EVALUATION OF DIFFERENT PROCESS STRATEGIES FOR THE PRODUCTION OF SURFACTIN

- INVESTIGATIONS ON PRODUCER STRAINS AND CONDITIONS -

zur Erlangung des akademischen Grades eines

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DISSERTATION

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"WE CAN ONLY SEE A SHORT DISTANCE AHEAD,

BUT WE CAN SEE PLENTY THERE THAT NEEDS TO BE DONE."

- Alan Turing -

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PREAMBLE

This thesis is based on peer reviewed research articles or work which is prepared for publication. All articles have been drafted during this work and describe the major research results of this dissertation concerning the microbial production of Surfactin with *Bacillus subtilis*. Chapters which are based on a publication or are arranged for submission at a research journal are indicated as such at the beginning of the chapter. The text of these chapters is in large parts identical to the content of the publication. However, layout, citation style and figures have been changed to match the formatting of this dissertation. This thesis includes an introduction presenting the essentials of the research topic, four chapters describing the main results of this dissertation and finally a brief conclusion.

Chapter 2 presents the results of a screening searching for Surfactin producer strains and a following analysis of their Surfactin productivity in a model fermentation process with integrated foam fractionation. This study is based in great extent on the publication:

"Evaluation of different Bacillus strains in respect of their ability to produce Surfactin in a model fermentation process with integrated foam fractionation"

Applied Microbiology and Biotechnology, 2014

Chapter 3 illustrates the results of a study combining an anaerobic and foam-free fermentation approach for the production of Surfactin. The findings of this work present a great effectiveness and introduce a novel way to avoid foam formation during production of microbial surfactants. This study is based on the publication:

"Foam-free production of Surfactin via anaerobic fermentation of Bacillus subtilis DSM 10^{T}"

AMB Express, 2015

Chapter 4 describes the optimization of the medium which is usually employed for the production of Surfactin with *B. subtilis*. The results present a significant impact on Surfactin production after decreasing the initial glucose concentration and introduce another fermentation approach. The findings of this study are published in AMB Express.

"Enhancement of Surfactin yield by improving the medium composition and fermentation process"

AMB Express, 2015

Chapter 5 outlines the results of the genetic transformation of *B. subtilis* with the aim to increase Surfactin productivity by promoter exchange in front of the *srfA* operon. The results of this study are submitted for publication.

LIST OF PUBLICATIONS

Peer reviewed original papers

2014

EVALUATION OF DIFFERENT *BACILLUS* STRAINS IN RESPECT OF THEIR ABILITY TO PRODUCE SURFACTIN IN A MODEL FERMENTATION PROCESS WITH INTEGRATED FOAM FRACTIONATION

Judit Willenbacher, Michaela Zwick, Teresa Mohr, Ferdinand Schmid, Christoph Syldatk and Rudolf Hausmann

Applied Microbiology and Biotechnology, 98:9623-9632

2015

FOAM-FREE PRODUCTION OF SURFACTIN VIA ANAEROBIC FERMENTATION OF *BACILLUS SUBTILIS* DSM 10^T

Judit Willenbacher, Jens-Tilman Rau, Jonas Rogalla, Christoph Syldatk and Rudolf Hausmann

AMB Express 5:21

TEACHING BIOPROCESS ENGINEERING TO UNDERGRADUATES: MULTIDISCIPLINARY HANDS-ON TRAINING IN A ONE-WEEK PRACTICAL COURSE

Marius Henkel, Michaela Zwick, Janina Beuker, Judit Willenbacher, Sandra Baumann, Florian Oswald, Anke Neumann, Martin Siemann-Herzberg, Christoph Syldatk and Rudolf Hausmann

Biochemistry and Molecular Biology Education, 43:189-202

ENHANCEMENT OF SURFACTIN YIELD BY IMPROVING THE MEDIUM COMPOSITION AND FERMENTATION PROCESS

Judit Willenbacher, Wladimir Yeremchuk, Teresa Mohr, Christoph Syldatk and Rudolf Hausmann

AMB Express 5:57

SUBSTITUTION OF THE NATIVE *SRFA* PROMOTER BY CONSTITUTIVE P_{VEG} IN TWO *B. SUBTILIS* STRAINS AND EVALUATION OF THE EFFECT ON SURFACTIN PRODUCTION

Judit Willenbacher, Teresa Mohr, Marius Henkel, Susanne Gebhard, Thorsten Mascher, Christoph Syldatk and Rudolf Hausmann

