the absence of oxygen emanating from a promoter located in between *nasC* and *nasD* (Nakano et al. 1998). Hence, both P_{narG} and P_{nasD} are involved in anaerobic nitrate respiration, although also the availability of nitrogen sources plays an important role in this issue. In addition to these anaerobically inducible promoters, *B. subtilis* exhibits more regulatory networks that allow induction of gene expression under oxygen limitation. An overview of *B. subtilis* transcriptome under anaerobic growth was provided by Nicolas et al. (2012). Hence, further promising promoters for anaerobic induction of gene expression are P_{lctE}, P_{alsS} and P_{hmp}.

We hypothesized that the presented self-inducible system poses an interesting novel cultivation strategy to synthesize surfactin under oxygen-limited conditions resulting in reduced foam formation. The proposed system is not only promising for foaming agents like surfactin, but also for other oxygen-sensitive target products. On top, preliminary investigations in bioreactor cultivations to establish a simple and robust cultivation strategy using a native *B. subtilis* surfactin producer strain and derivative strains with promoter exchanges are presented. Emerging limitations of bioreactor cultivations will be emphasized that display a promising starting position for further optimizations.

Materials and methods

Chemicals and materials

All chemicals (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were of analytical grade. The reference standard for surfactin (≥ 98% purity) and glucose were from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany).

Microorganisms and genetic engineering

All strains are listed in Table 1 including strain *B. subtilis* JABs24 (Geissler et al. 2019b) and derivatives thereof. Chemical competent *E. coli* BL21-Gold strains

Table 1 Overview of strains used in the current study

Strain	Genotype or description	References
B. subtilis		
JABs24	*trp*$^+$ *sfp*$^+$ Δ*manPA*	Geissler et al. (2019b)
MG1	*trp*$^+$ *sfp*$^+$ Δ*manPA amyE*::[P_{narG}-*lacZ*, *spcR*]	Hoffmann et al. (2020)
MG5	*trp*$^+$ *sfp*$^+$ Δ*manPA amyE*::[P_{nasD}-*lacZ*, *spcR*]	Hoffmann et al. (2020)
KM1016	*trp*$^+$ *sfp*$^+$ Δ*manPA amyE*::[P_{srfA}-*lacZ*, *spcR*]	This study
MG11	*trp*$^+$ *sfp*$^+$ Δ*manPA* P_{srfA}::P_{narG}	This study
MG12	*trp*$^+$ *sfp*$^+$ Δ*manPA* P_{srfA}::P_{narG} *amyE*::[P_{narG}-*lacZ*, *spcR*]	This study
MG13	*trp*$^+$ *sfp*$^+$ Δ*manPA* P_{srfA}::P_{nasD}	This study
MG14	*trp*$^+$ *sfp*$^+$ Δ*manPA* P_{srfA}::P_{nasD} *amyE*::[P_{nasD}-*lacZ*, *spcR*]	This study
E. coli		
BL21-Gold (DE3)	*F*$^-$ *ompT hsdS*(rB^- mB^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte	Agilent, Waldbronn, Germany

were used for plasmid propagation. Plasmids and primers (Eurofins Genomics GmbH, Ebersberg, Germany) used for strain construction are summarized in Additional file 1: Table S1 and Table S2. Transformants were selected on LB agar plates supplemented with either ampicillin (100 µg/mL), spectinomycin (125 µg/mL) or erythromycin (10 µg/mL for *E. coli*, 5 µg/mL for *B. subtilis*).

For promoter exchange studies, strain JABs24 was used to replace the native promoter P_{srfA} markerless by either P_{nasD} or P_{narG} similar to the protocol described by Vahidinasab et al. (2020). Briefly, generated fragments upstream(P_{srfA})-P_{narG}-downstream(P_{srfA}) and upstream(P_{srfA})-P_{nasD}-downstream(P_{srfA}) were ligated into SmaI digested plasmid pJOE4786.1 resulting in plasmids pRIK2 for P_{srfA}::P_{narG} and pPB1 for P_{srfA}::P_{nasD}. After confirmation by sequencing, fragments of interest were digested of pRIK2 and pPB1 by HindIII and were ligated into plasmid pJOE6743.1 resulting in plasmids for chromosomal promoter exchange, called pRIK4 (P_{srfA}::P_{narG}) and pPB2 (P_{srfA}::P_{nasD}). These plasmids were isolated and transformed in natural competent *B. subtilis* JABs24 cells. A markerless promoter exchange of P_{srfA} was ensured by mannose counterselection as described by Wenzel and Altenbuchner (2015). Final validation of successful integration was performed by sequencing and resulted in strains MG11 for P_{srfA}::P_{narG} and MG13 for P_{srfA}::P_{nasD}. Using plasmids pKAM446 (P_{srfA}-*lacZ*), pKAM452 (P_{narG}-*lacZ*) and pSHX2 (P_{nasD}-*lacZ*) for construction of reporter strains and the protocol described in Hoffmann et al. (2020), the integration of promoter-*lacZ* fusion into *amyE* locus was performed for strains JABs24, MG11 and MG13. This resulted in strains KM1016 (*amyE*::[P_{srfA}-*lacZ, spcR*]), MG12 (P_{srfA}::P_{narG}; *amyE*::[P_{narG}-*lacZ, spcR*]) and MG14 (P_{srfA}::P_{nasD}; *amyE*::[P_{nasD}-*lacZ, spcR*]), respectively.

Cultivation medium and conditions

Main cultivations were performed in 500 mL baffled shake flasks with relative filling volumes (Rv) of 10% (=0.1 mL/mL), 50% (=0.5 mL/mL) and 100% (=1 mL/mL) at 120 rpm. This approach results in different oxygen availabilities in shake flasks (Heyman et al. 2019; Schiefelbein et al. 2013). In any case, even at 100% Rv, overflowing during shaking did not occur as the shake flask neck posed a sufficient barrier. Cultivations in bioreactors were performed in custom-built bioreactors (ZETA GmbH, Graz/Lieboch, Austria) equipped with foam centrifuge as described in Hoffmann et al. (2020) at 37 °C. Due to the installation and feed-program of the bioreactors, batch and feed media were prepared as g/kg respectively mol/kg.

Preparation of LB medium for the first pre-culture and a modified mineral salt medium for all other shake flask and bioreactor cultivations was performed as described in Hoffmann et al. (2020). The concentrations of the buffer, $MgSO_4 \cdot 7H_2O$ and trace elements were the same, while the glucose concentration was increased to 40 g/L for shake flask cultivations and 20 g/kg for the bioreactor batch medium. The initial nitrogen source was 0.1 mol/L (or mol/kg) $NaNO_3$ and 0.1 mol/L (or mol/kg) $(NH_4)_2SO_4$, if not indicated otherwise in the results part. For bioreactor cultivations, a feed solution was prepared containing 400 g/kg glucose as well as 20 mL TES/kg and 3.69 g/kg $MgSO_4 \cdot 7H_2O$.

Pre-cultures and the main culture were prepared as described in Hoffmann et al. (2020). The second pre-culture was inoculated for 12–16 h. Bioreactor cultivations had an initial batch volume of 20 kg. The agitation rate was fixed at 300 rpm throughout cultivation. A constant aeration rate of 1.2 L/min, equal to 0.06 vvm at T_0 and the lowest possible aeration rate with the given technical equipment, was set without further pO_2 regulation. pH

was maintained at 7 by addition of 4 M NaOH and 4 M HNO_3, the latter one serving as self-regulated nitrate-feed due to the basic pH shift caused by anaerobic nitrate respiration. The foam centrifuge run at 2790 rpm when activated by a sensor in the headspace of the bioreactor vessel. The feed was started when glucose became depleting with a set growth rate of 0.03 1/h and an initial feed addition of 0.04 kg/h. The feed was limited to 2 kg.

Sampling and sample analysis

After measuring the OD_{600} (Biochrom WPA CO8000, Biochrom Ltd., Cambridge, UK) of the samples, cell-free supernatants were stored at − 20 °C. Cell dry weight (CDW) was calculated by dividing the OD_{600} by the factor 3.762, which was established previously (Geissler et al. 2019b). Glucose and surfactin concentrations were determined using an HPTLC method as specified in Geissler et al. (2017, 2019b). Nitrate, nitrite and ammonium concentrations were analyzed with spectrophotometric assays (nitrate: Cat. No. 1.09713.0001, nitrite: Cat. No. 1.14776.0001, ammonium: Cat. No. 1.14752.0001, Merck KGaA, Darmstadt, Germany). Miller units (MU) as indicator for the promoter activity of the *lacZ*-fused promoters were determined by the β-galactosidase assay as described in Hoffmann et al. (2020).

Data analysis

Process parameters were calculated in the same approach, means Δt yields and overall yields, as explained in Hoffmann et al. (2020). For bioreactor cultivations, all concentrations were converted to absolute values by multiplying the respective value by the weight of the medium. This allowed to compensate for the dilution occurred due to the addition of feed, acid and base solutions. All experiments were performed in duplicates. Depending on the aim of the respective experiment, either strain JABs24, KM1016, MG1 or MG5 was used as reference strain.

Results

Influence of different oxygen availabilities on nitrogen metabolism

Varying filling volumes in shake flasks are associated with different oxygen availabilities for cells. In case of *B. subtilis*, altered patterns in nitrogen consumption were expected due to assimilatory and dissimilatory pathways. More precisely, low filling volumes result in highest oxygen availability (Heyman et al. 2019; Schiefelbein et al. 2013) and hence aerobic growth is predominant. In this sense, ammonium should be identified as preferred nitrogen source for biomass assimilation, when both ammonium and nitrate are present. High filling volumes result in low oxygen availability (Heyman et al. 2019; Schiefelbein et al. 2013) and nitrate consumption increases due to anaerobic nitrate respiration. To validate these theses, *B. subtilis* JABs24 was cultivated in 10%, 50% and 100% relative filling volume (Rv). Figure 1 displays the corresponding CDW and the concentrations of nitrate, nitrite and ammonium during the time course of cultivation. CDW_{max} was reached after 44 h, 48 h and 36 h with 5.85 ± 0.66 g/L, 5.12 ± 0.20 g/L and 1.21 ± 0.01 g/L at increasing cultivation volumes. Additional glucose measurements excluded carbon limitation (data not shown). Nitrate concentrations were relatively constant at 10% and 50% Rv, with a slight decrease detectable in the last-mentioned. On the contrary, nitrate limitation occurred in 100% Rv after 36 h. Hence, growth was limited by nitrate depletion. As expected, the ammonium concentration decreased during cultivation with 10% Rv. At 50% Rv, ammonium initially decreased slightly before a relatively constant concentration could be measured. A continuous increase in ammonium was detected in the cultivation using 100% Rv of the flask capacity. Nitrite, the intermediate of nitrate respiration, was not detected in cultivations using 10% Rv. Nitrite peaked to values of 4.11 ± 1.17 mmol/L and 4.95 ± 0.03 mmol/L after 20 h of cultivation in both 50% and 100% Rv, respectively. Those

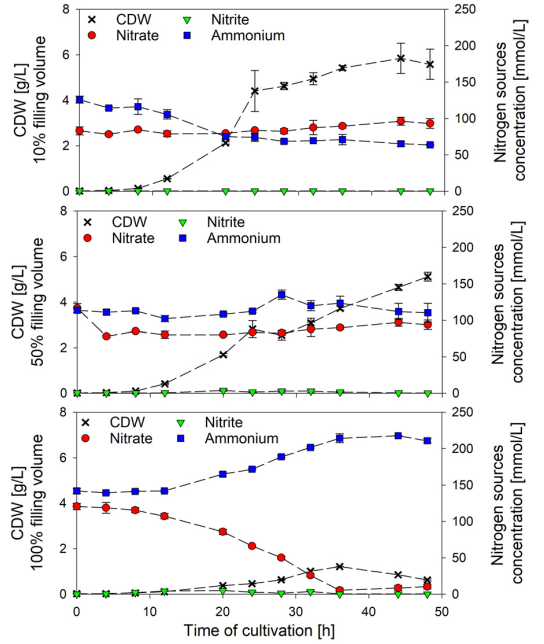

Fig. 1 Cell dry weight (CDW) (g/L) and concentrations of nitrate, nitrite and ammonium (all [mmol/L]) of strain *B. subtilis* JABs24 cultivated in a mineral salt medium employing 40 g/L glucose, 0.1 mol/L NH_4^+ and 0.1 mol/L NO_3^- in baffled shake flasks with three different relative filling volumes of 10%, 50% and 100% representing different oxygen availabilities

nitrite concentrations particularly affect a prolonged lag phase of cultivation of anaerobically growing *B. subtilis* cultures, while the values of maximum specific growth rates μ_{max} remain unchanged (Hoffmann et al. 2020). However, concentrations further declined until CDW_{max}. Hence, the time course of nitrogen sources indicated that higher filling volumes resulted in a converged nitrogen consumption pattern. More precisely, nitrate respiration was preferred with increasing Rv and hence lower oxygen availabilities can be assumed.