Submitted for publication

Poster Presentation

2014

PRODUCTION OF SURFACTIN WITH *BACILLUS SUBTILIS* IN A FERMENTATION PROCESS WITH INTEGRATED FOAM FRACTIONATION

Judit Willenbacher, Christoph Syldatk and Rudolf Hausmann

DECHEMA Jahrestagung, 2014

ABSTRACT

Surfactants are employed in a variety of different industries e.g., in washing, textile, and food industry. Additionally, surfactants play an important part in the production of pharmaceuticals and cosmetics due to their emulsifying properties. Consequently, a large diversity of surfactants is required to cover all demands. Microbial surfactants are possibly the most sustainable surfactants as they are produced by microorganisms under mild conditions and on basis of renewable resources. Furthermore, it is often claimed that they are biodegradable and therefore not harmful to the environment. A vast variety of microbial surfactants are already identified and characterized. However, Surfactin, a lipopeptide produced by the well-established industrial strain *Bacillus subtilis* is one of the most noticeable biosurfactants as it exhibits an extraordinary surface activity even in comparison to conventional surfactants. At this time, the production of Surfactin is almost limited to research purposes as high production costs and low production rates prevent an application in industrial scale. Based on the numerous characteristics of Surfactin (strong surface activity, induction of plant systemic resistance, antiviral and insecticidal activity, etc.) it is reasonable to further investigate the essentials of its production to promote future industrial applications.

Within this thesis the microbial production of Surfactin was analyzed focusing on production strains and cultivation methods to create a comprehensive investigation. The first study was performed in order to evaluate Surfactin producer strains in a classical foam separation fermentation process. Therefore, a screening was conducted investigating the Surfactin productivity of various *Bacillus* strains. The ability to produce Surfactin was confirmed for six *Bacillus* strains (DSM 10^{T}, ATCC 21332, DSM 3256, DSM 3258, DSM 1090 and DSM 28227). Interestingly, the Surfactin productivity of *Bacillus* sp. DSM 28227 and *Bacillus subtilis* DSM 1090 has not been reported before. In the following, an evaluation of the six Surfactin producers was conducted to investigate their Surfactin productivity in comparison to each other. A comparative study addressing the productivity while employing identical cultivation conditions was previously unreported. A model fermentation process applying foam fractionation was conducted to handle the severe foam formation and to gain numerous data concerning the Surfactin productivity. The investigation revealed only slight differences between the Surfactin producer strains but outlined the characteristic features of every strain which can now be employed according to their distinctive

features. Additionally, the approach of foam fractionation proved to be a useful tool to cope with foam formation and was also able to improve Surfactin yields as it enabled product enrichment and *in situ* product recovery.

An additional way to handle foam formation was investigated during the second study of this thesis. The formation of foam is usually one of the greatest challenges during the microbial production of surfactants. The most sophisticated solution is to avoid foam formation instead of destroying the foam because this is commonly accompanied by high expense. As a result, a foam-free fermentation approach was established during this thesis. The fermentation of *B. subtilis* under anaerobic conditions, due to the exclusion of any gas-flow through the liquid medium, was performed for the first time. This technique proved to be very efficient as the microorganisms produced much more Surfactin per cell dry weight in comparison to other foam-free fermentation approaches. Interestingly, the initial glucose concentration plays an important part as preferably low concentrations supported high specific production rates. This fermentation approach is conducted without the need of expensive constructions introducing an economically reasonable alternative to other fermentation processes.

The medium composition used for the cultivation of *B. subtilis* was focused in the third study of this thesis. The well-established Cooper medium is usually employed during microbial production of Surfactin. Instead of the commonly used 40 g/L glucose various lower glucose concentrations were tested. Surprisingly, *B. subtilis* produced 25 % more Surfactin when lower glucose concentrations were applied. The medium was accordingly adjusted and additionally an alternative nitrogen source and an environmentally friendly chelating agent were introduced. The resulting medium optimized the Surfactin productivity during cultivation of *B. subtilis* DSM 10^{T}. In order to investigate a general improvement five other Surfactin producer strains were cultivated in the optimized medium revealing almost every time an enhancement in Surfactin productivity. Subsequently, the enhanced medium was applied during fermentation with integrated foam fractionation. As the much lower initial glucose concentration limited cultivation time a fed-batch fermentation approach was introduced were glucose was manually added after its depletion. In comparison to the primarily applied fermentation approach 30 % more Surfactin was obtained after these adjustments.

The final study of this thesis focused on the genetic optimization of *B. subtilis*. Genetically modified strains are often employed in industrial processes to increase product yield. Interestingly, *B. subtilis* is especially suited for genetic modification which is shown by its application as model organism of gram-positive bacteria. A markerless transformation increasing Surfactin yield, as was performed