Suitability of the promoters P_{narG} and P_{nasD} for gene expression under anaerobic conditions

The nitrogen patterns of the preliminary cultivations have indicated that different oxygen availabilities are present in cultivations with 10%, 50% and 100% Rv. Further cultivations aimed at investigating the suitability and expression of P_{narG} and P_{nasD} as anaerobically inducible promoters. Therefore, strains KM1016, MG1 and MG5 carrying P_{srfA}-*lacZ*, P_{narG}-*lacZ* and P_{nasD}-*lacZ*-fusions, respectively, were cultivated in shake flasks with 10%, 50% and 100% Rv. Comparable growth curves were observed as described in Fig. 1. Figure 2 displays the promoter activities as determined by Miller units (MU) during cultivations.

The P_{srfA} expression was comparatively congruent amongst the different Rv values tested. Altogether, a marginal tendency to higher mean MU with increasing Rv was noticed. In general, P_{srfA} expression dropped from ~300 MU to ~125 MU after 8 h of cultivation. Another slight increase in expression was followed by a subsequent decline to 70–100 MU at CDW_{max}. For both P_{narG} and P_{nasD} expressions, MU increased with increasing Rv. At 10% Rv, LacZ activity was not detectable for transcriptional fusion with P_{narG} and expression did not surpass 10 MU for P_{nasD}. At 50% Rv, both expression levels of P_{narG} and P_{nasD} increased to a maximum after 12 h of cultivation with ~40 MU and ~270 MU, respectively, with an ensuing decrease to ~15 MU and ~140 MU until CDW_{max}. Highest expression levels were monitored at 100% Rv. For P_{narG}, expression increased to ~180 MU after 12 h of cultivation before a decline to ~80 MU was detectable. In contrast to that, maximum expression of P_{nasD} was ~10-fold higher and reached ~2000 MU after 20 h of cultivation. Noticeably, comparable to P_{narG} expression, a reduction of MU values to ~800 MU could be observed at CDW_{max}. In this context, as already described by Hoffmann et al. (2020), a strong P_{nasD} activity is detectable, although the overflow metabolite acetate increases with higher glucose amounts in anaerobic cultivations. In summary, while P_{srfA} expression was relatively constant amongst the different Rv tested, both P_{narG} and P_{nasD} expressions were induced at higher Rv and hence

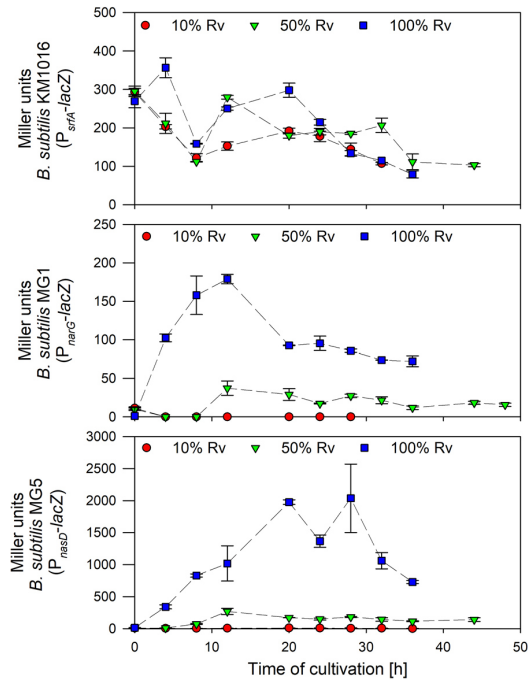

Fig. 2 Time course of Miller units determined by the Miller Assay representing expression of promoters P_{srfA}, P_{narG} and P_{nasD}. Data recorded until CDW_{max} of cultivation of strains *B. subtilis* KM1016 (P_{srfA}-*lacZ*), MG1 (P_{narG}-*lacZ*) and MG5 (P_{nasD}-*lacZ*) in a mineral salt medium employing 40 g/L glucose, 0.1 mol/L NH_4^+ and 0.1 mol/L NO_3^- in baffled shake flasks with three different relative filling volumes (Rv) of 10%, 50% and 100% representing different oxygen availabilities

lower oxygen availabilities. More specifically, P_{nasD} revealed the overall highest expression values.

To apply P_{narG} and P_{nasD} promoter systems for bioprocesses, more information about *B. subtilis* physiology is important. In brief, *B. subtilis* harbors two nitrate reductases with the genes *nasBC* and *narGHI* encoding for the assimilatory and dissimilatory nitrate reductase, respectively. The transcription of the latter one, *narGHI*, is thereby repressed in the presence of oxygen (Hoffmann et al. 1995; Nakano et al. 1996). On the contrary, *nasDE* encodes for both assimilatory and dissimilatory nitrite reductase and transcription is hence also feasible aerobically (Nakano et al. 1998). To demonstrate that the P_{nasD} expression is dependent on the presence of ammonium or nitrate, respectively, *B. subtilis* MG5 was cultivated in media containing either NH_4^+ or NO_3^- in 10% Rv. As expected, mean MU for P_{nasD} expression was below 10 MU in the medium containing only NH_4^+, whereas much higher MU values (continuously ~200 MU) were detected in NO_3^- supplemented medium (Additional

file 1: Fig. S1). In addition, in media supplemented with either 0.005 or 0.01 mol/L NH_4^+, a time-delayed expression of P_{nasD} was monitored (Additional file 1: Fig. S1) which was triggered by depletion of NH_4^+ (data not shown). Hence, it can be concluded that P_{nasD} is a strong, anaerobically inducible promoter, but the effect of anaerobic induction is dependent on the availability of NH_4^+ during aerobic growth.

Investigation of strains *B. subtilis* MG12 and MG14 carrying promoter exchanges P_{srfA}::P_{narG} and P_{srfA}::P_{nasD}

Growth behavior and surfactin synthesis

The promoter exchange strains MG12 (P_{srfAA}::P_{narG}; *amyE*::[P_{narG}-*lacZ, spcR*]) and MG14 (P_{srfA}::P_{nasD}; *amyE*::[P_{nasD}-*lacZ, spcR*]) were cultivated in shake flasks with 10%, 50% and 100% Rv. An overview of CDW and surfactin concentrations of these strains, as well as the reference strain JABs24, are displayed in Fig. 3a and b. In 10% Rv, strains MG12 and MG14 reached CDW_{max} after 24 h and 20 h with 3.93 ± 0.11 g/L and 4.45 ± 0.07 g/L, respectively. CDW remained constant until the end of cultivation. CDW_{max} for strain JABs24 was reached after 44 h with 5.85 ± 0.66 g/L. In 50% Rv, all strains reached CDW_{max} after 44 to 48 h with 5.12 ± 0.20 g/L, 5.78 ± 0.07 g/L and 5.98 ± 0.30 g/L for strains JABs24, MG12 and MG14, respectively. In contrast, CDW_{max} was much lower in cultivations with 100% Rv and did not surpass 1.21 ± 0.01 g/L, 1.12 ± 0.03 g/L and 0.69 ± 0.00 g/L for strains JABs24, MG12 and MG14 after 36 h, 32 h and 44 h, respectively, before the concentration of biomass dropped. In addition, cell agglutination was visible with progressive cultivation causing difficulties in OD_{600} measurements. However, strong induction of P_{nasD} activity was detected, suggesting that cell agglutination does not counteract *nasD* expression.

As expected, strains MG12 and MG14 did not synthesize surfactin at 10% Rv (Fig. 3b). For the reference strain JABs24, the maximum surfactin concentration was measured after 20 h of cultivation with 1668 ± 87 mg/L. During subsequent bacterial growth, the concentration of surfactin declined to 146 ± 118 mg/L at CDW_{max}. The surfactin concentration of the reference strain was even higher with 2806 ± 68 mg/L in 50% Rv. Under this condition, strain MG12 produced up to 215 ± 69 mg/L and strain MG14 up to 1189 ± 15 mg/L. In 100% Rv, lowest surfactin titer was detected for strain MG12 with 193 mg/L, $surfactin_{max}$ of strain MG14 surpassed the titer of