during this study, was previously unreported. The synthesis of Surfactin is realized by a large multi-enzyme complex called Surfactin synthetase. This nonribosomal peptidesynthetase is coded by a large operon which contains several subsequent open reading frames. The promoter initiating the expression of this operon (*srfA*) is influenced by the surrounding cell density. The aim of this study was the markerless exchange of the native promoter in front of the *srfA* operon in order to achieve a constitutive expression of the Surfactin synthetase. This was successfully conducted using the cloned plasmid pMAD Pveg srfA. The transformation of *B. subtilis* 3A38 (originally yielding minor Surfactin concentrations) led to creation of *B. subtilis* JWSurf1 and JWSurf2, whereas after transformation of *B. subtilis* DSM 10^T (initially producing high amounts of Surfactin) the strain *B. subtilis* JWSurf3 was obtained. The production of Surfactin was several-fold increased after the promoter exchange in 3A38 (JWSurf1 and JWSurf2) but did not achieve an enhancement in strain DSM 10^T (JWSurf3). As a consequence, a promoter replacement in order to uncouple Surfactin synthesis from quorum sensing control does not generally enhance Surfactin yields. This implicates a more complex process of the biosynthesis of Surfactin then initially thought. However, subsequent investigations are necessary to fully understand the limiting factors in Surfactin synthesis and to find a solution to further increase Surfactin yields in naturally strong Surfatin producer strains.

This dissertation engaged the biotechnological process for the production of Surfactin from its initial point by identifying Surfactin producer strains and approaching a genetic optimization. Additionally, the medium composition and fermentation approach were examined yielding a significant increase in productivity. In summary, this thesis investigated the production of Surfactin on microbial and procedural level by evaluating various wild type and transformed *Bacillus* strains concerning their Surfactin productivity in both aerobic and anaerobic fermentation processes.

ZUSAMMENFASSUNG

Tenside finden ein breites Anwendungsspektrum, beispielsweise in der Waschmittel-, Textil- oder Lebensmittelindustrie. Außerdem werden sie in vielen pharmazeutischen und kosmetischen Produkten aufgrund ihrer emulgierenden Eigenschaften verwendet. Um die unterschiedlichen Anforderungen zu erfüllen wird eine große Anzahl verschiedener Tenside benötigt. Mikrobiell hergestellte Biotenside sind möglicherweise die nachhaltigsten Tenside, da sie von Mirkoorganismen unter milden Reaktionsbedingungen gebildet und auf Basis erneuerbarer Ressourcen produziert werden. Des Weiteren wird häufig behauptet sie seien vollständig biologisch abbaubar und daher nicht umweltschädlich. Eine große Vielfalt mikrobieller Biotenside wurde bereits identifiziert und charakterisiert. Jedoch gilt Surfactin, ein Lipopeptid produziert durch den bereits industriell verwendeten Stamm *Bacillus subtilis*, als eines der herausragensten Biotenside, da es eine ungewöhnlich hohe Oberflächenaktivität aufweist, die mit herkömmlich verwendeten Tensiden vergleichbar ist. Zum heutigen Zeitpunkt wird Surfactin fast ausschließlich im Labormaßstab hergestellt, da hohe Produktionskosten und geringe Produktausbeuten eine industrielle Herstellung bisher verzögern. Aufgrund der vielversprechenden Eigenschaften von Surfactin (starke Oberflächenaktivität, Induktion der pflanzlichen Immunantwort, antivirale und insektizide Wirkung, etc.) ist es jedoch sinnvoll weiter an den Grundlagen der mirkobiellen Produktion zu forschen, um eine mögliche industrielle Herstellung voranzutreiben.

Um einen breitgefächerten Überblick über die mikrobielle Produktion von Surfactin zu gewährleisten wurden innerhalb dieser Arbeit sowohl unterschiedliche Produktionsstämme als auch verschiedene Kultivierungsmethoden analysiert. Mehrere Stämme, welche Surfactin produzieren, wurden zu Beginn dieser Arbeit in einem klassischen Fermentationsprozess mit integrierter Schaumfraktionierung evaluiert. Hierfür wurde zunächst ein Screening durchgeführt, welches die Surfactin-Produktivität verschiedener *Bacillus*-Stämme untersuchte. Die Fähigkeit Surfactin zu produzieren konnte bei sechs verschiedenen Stämmen festgestellt werden (DSM 10^T, ATCC 21332, DSM 3256, DSM 3258, DSM 1090 und DSM28227). Interessanterweise wurden die Stämme *Bacillus* sp. DSM 28227 und *Bacillus subtilis* DSM 1090 erstmals als Surfactin-Produzenten identifiziert. Im weiteren Verlauf wurde eine Evaluierung der sechs Surfactin-Produzenten durchgeführt, innerhalb derer die Produktivität der verschiedenen Stämme direkt miteinander verglichen wurde. Eine Studie, welche Surfactin-Produktivitäten verschiedener Stämme unter

exakt gleichen Bedingungen untersucht, wurde zuvor nicht veröffentlicht. Für diese Untersuchung wurde ein Modell-Fermentationsprozess etabliert, welcher mithilfe integrierter Schaumfraktionierung die extreme Schaumproduktion handhabbar machte und die Sammlung vielerlei Prozessparameter erlaubte. Diese vergleichende Untersuchung demonstrierte nur geringe Unterschiede zwischen den einzelnen Stämmen, konnte jedoch spezifische Charakteristika der Stämme hervorheben, welche nun entsprechend ihrer Vorzüge gezielt eingesetzt werden können. Zusätzlich wurde durch die Verwendung der Schaumfraktionierung eine effiziente Methode zur Verarbeitung der Schaumproduktion vorgestellt, welche aufgrund von Produktanreicherung und Produktgewinnung eine Steigerung der Surfactin-Ausbeuten ermöglichte.