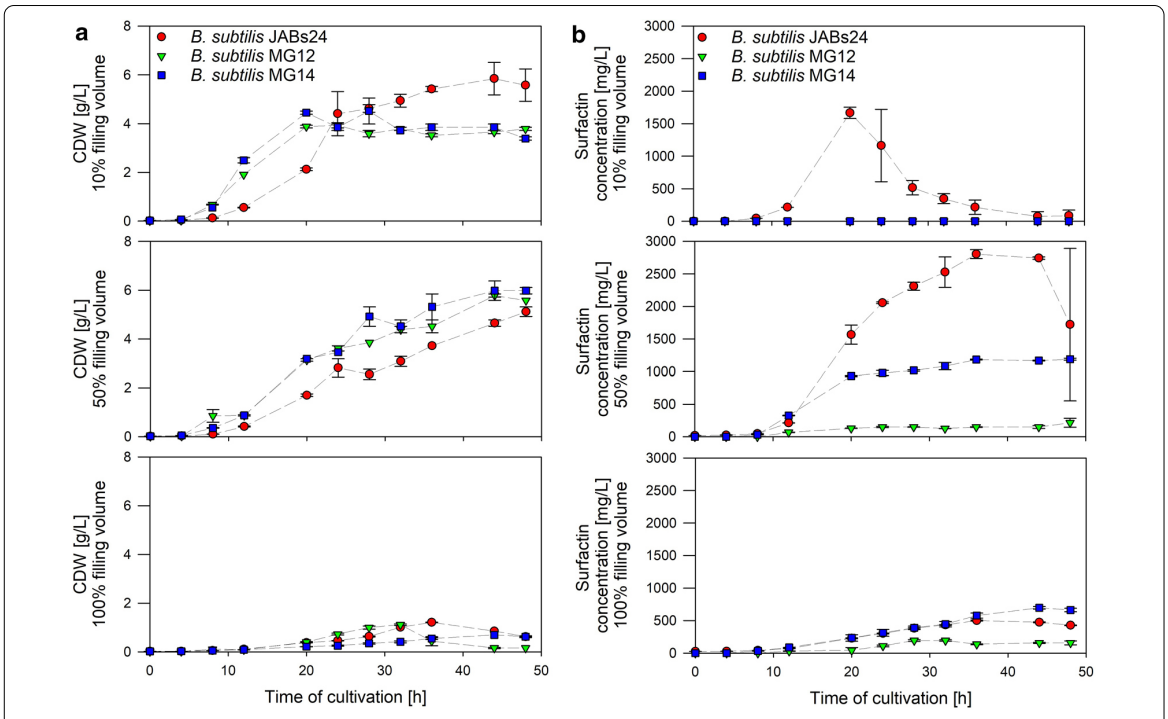

Fig. 3 Time course of cell dry weight (CDW) [g/L] (**a**) and surfactin concentration [mg/L] (**b**) of strains *B. subtilis* JABs24 (reference), MG12 (P_{narG}-*lacZ; amyE*::[P_{narG}-*lacZ, spcR*]) and MG14 (P_{srfA}::P_{nasD}; *amyE*::[P_{nasD}-*lacZ, spcR*]) cultivated in a mineral salt medium with 40 g/L glucose, 0.1 mol/L NH_4^+ and 0.1 mol/L NO_3^- employing three different relative filling volumes of 10%, 50 and 100% representing different oxygen availabilities

strain JABs24 by ~ 200 mg/L with 696 ± 19 mg/L. With respect to foam formation, this issue could be observed for strain JABs24 from the beginning in 10% and 50% Rv, while foam formation was time-delayed for strains MG12 and MG14. At 100% Rv, foam was not present in any cultivation due to the high fluid level and low turbulences.

The corresponding Miller units of strains MG12 and MG14 are displayed in Additional file 1: Fig. S2. In sum, MU values of strain MG12 were similar to strain MG1 (Fig. 2). For strain MG14, MU values in 10% and 50% Rv were also comparable to the cultivation of strain MG5 (Fig. 2). At 100% Rv, highest MU values for MG14 did not surpass ~ 1100 MU and remained relatively constant until CDW_{max}.

In all cultivations, glucose was not depleted. With respect to the nitrogen sources, displayed in Additional file 1: Fig. S3, the patterns were similar as illustrated in Fig. 1 with one exception. In 50% Rv, nitrate consumption and hence increase in ammonium were more conspicuous in strains MG12 and MG14 than for the reference strain. After ~ 32 h of cultivation, nitrate was almost depleted and until this time point ammonium increased.

Strain B. subtilis MG14 reaches remarkable $Y_{P/X}$ values

All yields and growth rates of the cultivation in 10%, 50% and 100% Rv are displayed in Table 2. *B. subtilis* MG12 reached highest overall and maximum growth rates in all cultivations. In 10% and 50% Rv, JABs24 had lowest overall and maximum growth rates. Using 100% Rv, however, lowest growth rates were monitored for strain MG14. A trend amongst the strains for the $Y_{X/S}$ was not identified. Highest Rv and hence lowest oxygen availability resulted in lowest $Y_{X/S}$. As no surfactin was produced in 10% Rv for strain MG12 and MG14, no data could be calculated for $Y_{P/X}$, $Y_{P/S}$ and q. In accordance with surfactin concentrations, strain JABs24 was superior with respect to $Y_{P/X}$, $Y_{P/S}$ and q in cultivations using 50% Rv. Noticeable, strain MG14 reached an overall $Y_{P/X}$ of 1.007 ± 0.028 g/g in 100% Rv, representing an excellent result. In addition, the overall and maximum productivity q was the highest for strain MG14 in 100% Rv with 0.023 ± 0.001 g/(g h) and 0.209 ± 0.012 g/(g h), even surpassing the productivities of strain JABs24 in 10% and 50% Rv.

Bioreactor cultivations

As a rule of thumb, filling volume in shake flasks should not exceed 10% (Rv = 0.1 mL/mL) of flask nominal

Table 2 Comparison of calculated overall and Δt_{max} yields and specific growth rates μ of shake flask cultivations with different relative filling volumes employing strains *B. subtilis* JABs24 (reference), MG12 (P_{srfA}::P_{narG} *amyE*::[P_{narG}-*lacZ*, *spcR*]) and MG14 (P_{srfA}::P_{nasD} *amyE*::[P_{nasD}-*lacZ*, *spcR*])

Relative filling volume	*B. subtilis* strain	Overall yield to CDW_{max}				
		$Y_{P/X}$ [g/g]	$Y_{X/S}$ [g/g]	$Y_{P/S}$ [g/g]	q [g/(g h)]	μ [1/h]
10%	JABs24	0.021 ± 0.001	0.216 ± 0.026	0.005 ± 0.001	0.001 ± 0.000	0.144 ± 0.013
	MG12	0.000 ± 0.000	0.175 ± 0.030	0.000 ± 0.000	0.000 ± 0.000	0.223 ± 0.001
	MG14	0.000 ± 0.000	0.239 ± 0.055	0.000 ± 0.000	0.000 ± 0.000	0.185 ± 0.002
50%	JABs24	0.346 ± 0.242	0.129 ± 0.000	0.044 ± 0.031	0.007 ± 0.005	0.116 ± 0.002
	MG12	0.026 ± 0.004	0.183 ± 0.012	0.005 ± 0.001	0.001 ± 0.000	0.130 ± 0.000
	MG14	0.191 ± 0.009	0.181 ± 0.019	0.035 ± 0.002	0.004 ± 0.000	0.122 ± 0.005
100%	JABs24	0.413 ± 0.002	0.090 ± 0.006	0.036 ± 0.002	0.011 ± 0.000	0.111 ± 0.001
	MG12	0.173 ± 0.015	0.120 ± 0.064	0.020 ± 0.010	0.005 ± 0.000	0.126 ± 0.003
	MG14	1.007 ± 0.028	0.051 ± 0.006	0.053 ± 0.005	0.023 ± 0.001	0.079 ± 0.000
Relative filling volume	*B. subtilis* strain	Δt_{max}				
		$Y_{P/X}$ [g/g]	$Y_{X/S}$ [g/g]	$Y_{P/S}$ [g/g]	q [g/(g h)]	μ [1/h]
10%	JABs24	1.081 ± 0.039	0.535 ± 0.181	0.194 ± 0.001	0.141 ± 0.008	0.369 ± 0.022
	MG12	0.000 ± 0.000	0.289 ± 0.032	0.000 ± 0.000	0.000 ± 0.000	0.775 ± 0.006
	MG14	0.000 ± 0.000	0.339 ± 0.093	0.000 ± 0.000	0.000 ± 0.000	0.527 ± 0.005
50%	JABs24	1.273 ± 0.095	0.629 ± 0.156	0.217 ± 0.037	0.169 ± 0.002	0.343 ± 0.001
	MG12	0.080 ± 0.007	0.331 ± 0.059	0.304 ± 0.279	0.020 ± 0.002	0.833 ± 0.078
	MG14	0.476 ± 0.005	0.451 ± 0.130	0.629 ± 0.554	0.119 ± 0.001	0.471 ± 0.003
100%	JABs24	0.642 ± 0.015	0.208 ± 0.015	0.099 ± 0.017	0.090 ± 0.001	0.205 ± 0.007
	MG12	0.303 ± 0.030	0.291 ± 0.036	0.030 ± 0.012	0.076 ± 0.008	0.388 ± 0.015
	MG14	1.061 ± 0.179	0.161 ± 0.089	0.243 ± 0.135	0.209 ± 0.012	0.151 ± 0.002

volume to ensure sufficient oxygen supply. However, the previous shake flask cultivations have demonstrated that reference strain JABs24 reached highest surfactin titer at a 50% Rv, and even at 100% Rv both $Y_{P/X}$ and q were promising. In the latter condition, *B. subtilis* MG14 with P_{srfA}::P_{nasD} even surpassed the surfactin titers, $Y_{P/X}$ and q of the reference strain. Consequently, the next step aimed at transferring these results to bioreactor scale.

Reference process and preliminary pO_2 strategies

CDW, surfactin and pO_2 levels of different processes employing *B. subtilis* JABs24 or MG1 are given in Fig. S4. A conventional batch process with foam centrifuge employing 20 g/kg glucose was performed as control with an initial agitation and aeration rate of 300 rpm and 2.0 L/min and a pO_2 set-point of 20% (Additional file 1: Fig. S4a). CDW_{max} was reached after 24 h with 77.51 g prior to glucose depletion. Simultaneously, $surfactin_{max}$ was reached with 57.43 g. Nitrate was almost constant throughout cultivation, while ammonium was reduced by ~ 50%. In addition, slight persistent foaming occurred which was accompanied by an increase in the pressure due to blocked exhaust air filters. In a subsequent fed-batch approach, the aeration rate was stopped after 21 h of cultivation and switched to N_2 based ventilation (Additional file 1: Fig. S4b). At this time point, the pO_2 was already at 20% for ~ 5 h. When the process air was stopped, the pO_2 dropped to 0% within seconds. However, also the CDW dropped from 50.27 g to 3.67 g within 4.5 h. Surfactin reached 29.48 g prior to the change in pO_2 and was reduced slightly when CDW dropped. However, cells restored the growth and after 41.5 h of cultivation, a biomass of 24.1 g was obtained with a slight increase in surfactin to 32 g. In a next approach, the pO_2 was decreased stepwise to ideally avoid the observed drop of CDW by cell adaptation (Fig. S4c). Each pO_2 level of 20%, 10%, 5% and 0% was kept for 3 h before aeration was switched to N_2. In this approach, the CDW as well as surfactin still dropped, but to a much lower extent, from 80.74 g to 61.75 g, and 39.84 g to 34.80 g, respectively. Both CDW and surfactin increased afterwards and reached maximum values of 73.26 g and 41.91 g.

Comparison of strains *B. subtilis* KM1016 and MG14 with 1.2 L/min aeration rate employing a self-regulated HNO_3 feed

Due to the observed drops in CDW and the concomitant stagnating surfactin production, another approach was tested employing a constant aeration and agitation rate throughout the cultivation. This should result in a decline of available oxygen per cell and hence an adaptation of cells to microaerobic conditions. First, a fed-batch cultivation was performed with a constant aeration rate of 2 L/min employing the reporter strain MG1 to monitor P_{narG} expression, which is exclusively activated anaerobically. Cells grew without drop and reached a CDW_{max} of 196 g after 40 h. Surfactin values increased constantly during growth to a maximum of 104 g (Fig. S4d). Miller units revealed that only a small portion of cells adapted to anaerobic conditions. During Miller Assay, the yellow color as indicator to stop the reaction was visible in less than 3 min, but calculation revealed 5 MU, as the high OD_{600} of 30 is included in the calculation. Hence, it can be assumed that a high expression level of the promoter is present, but most cells did not grow by nitrate respiration. A cultivation with 1.2 L/min and strain MG1, which actually represents the lowest aeration rate that could be set with the given technical equipment, resulted in Miller units up to 35 for P_{narG} and was hence used as process to compare *B. subtilis* KM1016 and MG14 with P_{srfA}::P_{nasD}. Figure 4 exemplarily illustrates the CDW, glucose, surfactin, nitrate, nitrite and ammonium values of a cultivation with *B. subtilis* KM1016 (Fig. 4a) and *B. subtilis* MG14 (Fig. 4b). In addition, OD_{600} in correlation to Miller units and pO_2 are given in Fig. 4c and d for strains KM1016 and MG14, respectively. Comparable CDW_{max} values were reached for strain KM1016 and MG14 after 46 h with 140 g and 147 g, respectively, whereas the growth was terminated by glucose depletion. In contrast, different $surfactin_{max}$ were reached at CDW_{max} with 81 g for KM1016 and 35 g for MG14. Strain KM1016 consumed a total of 5.6 mol nitrate until CDW_{max}. Nitrate addition by HNO_3 was higher than consumption, resulting in a nitrate accumulation to 2.7 mol in the medium. Strain MG14 showed a similar behavior but consumed less nitrate. At CDW_{max}, total nitrate consumption was 4.5 mol and another 4.2 mol were detectable in the medium.