Eine weitere Möglichkeit zur Bewältigung von Schaumbildung wurde im zweiten Abschnitt dieser Arbeit untersucht. Die Bildung von Schaum ist meistens eine der größten Herausforderungen während der mikrobiellen Produktion von Tensiden. Eine raffinierte Lösung ist die Vermeidung von Schaumbildung anstelle der Schaumzerstörung, da diese häufig zur Steigerung der Produktionskosten führt. Daher wurde eine schaumfreie Kultivierungsmethode innerhalb dieser Arbeit etabliert. Eine anaerobe Fermentationsmethode, bei der jeglicher Gasfluss durch das Medium unterbunden wird, wurde zum ersten Mal durchgeführt. Diese Technik demonstrierte große Effizienz da wesentlich mehr Surfactin pro Biotrockenmasse gebildet wurde im Vergleich zu anderen schaumfreien Prozessen. Dabei scheint die Ausgangsglukosekonzentration eine wichtige Rolle zu spielen da niedrige Konzentrationen eine hohe Produktionsrate förderten. Hiermit wurde eine schaumfreie Fermentationsmethode vorgestellt, welche im Vergleich zu anderen schaumfreien Prozessen ohne aufwendige Konstruktionen auskommt.

Der dritte Abschnitt dieser Dissertation befasste sich mit der Zusammensetzung des Mediums, welches für die Kultivierung von *B. subtilis* verwendet wurde. Für die mikrobielle Produktion von Surfactin wird herkömmlich das seit langem etablierte Cooper-Medium verwendet. In dieser Studie wurden anstelle der normalerweise eingesetzten 40 g/L Glukose geringere Glukose-konzentrationen verwendet. Dabei wurde 25 % mehr Surfactin produziert bei der Verwendung von geringen Glukosekonzentrationen. Das Medium wurde dementsprechend abgeändert. Zusätzlich wurden eine alternative Stickstoffquelle und ein umweltfreundlicherer Komplexbildner eingeführt. Das resultierende Medium optimierte die Surfactin-Produktivität bei Kultivierungen von *B. subtilis* DSM 10^{T}. Um festzustellen ob eine generelle Steigerung der Surfactin-Produktion durch das optimierte Medium erreicht wird, wurden die übrigen fünf Surfactin-Produzenten ebenfalls in diesem Medium kultiviert. Hierbei wurde fast immer eine Steigerung der Surfactin-Produktion nachgewiesen. Im Folgenden wurde das optimierte Medium während einer Fermentation mit integrierter Schaumfraktionierung eingesetzt. Da die geringe

Glukosekonzentration die Kultivierungsdauer drastisch verkürzte, wurde ein Fed-Batch-Ansatz etabliert, bei welchem Glukose nach vollständigem Verbrauch manuell zugegeben wurde, um die Kultivierungszeit zu verlängern. Diese Veränderungen führten zu einer Steigerung der Produktausbeute um 30 % im Vergleich zu zuvor verwendeten Fermentationsverfahren.