The detection of ammonium showed an initial slight decrease before the amount increased steadily for both strains due to nitrate reduction and reached 5.8 mol and 6.7 mol for strains KM1016 and MG14, respectively. Nitrite concentrations increased until 22 h in both cultivations. After a subsequent decrease until ~ 30 h, nitrite again increased steadily and reached values of ~ 0.21 mol at CDW_{max}. The pO_2 declined faster in the cultivation of strain MG14 which correlated to higher growth rates (Fig. 4d). For strain KM1016, a pO_2 of ~ 14% was reached after ~ 15 h, and mean pO_2 of ~ 1.8% was measured after 25 h of cultivation (Fig. 4c). On the contrary, the pO_2 of strain MG14 fell to ~ 2% after 7 h of cultivation, and a mean pO_2 of ~ 1.2% was reached after ~ 15 h of cultivation. For both strains, the pO_2 increased drastically when cell growth was terminated. In terms of foaming, the foam centrifuge was activated right from the beginning for strain KM1016 and after ~ 6 h for strain MG14. With

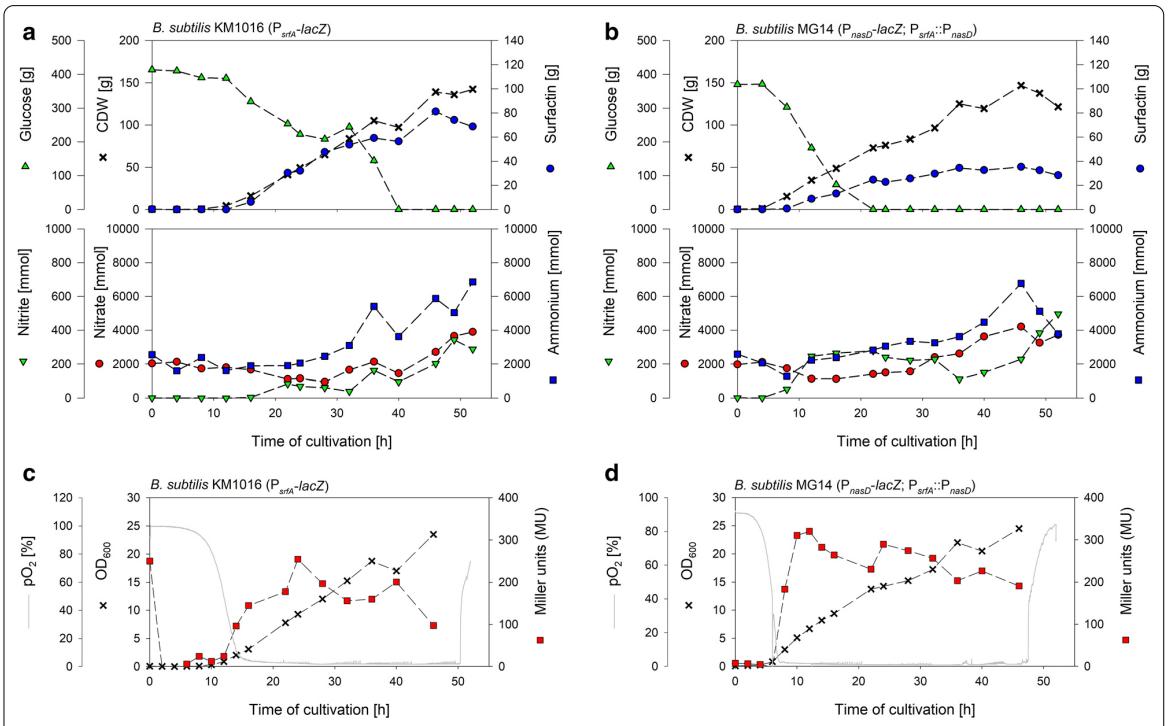

Fig. 4 Exemplary cultivation results of fed-batch bioreactor cultivations employing strains *B. subtilis* KM1016 (P_{srfA}-*lacZ*) (**a**, **c**) and *B. subtilis* MG14 (P_{srfA}::P_{nasD}; *amyE*::[P_{nasD}-*lacZ*, *spcR*]) (**b**, **d**). Displayed are absolute values of cell dry weight (CDW), glucose, surfactin (all [g]), nitrate, nitrite and ammonium (all [mmol]) (**a**, **b**), as well as the time course of OD_{600}, Miller units as determined by the Miller Assay to determine the expression of P_{srfA} and P_{nasD}, and pO_2 [%] (**c**, **d**)

respect to the Miller units, P_{srfA} expression was detectable as soon as cells grew after a short lag-phase. A maximum of 250 MU was determined after 24 h of cultivation and values slightly decreased afterwards (Fig. 4c). For P_{nasD}, induction levels were below 5 MU until 6 h of cultivation. In accordance with the pO_2, induction increased to 180 MU after 8 h when the pO_2 was < 2%. Highest expression was detected after 12 h with 320 MU before expression declined slightly (Fig. 4d).

Table 3 summarizes the main parameters and overall yields of the cultivations with strains KM1016 and MG14 employing an aeration rate of 1.2 L/min. For comparison, data of the reference process with *B. subtilis* JABs24 and the processes employing a constant aeration rate of 2 L/min with strain MG1 are included. Corresponding calculated Δt_{max} yields and growth rates are listed in Additional file 1: Table S3. For fed-batch processes and for better comparison to the reference batch cultivation, results are displayed as "after batch glucose consumption" calculations and "end of process" calculations. The fed-batch process employing the reference strain and 1.2 L/min reached only 82.14 g of surfactin, which resulted in a productivity q of 0.012 g/(g h). This value is similar to the 2 L/min fed-batch process with 0.013 g/(g h), where 104.57 g of surfactin were produced, but with biomass of 196.97 g. Comparing batch data, the reference batch process had a productivity q of 0.031 g/(g h). Here, the 2 L/min process was inferior with 0.022 g/(g h) and the 1.2 L/min process performed similar with 0.030 g/(g h). Comparing strains KM1016 and MG14, both processes reached comparable CDW of 147.52 g and 150.18 g, but the surfactin produced with strain MG14 with 31.56 g was ~2.5-fold lower. Hence, both productivities and yields were inferior for strain MG14 and solely the growth rates during the batch phase were superior.

Discussion

The current study aimed at introducing a promoter exchange strain that allows for self-inducible surfactin synthesis when oxygen-limiting, or even depleting, conditions occur and anaerobic nitrate respiration initiates. To mimic different oxygen availabilities in shake flask cultivations, either the filling volume or the agitation rate can be varied. This method was already used in other studies investigating surfactin synthesis in *B. subtilis* (Fahim et al. 2012; Jokari et al. 2013; Rangarajan

Table 3 Summary of main process data, calculated overall yields and specific growth rates of *B. subtilis* bioreactor cultivations

Strategy	Reference pO_2 set-point 20%	Fed-batch, constant aeration of 2 L/min		Fed-batch, constant aeration of 1.2 L/min		Fed-batch, constant aeration of 1.2 L/min	
Evaluation until	End of batch	After batch glucose consumption	End of process	After batch glucose consumption	End of process	After batch glucose consumption	End of process
B. subtilis strain	JABs24	MG1		KM1016		MG14	
Time of cultivation (h)	24	22	40	26	46	19	46
CDW_{max} (g)	77.51	104.55	196.97	61.54 ± 9.21	147.52 ± 8.22	55.78 ± 13.24	150.18 ± 3.33
$Surfactin_{max}$ (g)	57.43	51.65	104.57	48.47 ± 11.81	82.14 ± 0.91	15.49 ± 6.40	31.56 ± 3.74
$Y_{P/X}$ (g/g)	0.741	0.494	0.531	0.776 ± 0.088	0.557 ± 0.024	0.266 ± 0.039	0.211 ± 0.030
$Y_{X/S}$ (g/g)	0.145	0.249	0.195	0.161 ± 0.011	0.131 ± 0.005	0.134 ± 0.016	0.123 ± 0.003
$Y_{P/S}$ (g/g)	0.108	0.124	0.103	0.127 ± 0.022	0.073 ± 0.001	0.036 ± 0.010	0.026 ± 0.004
q (g/(g·h))	0.031	0.022	0.013	0.030 ± 0.003	0.012 ± 0.001	0.014 ± 0.002	0.004 ± 0.001
μ (1/h)	0.222	0.272	0.165	0.204 ± 0.009	0.134 ± 0.005	0.271 ± 0.010	0.128 ± 0.003

et al. 2015; Rocha et al. 2020). In the current study, different filling volumes were employed. Studies of Schiefelbein et al. (2013) and Heyman et al. (2019), investigating either the kLa value or oxygen transfer rate OTR, have demonstrated that higher filling volumes result in lower oxygen availability. Results from this study supported the hypothesis when nitrogen sources were analyzed at different Rv resulting in either aerobic growth on ammonium as preferred nitrogen source, or anaerobic growth by nitrate respiration.

Experiments have demonstrated that *B. subtilis* MG14 with P_{srfA}::P_{nasD} poses an interesting candidate for further bioreactor process developments targeting at self-inducible surfactin synthesis uncoupled from the native quorum-sensing system. In addition, this self-inducible system is advantageous compared to IPTG-induced promoters due to reduced production costs. Furthermore, foaming was also lowered, and time delayed in both shake flask and bioreactor cultivations. Interestingly, both strains MG12 (P_{srfA}::P_{narG}) and MG14 showed improved growth rates when no surfactin was produced in 10% Rv. At both 50% and 100% Rv, increased growth rates were obtained with decreasing surfactin titers. Hence, expression and presence of surfactin seems to result in reduced growth rates, which is in accordance with previous studies (Tsuge et al. 2001; Vahidinasab et al. 2020). Shake flask cultivations employing different Rv have been shown to be a suitable approach to investigate the activation of the promoters P_{narG} and P_{nasD}. In the presented set-up, the reference strains MG1 and MG5 reached Miller units of up to 2000 for P_{nasD} and 180 for P_{narG}, respectively. Hence, P_{nasD} expressions were even slightly higher than reported by Hoffmann et al. (2020) when cultivating in anaerobic serum flasks. On the contrary, P_{narG} expression levels were reported to reach up to 440 MU (Hoffmann et al. 2020). Consequently, as P_{narG} is an exclusively anaerobically activated promoter, the combination of 100% Rv at 120 rpm probably did not result in full anaerobic induction. One possibility to further decrease the oxygen input would be to vary the agitation rate as performed by Fahim et al. (2012). However, cell agglutination already occurred at 120 rpm and is expected to appear even earlier at lower agitation rates. Hence, as nitrate was often observed as growth limiting factor, increasing the nitrate concentration to elongate the cultivation would not be possible under these circumstances. One option would be to perform further strain engineering and delete genes responsible for biofilm formation and thus for cell agglutination (Pedrido et al. 2013). Assuming microaerobic conditions at 100% Rv and 120 rpm, strain MG14 reached up to ~ 3-fold higher P_{nasD} expression than P_{srfA} in the reference strain. On the contrary, the surfactin concentration was only 1.4-fold increased. Feedback mechanisms or nutrient limitations might result in this observed discrepancy, and further studies must investigate which optimizations have to be performed on a molecular level or in operational parameter to reach a 3-fold increase in surfactin titer to exploit the complete potential of P_{nasD}.

As reported by Hoffmann et al. (2020), high ammonium and glucose concentrations reduced expression of P_{narG}, but such a reductive effect was not observed for P_{nasD}. Still, fine-tuning of the medium is important to find a good balance between sufficient ammonium for aerobically growing cells, and cells performing nitrate respiration, hence providing ammonium. Particularly, the effect of different nitrate concentrations on the expression of both P_{narG} and P_{nasD} has not been examined so far. This is also of utmost importance when employing a self-regulated HNO_3-feed as pH regulator. Indeed, the amount

of HNO_3 added was higher than nitrate consumption due to the production of various metabolites that alkalize the medium on top to the pH shift caused by nitrate respiration, resulting in a continuous increase in nitrate. In addition, the amount of HNO_3 needed is three-times higher than for e.g. H_3PO_4 (monovalent vs. trivalent acid). This results in further limitations as the bioreactor capacity is exhausted earlier. With the current set-up it cannot be examined if both oxygen-depleted and nitrate-depleted conditions reach even higher $Y_{P/X}$ values as reported by Davis et al. (1999). For this purpose, mathematical modeling of the bioprocess combining a metabolic model of the nitrogen metabolism, oxygen availability and consumption, along with mechanisms of genetic regulation can be used to provide further insights into this complex interplay. Using this toolset, investigation and evaluation of a dual-limitation fed-batch process becomes feasible, which is a very demanding task for process control, as both limitations need to be tightly regulated (Noll and Henkel 2020).

With respect to bioreactor cultivations, the original intention was to perform so called switch-processes, with the first part aiming at biomass accumulation, and the second part resulting in surfactin production. However, a drastic decrease in biomass was observed when cells entered oxygen-limited or even depleted conditions too fast. It was recently reported that ~ 90% of cells died upon oxygen depletion, while the remaining cells maintain their viability (Arjes et al. 2020). Similar observations were made in the current employed processes with drop in CDW. Although cells restored their growth, the surfactin productivity of these cells was reduced. Consequently, when targeting at switch processes with different pO_2-profiles, it is of utmost importance to relate oxygen availability to adaptation of cells to anaerobic conditions. For example, Hoffmann et al. (1995) reported on a lag-phase of 24 to 36 h when aerobically growing cells were transferred to anaerobic conditions. This would result in time-consuming processes and the increased growth rates observed in aerobic non-surfactin producing cells would probably not compensate for these adaptation times. In a further approach, a cultivation with a constant agitation rate of 300 rpm and aeration rate of 1.2 L/min was tested. This process set-up was identified as easily operable and issues due to foam formation were drastically reduced compared to conventional batch and fed-batch processes with varying agitation and aeration rates due to pO_2 regulation. In addition, foaming occurred time-delayed in strain MG14 compared to the reference strain KM1016 which can be attributed in parts to the absence of surfactin during the first hours of cultivation. However, foam formation was yet present prior to the induction of surfactin production. In this sense, identification of other metabolites, such as proteins, in foam and deletion of corresponding genes might be a target in future works to further reduce the foam formation. Results of both surfactin titer and promoter activity of P_{nasD} also clearly indicated that the employed set-up more likely correlated to shake flask cultivations with 50% Rv. Also based on the Miller units obtained, it can be assumed that most cells still grew aerobically. This leads to the open question how many cells have already adapted to anaerobic conditions and it is crucial to further characterize the cell differentiation patterns in aerobically-anaerobically growing cells. Hence, further bioreactor cultivations must be performed with even lower aeration rates, either by applying air with a lower oxygen concentration (currently 16%), pure oxygen or, of course, by adjusting the technical equipment to allow for aeration rates below 1.2 L/min. Ideally, applying pure oxygen at much lower aeration rates (< 0.2 L/min) would result in two advantages. First, even less foam formation is expected to occur, and second, the autoinduction of surfactin synthesis is expected to be much higher as the available amount of oxygen per cell decreases faster. Another issue faced was the drastic accumulation of nitrite in bioreactor cultivations, which was not observed in shake flasks. At these concentrations, nitrite is reported to reduce and even inhibit cell growth (Hoffmann et al. 2020). Prior to further bioreactor process optimization, studies must address this limitation and investigate the defect of nitrite reduction to ammonium. Nevertheless, even under the current bioreactor process conditions, strain MG14 with a $Y_{P/X}$ of 0.211 g/g and productivity q of 0.004 g/(g h) was already superior to other non-conventional bioreactor processes reported by Chtioui et al. (2012) (Rotating disc bioreactor, $Y_{P/X} = 0.068$ g/g, $q = 0.001$ g/(g h)) Coutte et al. (2010b) (Membrane bioreactor, $Y_{P/X} = 0.078$ g/g, $q = 0.002$ g/(g h)). A comparative table was prepared previously in Hoffmann et al. (2020). A foam-free anaerobic cultivation was reported to reach a promising $Y_{P/X}$ with 0.278 g/g (Willenbacher et al. 2015), but as summarized by Hoffmann et al. (2020), complete anaerobic processes are time-consuming due to low growth rates and the overall surfactin titers are by far not competitive to conventional aerobic batch processes. With further appropriate regulation of the nitrogen sources and aeration, processes employing strain MG14 are expected to become superior to the reference strain JABs24 as demonstrated in shake flask cultivations with a $Y_{P/X}$ of 1.007 g/g and productivity q of 0.023 g/(g h). Reaching these yields in bioreactor cultivations, strain MG14 will also surpass reported yields of an IPTG-inducible promoter with $Y_{P/X}$ of 0.92 and q

of 0.025 g/(g h) (calculated based on data given in Jiao et al. (2017)), being additionally advantageous due to the proposed cheap, foam-reduced and self-inducible system.

To conclude, a self-inducible *B. subtilis* strain for surfactin synthesis induced upon oxygen-limitation has been developed. First, two interesting promoters, P_{narG} and P_{nasD} that are involved in anaerobic nitrate respiration, were tested. P_{narG} has the advantage of being an exclusively anaerobically activated promoter, but surfactin titers and yields were shown to not be competitive to the reference strain *B. subtilis* JABs24. However, strain MG14 with P_{srfA}::P_{nasD} surpassed the titer of strain JABs24 by 200 mg/L at low oxygen availability and reached an exceptionally high $Y_{P/X}$ of 1.007 g/g. Strain *B. subtilis* MG14 poses an interesting candidate for further surfactin production process development. Additional process developments are crucial to elaborate the high potential of strain *B. subtilis* MG14 as self-inducible surfactin producer in reduced foaming or even foam-free environments.

Supplementary Information

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Additional file 1. Information on plasmids and oligonucleotides used, main process data for bioreactor cultivations as well as the availability of nitrogen sources and their impact on target promoter expression during bioprocesses. **Figure S1**: Time course of CDW and P_{nasD}expression under different availability of nitrogen sources.**Figure S2**: Time course of P_{narG} and P_{nasD} expression in MG12 and MG14 under different relative filling volumes. **Figure S3**: Availability of nitrate, nitrite and ammonium during cultivation of MG12, MG14 and JABs24 under different relative filling volumes. **Figure S4**: Strategies for oxygen-mediated bioreactor switch-processes using JABs24 and MG1.

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Authors' contributions
MHo planned and executed the experiments, collected data, created the graphs and drafted the whole manuscript. AB and DSFC performed part of the experiments and collected and evaluated corresponding data. KR, PB, CT and PK were involved in strain engineering. MHe supported in interpretation of results. LL significantly contributed to conception and design of the study and interpretation of the experiments. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

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Availability of data and materials
All discussed data have been included into the manuscript or in the additional file. Please turn to the corresponding author for all other requests.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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The authors declare that they have no conflict of interests.

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Publisher's note

Chapter 3 General Discussion

3.1 Introductory remarks

Biosurfactants of microbial origin are often reported as environmentally friendly alternative to conventional petrochemically or oleochemically produced surfactants (Banat et al. 2000, Singh et al. 2019). The cyclic lipopeptide surfactin synthesized by *Bacillus subtilis* represents one candidate with excellent emulsifying properties (Hoffmann et al. 2021a). Numerous studies report on the high application potential (Chapter 1.2). Nevertheless, high-titer production strains are rare and conventional aerobic processes are accompanied by severe foaming as surfactin accumulates at air-water-interfaces. This poses a major hurdle for large-scale and high-titer bioreactor cultivations (Klausmann et al. 2021). The foam-free anaerobic cultivation to synthesize surfactin as reported by Willenbacher et al. (2015a) represents an interesting process strategy and was further examined within this study. More precisely, *B. subtilis* strains grew by anaerobic nitrate respiration. In this respiratory pathway, nitrate serves as electron acceptor and is reduced to ammonium via nitrite. This results in a proton gradient to generate ATP (Nakano and Hulett 1997). Indeed, to date, the anaerobic lifestyle of *B. subtilis* and genes and regulators involved are well explored, but only little studies report on this method as cultivation strategy to produce target products such as biosurfactants. In this thesis, different aspects and approaches were included, aiming at anaerobic process characterization and at the anaerobic production of surfactin as product of interest. The approaches can be categorized into three parts:

(1) Media and process parameter optimization
- Impact of different temperatures (Chapter 2.1)
- Influence of different ammonium and glucose concentrations (Chapter 2.2)
- Effect of altering oxygen availabilities (Chapter 2.3)

(2) Strain engineering
- Application of a genome reduced *B. subtilis* strain (Chapter 2.1)
- Promoter exchange to induce surfactin synthesis under oxygen limited conditions (Chapter 2.3)

(3) Novel process strategies
- Anaerobic cultivation as fed-batch process (Chapter 2.2)
- Aerobic-anaerobic switch processes (Chapter 2.3)
- Self-inducible surfactin synthesis upon oxygen limited conditions (Chapter 2.3)

3.2 Surfactin quantification as key method

In the current thesis, the surfactin analysis played a crucial role throughout all research approaches (Chapter 2.1, Chapter 2.2 and Chapter 2.3). A suitable HPTLC method for quantification as well as an appropriate protocol for sample preparation was established and validated previously (Geissler et al. 2017). This method was comparable to HPLC analysis (Willenbacher et al. 2014) and was hence applied in all experiments for surfactin quantification. Indeed, future studies should include MS analyses of the surfactin produced, as "surfactin" represents a lipopeptide family produced by *Bacillus* species and comprises a collection of congeners with structural similarities (Chapter 1.2). Previous studies also reported that the medium composition and process parameter have an impact on the surfactin congeners produced (Akpa et al. 2001, Bartal et al. 2018, Sun et al. 2019), but studies on the structure-activity relationship are rare. It can be assumed that the congener patterns change as well within this study applying different oxygen availabilities and nitrogen sources. In this context, more specific application potentials of the produced surfactin might be identified, as different congeners are attributed with different physico-chemical properties such as foam stability (Razafrindalambo et al. 1998, Kracht et al. 1999, Liu et al. 2009, Liu et al. 2015).