Der letzte Abschnitt dieser Arbeit befasste sich mit der genetischen Optimierung von *B. subtilis*. Genetisch modifizierte Stämme werden oft in industriellen Prozessen eingesetzt, um eine Steigerung der Produktausbeuten zu erzielen. *B. subtilis* ist besonders geeignet um genetische Modifikationen durchzuführen, was sein Verwendung als Modellorganismus grampositiver Bakterien untermauert. Jedoch ist bisher keine markerfreie Transformation zur Steigerung der Surfactin-Ausbeute bekannt, welche mit der hier ausgeführten Transformation gleichgesetzt werden könnte. Die Synthese von Surfactin wird durch einen Muti-Enzymkomplex namens Surfactin Synthetase katalysiert. Die nichtribosomale Peptidsynthetase wird durch ein großes Operon codiert, welches mehrere aufeinanderfolgende offene Leserahmen aufweist. Der Promotor welcher die Expression des Operons (*srfA*) initiiert ist von der Zelldichte anhängig. Das Ziel dieser Studie war der markerfreie Austausch des nativen Promotors vor dem *srfA*-Operon um eine konstitutive Expression der Surfactin Synthetase zu ermöglichen. Dies wurde erfolgreich durchgeführt mithilfe des klonierten Plasmids pMAD Pveg srfA. Die Transformation von *B. subtilis* 3A38, welcher ursprünglich nur sehr geringe Surfactin-Konzentrationen erzielte, führte zur Etablierung der *B. subtilis*-Stämme JWSurf1 und JWSurf2. Nach Transformation von *B. subtilis* DSM 10^T (welcher ursprünglich hohe Surfactin-Ausbeuten erzielte) wurde der Stamm *B. subtilis* JWSurf3 erhalten. Die Produktion von Surfactin wurde mehrfach erhöht durch den Promotoraustausch in *B. subtilis* 3A38 (JWSurf1 und JWSurf2), erzielte jedoch keine Steigerung der Surfactin-Ausbeuten in *B. subtilis* DSM 10^T (JWSurf3).Dies impliziert, dass ein Promotor-Austausch, mit dem Ziel der quorum sensing-Entkopplung, nicht generell zur Erhöhung der Surfactin-Konzentration führt. Daraus lässt sich schließen, dass die Biosynthese von Surfactin komplexer ist als bisher angenommen. Wie dem auch sei, zukünftige Untersuchungen sind notwendig um vollständig aufzuklären welche Faktoren die Surfactin-Synthese limitieren und auf welche Weise die Produktivität in bestehenden Produktionsstämmen erhöht werden kann.

Diese Dissertation befasste sich zunächst mit dem Ausgangspunkt eines biotechnologischen Prozesses indem Surfactin-Produzenten identifiziert und genetische Untersuchungen zur Verbesserung der Surfactin-Produktivität durchgeführt wurden. Zusätzlich wurde die Medium-Zusammensetzung und das verwendete Fermentationsverfahren analysiert, welches zu einer signifikanten Steigerung der Produktivität führte. Zusammengefasst, wurde in dieser Arbeit sowohl die mikrobielle Ebene als auch die verfahrenstechnische Ebene der Surfactin-Produktion

untersucht, indem die Produktivität verschiedene Wildtyp- und transformierter Stämme in aeroben und anaeroben Fermentationsprozessen evaluiert wurden.

TABLE OF CONTENTS

1. THEORETICAL BACKGROUND AND RESEARCH PROPOSAL

This chapter introduces the research field of microbial surfactants. It illustrates the motivation of sustainable biosurfactant production and characterizes some of the most important microbial surfactants originating from *Bacillus subtilis*. In the following the lipopeptide Surfactin is highlighted in particular. The aim of this chapter is to illuminate the uniqueness of this extraordinary biosurfactant and to outline the reasons motivating its production.

1.1 SURFACTANTS

Chemical compounds exhibiting an amphiphile nature, thanks to a hydrophilic head group and a hydrophobic tail, are called surfactants. Highly diluted surfactant solutions in aqueous systems form monodisperse layers and are concentrated at the interfaces by hydrophilic-hydrophobic oriented adsorption (Kosswig 2000). As a consequence they are able to reduce the surface tension of liquids or the interfacial tension between two condensed phases which allows the formation of dispersions. This quality is used in numerous applications in detergent and textile industry but also in food production, oil recovery, cosmetics and pharmaceuticals. Approximately 70 % of the worldwide surfactant market is located in North America and Western Europe. It is often claimed that the worldwide surfactant industry is valued several billion dollars per year (Soberón-Chávez and Maier 2011) which implies a financially worthwhile market.

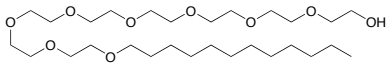
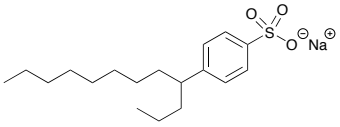
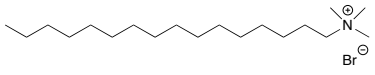
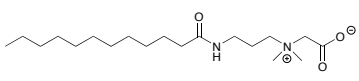
Surfactants are differentiated based on their head group because the occurring hydrophilic moieties are more divers in comparison to the hydrophobic tail groups. Consequently, the classification is divided in nonionic, anionic, cationic, and amphoteric surfactants (Tab. 1.1). Nonionic and anionic surfactants are the most commonly used surfactants today. Several characteristics are considered when surfactants are evaluated. For example their ability to reduce the surface tension of water. Water has a surface tension of 72.8 mN/m (at 20 °C, against air). Common surfactants reduce this surface tension to values between 40 mN/m and 20 mN/m (Kosswig 2000). Another characteristic is the critical micelle concentration (CMC). Usually, the surface tension of a liquid is continuously lowered by an increasing addition of surfactants to this liquid. The CMC is reached if the further addition does not lead to a further decrease of the surface tension and the surfactants start to form micelles. This describes a congregation in an entropy-governed manner in which the hydrophilic groups point towards the aqueous phase and the hydrophobic groups towards the interior of the micelle. A group of widely used surfactants for instance reaches CMC values of 0.5 g/L (Kosswig 2000).

The first employed surfactants were sodium or potassium salts of fatty acids. These compounds are also known as soap. With advances in chemical industry during the first half of the 20th century new surfactants were synthetized. The most common surfactant until the 1950s was tetrapropylenebenzyl sulfonate (TPS) which is a multiple branched alkylbenze sulfonate. This surfactant was found to be highly detrimental to the environment as it is only poorly biodegradable (Falbe and Gerike 1987). An increased usage of this detergent, especially in private homes, led to the formation of foam in natural aquatic habitats with disturbing consequences for the environment. As a result TPS production was significantly reduced. This gap in the market was mainly filled by linear alkylbenze sulfonates (LAS) which were found to be better degradable by

microorganisms (van de Plassche et al. 1999). LAS belong to the majorly produced surfactants worldwide (Scott and Jones 2000). However, effects of LAS have been intensely studied in the past years and it was found that biodegradation under laboratory condition is much better compared to field studies (Krueger et al. 1998). Additionally, biodegradation of LAS requires the presence of oxygen which prevents its depletion under anaerobic conditions (Scott and Jones 2000). This is a major disadvantage as detergents are often carried on with the water to end up under conditions with oxygen exclusion.

The vast majority of commonly produced surfactants today are based on either oleochemical or petrochemical compounds. The usage of both substrates is balanced out (Saouter et al. 2006) yielding 50 % surfactants originating from compounds made of plant and animal sources (fats and oils, oleochemicals) and 50 % surfactants originating from compounds made of crude oil (petrochemicals). The production of LAS is completely based on petrochemicals (Saouter et al. 2006) and

Tab. 1.1 Classification of surfactants Summarized are the main categories of surfactants including typical head groups and characteristic examples.

Head group	Typical head groups	Typical surfactants
Nonionic	-OH (alcohols) -O- (ethers)	Octaethylene glycol monododecyl ether 
Anionic	$-COO^-$ (carboxylic acids) $-SO_3^-$ (sulfonic acids)	Sodium dodecylbenzene sulfonate (LAS) 
Cationic	$R_4\text{-}N^+$ (quaternary ammonium)	Cetrimonium bromide (CTAB) 
Amphoteric	e.g. $R_4\text{-}N^+$ and $-COO^-$	Cocamidopropyl betaine (CAPB) 

involves the alkylation of benzene (Friedel – Crafts reaction), sulfonation of the resulting alkylbenzene and a following neutralization (Kosswig 2000). This production process requires (under inclusion of several precursors) partially exceedingly high temperatures, excess pressure and costly catalysts (Kosswig 2000). However, alternative substrates like oleochemicals are also controversial as they are often based on palm oil. The plantation of palm trees (in many cases accompanied by deforestation, Carlson et al. 2012) is the basis of an ongoing controversy about land grabbing and the sustainable production of chemicals (Pye and Bhattacharya 2013). These findings, together with the need for a variety of different surfactants and specific characteristics, support the urge to find alternative possibilities to produce surfactants.

1.2 GO GREEN: RETHINKING SURFACTANTS

1.2.1 Labelling surfactants: a controversial discussion

Surfactants are usually discriminated on the basis of their synthesis. As a result surfactants are either described as synthetic or natural which can sometimes be misleading. In fact the production of surfactants is based on petrochemicals, oleochemicals (from palm oil, tall oil, fish oil, lard etc.), enzymatic reactions (at the time restricted to ongoing research), extraction from natural sources or microbial cultivations. Synthetic production includes the industrial syntheses based on petrochemicals and oleochemicals (described in the previous paragraph) but also surfactants originating from enzymatic reactions as they all have an artificial origin and result in tailor-made molecules. Surfactants originating from natural sources are often described as natural surfactants or bio-based surfactants. This includes surfactants from oleochemicals, enzymatic reactions, extracted surfactants from natural sources and microbially produced surfactants because they all base on renewable resources. Obviously, some surfactants belong to both groups. It is therefore important to establish another term for the discrimination of these amphiphile molecules. Non artificial surfactants which are produced by biological systems are called biosurfactants. This includes surfactants which are obtained by extraction of natural sources (e.g. lecithin from soy seeds) or are produced by microorganisms (e.g. by yeasts or bacteria). Surfactants originating from enzymatic synthesis belong to a gray zone as they are indeed artificial but produced by enzymes with microbial origin and chemicals from renewable resources. Similar to alkyl polyglycosides, which are chemically produced by sugars and fats from renewable resources using high temperatures and pressures.