3.3 *B. subtilis* JABs24 as promising target strain for further surfactin process development

In a first approach, the genome reduced strain *B. subtilis* IIG-Bs20-5-1 was evaluated regarding its applicability as surfactin producer under both aerobic and anaerobic conditions at various temperatures (Chapter 2.1). As this strain lacks non-essential genes, amongst others genes involved in sporulation or encoding for toxins and prophages, it was hypothesized that the deletion of 10% of the genome saves metabolic resources and hence results in increased surfactin titers at a similar or even better growth behavior compared to the primary reference strain *B. subtilis* JABs24. Both strain IIG-Bs20-5-1 and JABs24 are derived from the established laboratory strain *B. subtilis* 168 and carry a functional *sfp* gene, which is amongst others necessary for surfactin synthesis (Chapter 1.2). Indeed, strain IIG-Bs20-5-1 reached similar or even higher biomasses than JABs24, reached overall higher specific growth rates μ and was superior with respect to glucose conversion into biomass $Y_{X/S}$. However, contrarily to the expectations, strain IIG-Bs20-5-1 was inferior regarding surfactin titers, $Y_{P/X}$, $Y_{P/S}$ and

productivity *q*. For example, at 37 °C, strain IIG-Bs20-5-1 produced 993.03 mg/L surfactin aerobically and 215.31 mg/L anaerobically at CDW_{max}. This accounts for 13.42% and 27.35% lower titers compared to strain JABs24, respectively. In fact, 37 °C was emphasized as optimal temperature under the tested conditions and was hence used for all further experiments described in Chapter 2.2 and Chapter 2.3.

In a recent study, more promising results were reported with a genome reduced *B. amyloliquefaciens* strain lacking 4.18% of the genome (Zhang et al. 2020). This strain, named GR167, had higher specific growth rates compared to the respective reference strain and reached approximately 9.7% elevated surfactin titers. Interestingly, an additional deletion of the operon encoding for NRPS synthesizing lipopeptides iturin and fengycin resulted in a further 10% increase in titer to ~30 mg/L and of 56% improvement in specific productivity. This was not observed within this thesis, although strain *B. subtilis* IIG-Bs20-5-1 also lacked the gene encoding for the NRPS for the lipopeptide plipastatin. Though, it must be emphasized that the obtained concentrations by Zhang et al. (2020) are overall comparatively low, suggesting that this strain is less suited for further high-titer surfactin production optimization than strain IIG-Bs20-5-1. Indeed, the same deletions as reported by Zhang et al. (2020) could be applied to strain 168 with functional *sfp* to allow for a distinct comparison with respect to surfactin titer improvement. In addition, different *Bacillus* species cannot be compared one-to-one. Besides, studies on the availability of amino acid or fatty acid precursors for surfactin synthesis or Sfp, which is crucial for surfactin synthesis (Chapter 1.2) could give further insights into the effect of the employed genome reduction. Interestingly, similar to the results reported by Zhang et al. (2020), a *B. subtilis* 168 derived strain lacking 3.8% of total genes reached a 3.3-fold increased surfactin titer of 1.7 g/L aerobically (Wu et al. 2019). The strain lacked biofilm-formation related genes and non-ribosomal peptide synthethase / polyketide synthase pathways. Hence, although all genome reduced strains lacked amongst others genes encoding for additional non-ribosomal peptide synthetases, strain IIG-Bs20-5-1 did not reach elevated surfactin titers. Further or other gene deletions might be feasible to create an adapted genome reduced strain based on *B. subtilis* JABs24, especially as this strain already reached more than 1 g/L surfactin aerobically without genome reduction. For example, similar to Zhang et al. (2020) and Wu et al. (2019), target genes for deletion could be *eps* and *capBCA* (synonym: *pgsBCA*) encoding for enzymes catalyzing extracellular polysaccharides and poly-γ-glutamate, as the synthesis is energetically demanding. These substances are involved in biofilm formation, and a deletion might also be beneficial for process control. For instance, as described

in Chapter 2.1, anaerobic serum flasks were incubated horizontally. An upright position resulted in cell agglutination with ongoing cultivation. This was also observed in the experiments presented in Chapter 2.3, when flasks were incubated with a relative filling volume of 1 mL/mL to mimic reduced oxygen availabilities. Contrariwise, depending on the process method applied, strong biofilm formation can also be a useful tool in, e.g., biofilm bioreactors (Brück et al. 2019, Brück et al. 2020).

Nevertheless, although the employed genome reduced strain IIG-Bs20-5-1 was inferior both aerobically and anaerobically, reference strain JABs24 was identified as a promising target strain for further process investigation and development as exceptional high titers of 1147.03 mg/L aerobically and 296.37 mg/L anaerobically were reached at 37 °C without further genetic modification. These values were even higher than for reported and promising surfactin producer strains *B. subtilis* DSM 10^T (Chapter 2.1, Willenbacher et al. 2014) and *B. subtilis* ATCC 21332 (Davis et al. 1999, Willenbacher et al. 2014). For comparison, strain DSM 10^T reached only 400 mg/L in aerobic and 158 mg/L in anaerobic shake flask cultivations employing 10 g/L glucose (Willenbacher et al. 2015a, Willenbacher et al. 2015b), which matches the carbon concentration employed in the experiments described in Chapter 2.1. Consequently, *B. subtilis* JABs24 was used for all further experiments presented in Chapter 2.2 and Chapter 2.3.

3.4 Drawbacks of anaerobic cultivations make this strategy a laborious approach to compete with aerobic processes

In Chapter 2.1, the anaerobic cultivation of *B. subtilis* JABs24 to synthesize surfactin was identified as promising strategy in terms of $Y_{P/X}$, an important factor in biotechnological processes. A high productivity of each single cell is desirable, as the cell itself serves as "catalyst" and the biomass is considered as waste stream which is, for example, further used as fertilizer. However, in direct comparison, the overall surfactin titers of anaerobically growing cells by nitrate respiration were much lower than in aerobic cultivations, which is a result of the reduced total biomasses obtained due to low growth rates and inferior $Y_{X/S}$ values (Chapter 2.1). As summarized in Chapter 2.2, studies on anaerobic growth either by fermentation or nitrate respiration generally did not focus on the production of a certain metabolite, but on fundamental research. Reported anaerobically achieved OD-values are mostly below three employing *B. subtilis*. A recent study targeting at anaerobic biosurfactant production reached an OD_{600} of

3.953 after 6 days of cultivation employing the isolate *B. subtilis* AnPL-1 and a medium containing sucrose and nitrate (Zhao et al. 2021). This, however, is still not even remotely comparable to the OD_{600} values of aerobic cultivations, although anaerobic nitrate respiration is the energetically most favorable form of respiration to generate ATP after aerobic growth with oxygen as terminal electron acceptor (Marino et al. 2001). For example, employing 10 g/L glucose strain *B. subtilis* JABs24 reached an OD_{600} of 10 aerobically and 1.2 anaerobically after 21 h and 78 h of cultivation, respectively (Chapter 2.1). Consequently, studies on the effect of various ammonium, nitrite and glucose concentrations were performed to evaluate if the presence of these media components have a negative impact on anaerobically growing cells and the observed discrepancies in growth (Chapter 2.2). For this purpose, reporter strains were used to monitor the promoter expression of either P_{narG} or P_{nasD}, promoters of nitrate reductase and nitrite reductase that are crucial for anaerobic nitrate respiration (Hoffmann et al. 1995, Nakano et al. 1998a). The reporter strains MG1 (P_{narG}-*lacZ*) and MG5 (P_{nasD}-*lacZ*) were also used for the experiments described in Chapter 2.3, as they were emphasized as a promising tool to rate the microbial performance during cultivations. Indeed, in Chapter 2.3, a reporter strain monitoring the expression of native surfactin operon promoter P_{srfA} was included as well, which would have been an interesting strain for the experiments described in Chapter 2.2. For example, increasing initial levels of ammonium resulted in decreased expressions of P_{narG} and especially reduced surfactin titers. Hence, it cannot be concluded if high ammonium concentrations resulted in lower P_{srfA} expression, or if other intracellular mechanisms caused a decrease in surfactin concentrations due to, e.g., lack of precursors. Next to ammonium, the impact of nitrite was investigated as well (Chapter 2.2). Increasing nitrite concentrations resulted in prolonged lag-phases, but growth rates were in a similar range during subsequent exponential growth, even when nitrite was still present. Indeed, nitrite can also serve as alternative electron acceptor instead of nitrate. Studies investigating anaerobic growth on either nitrate or nitrite reported on similar OD_{max} values (Hoffmann et al. 1998, Cruz Ramos et al. 2000, Marino et al. 2001). Consequently, further studies on the regulatory mechanism and interdependency of nitrogen sources are needed to fully understand anaerobic growth by nitrate respiration, allowing to optimally set cultivation parameters.

With respect to glucose, Willenbacher et al. (2015a) reported on superior $Y_{P/X}$ values at low glucose levels employing strain *B. subtilis* DSM 10^T. As strain *B. subtilis* JABs24 was identified as more promising in terms of anaerobic cultivations targeting at surfactin production than DSM 10^T (Chapter 2.1), strain JABs24 was cultivated employing different glucose levels

as well ranging from 2.5 g/L to 10 g/L (Chapter 2.2). Additionally, as the $Y_{X/S}$ is rather low anaerobically, it was hypothesized that glucose might be converted into acetate due to overflow metabolism at high glucose concentrations. For both surfactin and acetate, a slight trend to increased titers with increasing glucose levels was observed but the $Y_{surfactin/S}$ decreased. However, acetate concentrations were more than 10-fold higher than surfactin titers ranging from 1.15 to 2.17 g/L at CDW_{max}. In bioreactor batch and fed-batch processes, acetate production was also identified as limitation and the synthesis was independent of the performed cultivation strategy. Interestingly, surfactin production was highly similar in both processes and hence independent of the applied strategies as batch and fed-batch process. Indeed, as an increase in glucose resulted in reduced growth rates and P_{narG} expression, ongoing process development should be performed as fed-batch to keep the glucose concentration low. Acetate, added at different concentrations, was furthermore identified as overall and maximum growth rate reducing metabolite. Future studies must address this issue to establish anaerobic processes that maintain both the growth rate and surfactin productivity. One option to further improve the performance of anaerobic cultures would be media optimization. For example, Zhao et al. (2021) investigated the effect of different carbon sources on anaerobic biosurfactant production, with the biosurfactant being identified as surfactin. Sucrose was thereby reported to reach at least a 1.5-fold higher biosurfactant titer compared to glucose, molasses, corn starch and glycerol. An explanation could be that one molecule of sucrose provides two molecules of glucose to enter glycolysis. Hence, also *B. subtilis* JABs24 could be cultivated on different carbon sources to investigate the effect on growth, surfactin synthesis and acetate formation. Ideally, the optimal production process in terms of circular bioeconomy contains alternative media feedstocks derived from agricultural or food waste streams (Carus and Dammer 2018, Kardung et al. 2021). However, in the current stage of research, defined media are highly beneficial as to identify the impact of individual components as demonstrated for ammonium, nitrite, glucose and of course acetate (Chapter 2.2).