The production of biosurfactants by extraction from plant or animal resources is well established as the industrial production of lecithin from soy beans and egg yolk shows. Already millions of tons

are produced per year and used as emulsifiers in food products (van Nieuwenhuyzen 2010). But the extraction depends on large amounts of organic solvents and can therefore not be accounted as sustainable. Despite their origin from renewable resources it is therefore necessary to investigate in further opportunities to produce surfactants. The probably most ecofriendly process for the production of surfactants is the microbial fermentation. Since the cultivation of microorganisms does not depend on the usage of organic solvents, high temperatures, excess pressure or costly catalysts. Furthermore, does the microbial production of surfactants not compete with the production of food. As a result microbial surfactants gain more and more attention.

1.2.2 Benefits from microbial surfactants and possible applications

The general demand for sustainably, ecologically and economically well designed industrial processes increases since the sources of crude oil will eventually be exhausted and the current state of the worldwide environmental pollution is dramatic. Therefore, microbial surfactants, which are often claimed to be biodegradable (Hirata et al. 2009, Mohan et al. 2006) and are mostly considered to be non-toxic (Flasz et al. 1998), increasingly gain attention. Additionally, biosurfactants display a wide range of chemical structures and exhibit various biological activities (Soberón-Chávez and Maier 2011). This led to numerous publications focusing on possible applications. Naturally, microbial surfactants could be employed in washing detergents or during procedural steps in textile industries. Studies investigating this topic have been conducted (Mukherjee 2007). Additionally, the usage of microbial surfactants as multipurpose ingredients or additives in food products has been analyzed and was highly valued thanks to emulsifying, antiadhesive and antimicrobial activities (Nitschke and Costa 2007). Especially surfactants originating from GRAS-organisms (generally regarded as safe) are considered as possible candidates as their production harbors few risks and is less complicated. But a replacement of the established surfactants mainly used in these sectors (detergents: LAS, food industries: lecithin) is difficult to realize as microbial surfactants are not yet able to compete with these mass-produced products. It is therefore more likely that microbial surfactants will initially be employed as specialized niche products. For example for the remediation of organic or inorganic compounds in polluted soils (Banat et al. 2010). There are also studies investigating microbial enhanced oil recovery. Here, solutions of microbial surfactants are used to gain oil residuals from the ground (Banat et al. 2010). Furthermore, their attribute to affect the adhesion properties of microorganisms or their ability to disrupt membranes could be used in biomedical applications. For example their antimicrobial activity could be employed in alternatives to synthetic antimicrobial agents (Banat et al. 2000). The prevention of biofilm formation could be applied on medical devices (Harriott and Noverr 2009) or

they could play a role in antitumor pharmaceuticals (Cao et al. 2010). Next to medical applications an employment in cosmetic products is also imaginable owing to the skin compatibility of biosurfactants (Brown 1991). Additionally, many studies investigate the usage of biosurfactants in agriculture, e.g. to support the systemic resistance of plants or as plant disease biocontrol agents (Ongena and Jacques 2008; Ongena et al. 2007).

The main drawback of microbial surfactants today is the high production costs as a result from comparably low production rates and relatively high priced medium components. However, the increase on publications on this topic with the aim to overcome these obstacles proves the promising qualities of microbial surfactants (Soberón-Chávez and Maier 2011). Further, substrate costs are addressed by the employment of low prized carbon sources or byproducts from other processes (Freitas de Oliveira et al. 2013; Makkar and Cameotra 2002; Nitschke and Pastore 2004).

1.2.3 Classification of microbial surfactants

Microbial surfactants can be divided in glycolipids, lipopeptides, fatty acids, biopolymers and emulsifying proteins (Kosaric et al. 2015a; Sen 2010, Tab. 1.2). The most common glycolipids are trehalose lipids, rhamnolipids, sophorolipids and mannosylerythritol lipids (MELs) which all contain the according sugar part and an attached hydrocarbon moiety. They are produced by various different microorganisms like *Rhodococcus*, *Pseudomonas*, *Torulopsis* and *Candida*. Interestingly, the modification of these microbial surfactants (as well as their enzymatic syntheses) seems to become a new trending topic in industrial biocatalysis (Pöhnlein et al. 2015) exploring novel ways to produce tailor-made biosurfactants. The second class of microbial surfactants displays lipopeptides. The most common investigated are Surfactin, Iturin, Fengycin and Lichenysin. Lipopeptides are majorly produced by *Bacillus* species although other classes of bacteria like e.g. *Actinobacteria* are described as producers, too (Kügler et al. 2015). They are composed of a peptide ring attached to a variable fatty acid. Their occurrence and typical characteristics is investigated since the mid-20th-century (Soberón-Chávez and Jacques 2011). Additionally, fatty acids themselves can sometimes be accounted as biosurfactants like some extracellular fatty acids (C_{12}-C_{14}) and complex fatty acids with hydroxyl groups or alkyl branches from *Nocardia* and *Rhodococcus*. In contrast, polymeric biosurfactants are composed of amphiphile monomer units which exhibit high molecular weights and properties like high viscosity and tensile strength. Typical examples are Emulsan, Biodispersan, Alasan and Liposan which are produced by *Acinetobacter* or *Candida*. The last group of microbial surfactants is described as emulsifying proteins which are characterized by a hydrophilic part exhibiting both peptide and sugar moieties. Great examples are peptidoglycolipids from *Pseudomonas aeruginosa*.