With respect to bioreactor cultivations, several further drawbacks were identified and described in Chapter 2.2. Amongst others, anaerobic nitrate respiration results in a basic pH shift (Willenbacher et al. 2015a). However, the scale-up bioreactor process presented in Chapter 2.2 caused an acidic pH shift, presumably due to accumulated CO_2 that converts to H_2CO_3, which is in equilibrium with the carbonic acid ions, when the process is performed without nitrogen flow through the medium. Indeed, applying a nitrogen flow through the medium resulted in the expected acidic pH shift (data not shown), and furthermore ensures anaerobic conditions

throughout the cultivation as reflow is prevented. On the contrary, similar to an aeration with air or pure oxygen, foaming is induced and the idea of foam-free anaerobic surfactin production is unfeasible. Altogether, in Chapter 2.1 and Chapter 2.2 the foam-free synthesis of surfactin by anaerobic nitrate respiration was identified as viable, but also as a laborious, time-consuming cultivation approach reaching surfactin titers that are not competitive to aerobic cultivations although high $Y_{P/X}$ values can be achieved. As reported in Chapter 2.2, *B. subtilis* JABs24 reached ~2 g surfactin (0.101 g/L) within 63.5 h in an anaerobic fed-batch cultivation. This amount was already achieved within less than 10 h aerobically in a conventional batch process (Chapter 2.3, Supplemental Material). In addition, many limitations such as the presence of acetate, and hence loss of glucose to be converted into biomass and surfactin, must be overcome to make this approach comparable to aerobic cultures. In addition, the scale-up from the foam-free process with an acidic pH shift as reported by Willenbacher et al. (2015a) was not achieved (Chapter 2.3) and further studies must investigate if longer cultivation times without nitrogen flow are operable.

3.5 Aerobic-anaerobic switch processes with self-inducible surfactin synthesis as promising cultivation strategy

With respect to the knowledge gained in Chapter 2.1 and Chapter 2.2, a novel process strategy was investigated and presented in Chapter 2.3 aiming at combining advantages of both aerobic and anaerobic cultivations. As demonstrated, aerobic cultures have both higher specific growth rates and surfactin titers, anaerobic cultivations reach promising $Y_{P/X}$ values (Chapter 2.1) and result in foam-free or reduced foaming environments if cultivated without nitrogen flow (Chapter 2.2) or with a rather low nitrogen flow, respectively. Consequently, the aim was to establish so-called switch processes with an initial aerobic growth phase to accumulate biomass, followed by an anaerobic surfactin production phase. In addition, it was hypothesized that the substitution of the native promoter P_{srfA} by either P_{narG} or P_{nasD} to induce surfactin synthesis anaerobically is even more beneficial as foaming during the aerobic phase with aeration is reduced due to the absence of surfactin. Moreover, especially P_{nasD} is highly expressed anaerobically, assuming the titers obtained surpass those of native *srfA* operon expression (Chapter 2.2). Indeed, promoter exchange studies are an often-applied technique to increase the surfactin titers. Regarding this approach, either inducer-specific promoters (Sun et al. 2009, Jiao et al. 2017, Wang et al. 2018), constitutive promoters (Coutte et al. 2010,

Willenbacher et al. 2016) or auto-inducible promoters can be used. Although inducible promoters are often used, the addition of the inducer such as IPTG significantly increases large-scale production costs. Contrarily, auto-inducible promoters, such as the promoters P_{narG} and P_{nasD} investigated in Chapter 2.3, do not require a specific inducer and induction is generally easily realizable in cultivation processes by controlling the respective environmental parameter such as nutrient limitation, temperature, or oxygen availability as in the current study.

For the proof of concept, reporter strains *B. subtilis* MG1 and MG5, introduced in Chapter 2.2, as well as the reporter strain KM1016 monitoring natural expression of P_{srfA}, were cultivated in shake flasks with different filling volumes. This is an easy approach to investigate different oxygen availabilities. In contrast to serum flask cultivations (Chapter 2.1 and Chapter 2.2), the medium was not flushed with nitrogen to have anaerobic conditions directly at inoculation and the flasks were not sealed airtight. The available oxygen in the medium was hypothesized to improve bacterial growth and depletion of oxygen is faster at higher filling volumes, as the transfer rate of oxygen into the medium is reduced. In addition, also the concentrations of nitrate, nitrite and ammonium allowed to rate if cells grow aerobically with ammonium as preferred nitrogen source or anaerobically by nitrate respiration. With the described set-up, cells entered microaerophilic or anaerobic conditions after different times of cultivation. Indeed, in future studies, a method to measure the oxygen in shake flasks must be provided to allow for more precise correlations of the data collected. As expected, the expression of both P_{narG} and P_{nasD} increased with decreasing oxygen availabilities. In addition, P_{srfA} showed rather similar expressions amongst the different set-ups tested. The expression of P_{narG} was furthermore similar to P_{srfA} at lowest oxygen availability, while P_{nasD} reached a 5.5-fold higher expression. The data were well in accordance with those presented in Chapter 2.2. Consequently, the promoter exchange strains *B. subtilis* MG12 with P_{srfA}::P_{narG} and MG14 with P_{srfA}::P_{nasD} were cultivated using the same shake flask set-up. As hypothesized, surfactin production was not detected at lowest, and increased with decreasing oxygen availabilities. Surprisingly, although having similar promoter expressions, strain MG12 did produce ~300 mg/L less surfactin with 193 mg/L at lowest oxygen availability than the reference strain. However, strain MG14 surpassed the titer of the reference strain and produced 696 mg/L surfactin. In addition, an exceptionally high $Y_{P/X}$ and productivity q were obtained with 1.007 g/g and 0.023 g/(g·h). These values even surpass those of the reference strain under aerobic conditions, making this strategy of oxygen-depletion an interesting competitor to conventional processes and long-lasting anaerobic cultivations with low productivity q of 0.005 g/(g·h) (Chapter 2.2).

Nevertheless, there was no clear correlation of promoter expression and surfactin titer amongst all tested strains. However, due to the increased surfactin titers, the promoter exchange strain MG14 was identified as a promising target for further bioreactor process development. Indeed, the oxygen depletion in the shake flask set-up follows a reverse sigmoidal curve. According to the intended switch processes, the phase with high oxygen availability must be prolonged to accumulate more biomass, which can be used as catalyst when oxygen depletes. Though, bioreactor cultivations with defined oxygen profiles have demonstrated that the amount of biomass was reduced to a great extent when the switch from aerobic to anaerobic conditions was performed too fast. According to literature, 90% of cells lyse upon oxygen depletion (Arjes et al. 2020) and the process of cell anaerobization can last 24 to 36 h (Hoffmann et al. 1995). Hence, bioreactor cultivations performed have shown improvements with reduced cell lysis when the oxygen levels were decreased slowly and when each pO_2 level was maintained for 3 h (Chapter 2.3), but more experiments are needed to gently switch aerobic non-producer cells to anaerobic surfactin-producer cells.

Interestingly, results of Chapter 2.3 have shown that the growth rate correlates with surfactin synthesis and cells not producing surfactin have higher growth rates. To further reduce the process times of the switch processes, the initial aerobic phase could also be run at 40 °C, which additionally yields increased growth rates (Chapter 2.1). At 40 °C, both the surfactin titer and productivity were lower for strain JABs24 than at 37 °C, which would not pose an issue in the switch process as surfactin is not produced anyway in the first phase. Consequently, future studies might not only focus on the optimization of the pO_2 profile to prevent the observed cell lysis (Chapter 2.3), but also on an adaptive temperature-profile. In comparison to the reference strain, foaming with the promoter exchange strain MG14 with P_{srfA}::P_{nasD} started 6 h later in a bioreactor cultivation with a continuous low aeration rate which constantly decreased the available oxygen per cell due to cell growth. However, foaming still occurred during the aerobic phase and further process optimization such as reduced aeration rates are crucial. In addition, when cells are sufficiently anaerobized, aeration ideally should be stopped to result in a foam-free environment, as reported in Chapter 2.2.

Continuative, the promoter exchange P_{srfA}::P_{nasD} described in Chapter 2.3 could also be applied to the genome reduced strain *B. subtilis* IIG-Bs20-5-1 presented in Chapter 2.1. As reported by Zhang et al. (2020), a promoter exchange in a genome reduced *B. amyloliquefaciens* had a stronger effect compared to the reference strain, and this observation could be investigated with respect to its transferability to a *B. subtilis* strain. Hence, although strain IIG-Bs20-5-1 of this

study was slightly inferior to reference strain JABs24 in terms of surfactin titer (Chapter 2.1), a promoter exchange could have more pronounced effects, making the genome reduced strain superior. Furthermore, due to the absence of the gene encoding for the lipopeptide plipastatin in strain IIG-Bs20-5-1, foam formation might be reduced as well during the aerobic growth phase.

3.6 Nitrogen source availabilities as crucial parameter for ongoing research

Data of all Chapters emphasized that nitrogen sources have a significant impact on surfactin production, both aerobically and anaerobically. Hence, the availability and time courses of nitrogen sources pose a crucial target for subsequent works. According to results obtained in Chapter 2.2 and Chapter 2.3, also the cultivations presented in Chapter 2.1 could be performed with different nitrogen source compositions to clearly show benefits of anaerobic cultivations. This means that the medium of both aerobic and anaerobic cultivations should contain either only NH_4^+, only NO_3^- or a mixture of both nitrogen sources. For example, in Chapter 2.2 a $Y_{P/X}$ value of 0.466 g/g and 0.668 g/g was reported for aerobic growth either on ammonium or nitrate as sole nitrogen source in a cultivation with *B. subtilis* JABs24. Consequently, it can be hypothesized that the aerobically obtained $Y_{P/X}$ values of Chapter 2.1 would be higher in a medium containing high levels of nitrate. Indeed, also with respect to the promoter expression of P_{srfA} presented in Chapter 2.3, the anaerobically superior $Y_{P/X}$ value is not only dependent on the promoter expression, which was very similar amongst the different oxygen availabilities tested. It can be postulated that the presence of nitrate positively triggers surfactin synthesis independent of the availability of oxygen, and further studies in this field are crucial to fully understand the correlation of nitrogen source availability and surfactin synthesis.

With respect to nitrite, increasing concentrations up to 10 mmol/L resulted in prolonged lag-phases, but growth rates were comparable afterwards (Chapter 2.2). As presented in Chapter 2.2 and Chapter 2.3, nitrite peaks were observed within the first hours of cultivations. However, employing a fed-batch cultivation with elongated process times due to elevated glucose addition, a second nitrite increase was observed until the end of process (Chapter 2.3), which was not observed in Chapter 2.2 employing anaerobic batch and fed-batch processes. The nitrite accumulated reached concentrations of more than 10 mmol/L, which was shown to have a

negative impact on the growth behavior (Chapter 2.2). Therefore, further studies must address the imbalance of nitrate conversion to ammonium via nitrite by, e.g., appropriate nitrate-feeding profiles to avoid nitrite accumulation. Additionally, as reported by Espinosa de los Monteros (2001), high nitrate levels resulted in acetate and acetoin synthesis, while low nitrate levels went along with the synthesis of lactate and butanediol. The inhibitory effect of acetoin, lactate and butanediol must be investigated as described for acetate (Chapter 2.2). According to these results, metabolic engineering to delete respective genes or further feed strategies with, e.g., nitrate limited feeding could be applied. Concomitant with those results, and although posing an elegant nitrate-feeding strategy, the self-regulated HNO_3 pH-feed to provide NO_3^- due to the switch in pH as presented in Chapter 2.3 might be inapplicable as this strategy resulted in a continuous increase in nitrate. In addition, high ammonium concentrations resulted in a decrease in surfactin titer (Chapter 2.2). As reported in Chapter 2.2 for anaerobic processes, the optimal balance of initially added ammonium and nitrate must be identified in the proposed and promising switch processes (Chapter 2.3) for optimal surfactin production.

3.7 Next step: Foam fractionation as promising tool to overcome reported challenges

Continuative, the application of foam fractionation poses an interesting target for subsequent optimization based on the described processes in Chapter 2.2 and Chapter 2.3. Foam fractionation is a method that allows to recover surfactin from the culture broth. It takes advantage of the ability of surfactin to accumulate at the air-water interface. In literature, this approach was already described for cultures of *B. subtilis* to recover surfactin (Davis et al. 2001, Willenbacher et al. 2004, Chen et al. 2006). However, in these studies, the foam fractionation column was attached to the exhaust gas line and foaming was hence dependent on both the agitation and aeration and by that often uncontrollable. In this sense, a foam fractionation column suitable for the switch processes described in Chapter 2.3 must be operatable independently. Such a column which is attached aside the bioreactor was designed and tested in several preliminary cultivations. More precisely, culture broth from the bottom of the bioreactor was passed into the foam fractionation column via a peristaltic pump, allowing to accurately set the amount of culture broth to be subjected to foaming. Foaming inside the column was induced by an individually adjustable nitrogen or process air flow. Similar to the observations reported by Willenbacher et al. (2014), surfactin was enriched in the foam. In

addition, in comparison to a process without foam fractionation, the overall surfactin concentrations were higher in the process where cells were subjected to foaming (exemplarily shown in Figure S1 and Figure S2). This is in agreement with Alonso and Martin (2016), but the metabolic cause is not explored yet and poses an important target for further studies. In addition, it is hypothesized that a controllable foam fractionation column has further advantages. For example, the culture broth passed into the column can be adapted to the feed profiles, allowing to elongate the cultivation times which is an issue in high-titer fed-batch processes (Klausmann et al. 2021). In addition, next to surfactin, also other metabolites such as nitrite, ammonium and acetate, which were reported to have a negative impact on growth, promoter expressions and / or surfactin synthesis (Chapter 2.2), can be removed as well. Moreover, if the finally designed aerobic-anaerobic switch process presented in Chapter 2.3 results in a foam-free phase without any air or nitrogen flow, accumulated CO_2 as described in Chapter 2.2 can also be flushed by foaming. Nevertheless, optimal parameter settings of both the cultivation process and the foam column must be identified to make this strategy not only a tool to enrich surfactin, but also to improve overall cultivation performance by diminishing accumulated metabolites.

Chapter 4 Concluding Remarks

This study aimed at investigating, characterizing and optimizing the production of surfactin in *B. subtilis* cultures targeting at a foam-free production. Presented approaches included “media and process parameter optimization”, “strain engineering” and “investigation of novel process strategies”. Furthermore, the anaerobic cultivation by nitrate respiration run like a common thread through all Chapters, as this strategy was previously reported to be a promising foam-free approach and alternative to conventional aerobic processes.

Some limitations of anaerobic growth by nitrate respiration as process strategy were described, such as the time-consuming cultivations reaching only low absolute surfactin amounts. Moreover, nitrogen sources were identified to play a crucial role. Certainly, the identified issues, e.g., acetate accumulation, as well as nitrogen metabolism and its impact on surfactin synthesis, pose an interesting basis for further research. Nevertheless, results demonstrated the general feasibility of surfactin production in anaerobic and foam-free fed-batch processes by nitrate respiration. In addition, the presented anaerobically self-inducible process strategy employing the promoter P_{nasD} was emphasized as a highly promising starting point for further experiments. Future studies targeting at aerobic-anaerobic self-inducible switch processes that combine advantages of both aerobic growth and anaerobic growth by nitrate respiration show great promise. A further integration of a foam fractionation column in these switch processes additionally has the potential to become a coequal or even superior surfactin production process compared to currently employed conventional process strategies. On top, it must be emphasized that the proposed methods are not only suited for surfactin, but also for other target metabolites prone to foaming or sensitive to oxygen.

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List of Figures

Abbreviations and symbols

ATP	Adenosine triphosphate
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
B. licheniformis	*Bacillus licheniformis*
B. subtilis	*Bacillus subtilis*
capBCA	Gene for capsular polyglutamate
CDW	Cell dry weight
CDW_{max}	Maximum cell dry weight
CO_2	Carbon dioxide
e.g.	lat. exempli gratia (for example)
eps	Gene for extracellular polysaccharide
H_2CO_3	Carbonic acid
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
lacZ	Gene for ß-galactosidase
MS	Mass spectrometry
NarGHI	Nitrate reductase
NasDE	Nitrite reductase
NH_4^+	Ammonium
NO_3^-	Nitrate
NRPS	Nonribosomal peptide synthetase

OD	Optical density
OD_{600}	Optical density at a wavelength of 600 nm
pgsBCA	Gene for capsular polyglutamate
P_{narG}	Native promoter of nitrate reductase genes
P_{nasD}	Native promoter of nitrite reductase genes
pO_2	dissolved oxygen (%)
P_{srfA}	Native promoter of surfactin operon
Sfp	4-phosphopantetheinyl transferase
srfA	Surfactin operon
USD	US-Dollar

q	Specific productivity
$Y_{P/S}$	Yield coefficient product to substrate
$Y_{P/X}$	Yield coefficient product to biomass
$Y_{surfactin/S}$	Yield coefficient surfactin to substrate
$Y_{X/S}$	Yield coefficient biomass to substrate
μ	Specifc growth rate

Appendix

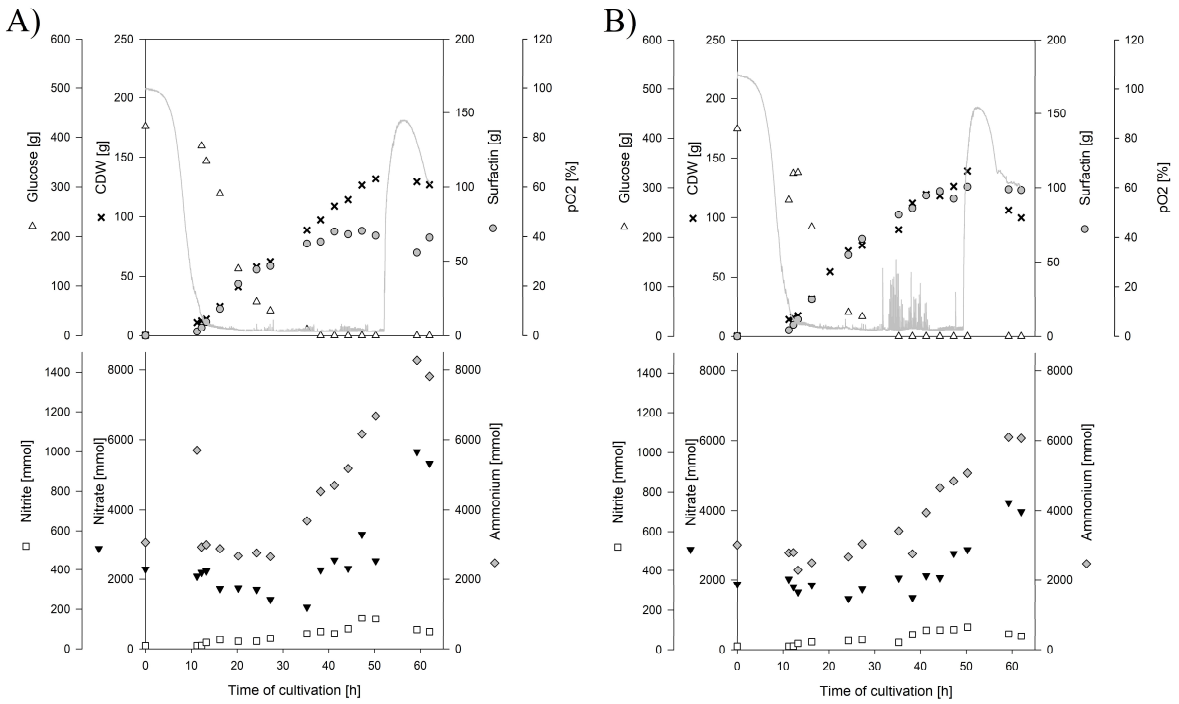

Figure S1: Bioreactor fed-batch cultivations with strain *B. subtilis* MG1 without (A) and with (B) foam fractionation.

In both processes, a constant aeration of 1.2 L/min was set resulting in a reduction in available pO_2 during the time course of cultivation. Foam fractionation was started after 13 h of cultivation (B) with a constant culture broth flow of 20 mL/min and an aeration rate of 3 L/min inside the column. Displayed are the absolute values for glucose [g] (open triangle), CDW [g] (cross), surfactin [g] (grey circle), pO_2 [%] (grey line), nitrite [mmol] (open square), nitrate [mmol] (black triangle) and ammonium [mmol] (grey rhomb).

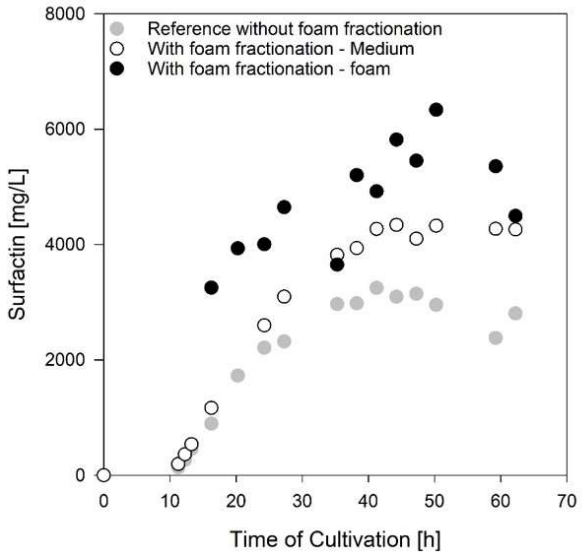

Figure S2: Surfactin concentrations [mg/L] during the time course of cultivation in a process without foam fractionation and with foam fractionation.

The fed-batch cultivations were performed with strain *B. subtilis* MG1. In both processes, a constant aeration of 1.2 L/min was set resulting in a reduction in available pO_2 during the time course of cultivation. The foam fractionation was started after 13 h of cultivation with a constant culture broth flow of 20 mL/min and an aeration rate of 3 L/min inside the column. The surfactin concentration of the reference process without foam fractionation are displayed in grey. For the foam fractionation process, concentrations of surfactin in the medium (white) and foam (black) are displayed.

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Exploiting novel strategies for the production of surfactin in Bacillus subtilis cultures

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

_____ _____

Place, Date Signature