Tab. 1.2 Classification of microbial biosurfactants Listed are the different groups of biosurfactants, some exemplary molecules belonging to the classes and the producing microorganisms (Sen 2010).

Biosurfactants	Examples	Microorganism	Study
Glycolipids	Trehalose lipids	*Rhodococcus* sp.	Philp et al. 2002
	Rhamnolipids	*Pseudomonas aeruginosa*	Monteiro et al. 2007
	Sophorolipids	*Torulopsis bombicola*	Tullock et al. 1967
	Mannosylerythritol lipids	*Candida antarctica*	Crich et al. 2002
Lipopeptides	Surfactin	*Bacillus subtilis*	Arima et al. 1968
	Iturin	*Bacillus subtilis*	Peypoux et al. 1978
	Fengycin	*Bacillus subtilis*	Wang et al. 2004
	Lichenysin	*Bacillus licheniformis*	McInerney et al. 1990
Fatty acids	Saturated fatty acids (C_{12}-C_{14})	*Nocardia* sp.	MacDonald et al. 1981
	Fatty acids with hydroxyl groups	*Rhodococcus erythropolis*	Kretschmer et al. 1982
Biopolymers	Emulsan	*Acinetobacter calcoaceticus*	Rosenberg et al. 1979
	Biodispersan	*Acinetobacter calcoaceticus*	Rosenberg et al. 1988
	Alasan	*Acinetobacter radioresistent*	Navonvenezia et al. 1995
	Liposan	*Candida lipolytica*	Cirigliano and Carman 1984
Emulsifying proteins	Peptidoglycolipids	*Pseudomonas aeruginosa*	Koronelli et al. 1983

To this day only few microbial surfactants are commercially available. Rhamnolipids are produced by several companies e.g. by Jeneil Biotech Inc. (Saukville, Wisconsin, USA) and Rhamnolipids Companies Inc. (Saint Petersburg, Florida, USA) but are not always readily available for purchase (Soberón-Chávez and Maier 2011). The MEL Surfmellow® is offered by Toyobo Co. (Okasaka, Japan). Some lipopeptides originating from *Bacillus* can also be obtained by either Sigma-Aldrich (St. Louis, Missouri, USA) or Lipofabrik (Villeneuve-d'Ascq, France). However, these microbial surfactants are fine chemicals primarily used for research purposes. An exception makes the lipopeptide Daptomycin from *Streptomyces roseosporus* (Debono et al. 1987). It is already produced

in industrial-scale based on its application as an antibiotic against gram-positive bacteria. However, most applications discussed in the previous paragraph still have to be established.

1.3 A GENEROUS HOST: *BACILLUS SUBTILIS*

1.3.1 *Bacillus subtilis* in industrial production

The bacterium *Bacillus subtilis* is a ubiquitous soil bacterium discovered in 1834 by C. Ehrenberg (*Vibrio subtilis*) and was later renamed by F. Cohn in 1872. *B. subtilis* was long time believed to be a strict aerobe. Meanwhile it was demonstrated that *B. subtilis* is also able to live under complete anaerobe conditions either by using nitrate or nitrite as terminal electron acceptor or by fermentation (Nakano and Zuber 1998). The gram-positive bacterium which originates from the phylum *Firmicutes* is able to form spores and does not require complex media for cultivation. The physiology and genetics of *B. subtilis* have been extensively studied leading to the complete sequencing of its genome (Kunst et al. 1997). Today it is the best characterized gram-positive bacterium and is therefore often referred as the model organism of gram-positive bacteria. Many studies concerning general characteristics like natural competence, spore formation or genetics and proteomics are conducted with this bacterium (Dubnau 1991; Setlow 2006; Völker and Hecker 2005).

Many products (especially enzymes) deriving from *B. subtilis* are considered as generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA) because they are harmless and can be used in food products and pharmaceuticals without any concerns. As a consequence, *B. subtilis* is often itself labelled as a GRAS organism. It is commonly regarded as non-toxic which is confirmed by its consumption in large quantities through Nattō, a Japanese dish made of fermented soy beans (Schallmey et al. 2004). This results in biotechnological applications even in tightly controlled sectors like the production of pharmaceuticals, cosmetics or food products. The genus *Bacillus* is already commonly used in industrial processes because these microorganisms are able to secrete a substantial amount of proteins e.g. proteases and amylases (Gupta et al. 2002). One of the most important commercially employed proteases is Subtilisin which is produced by *Bacillus subtilis* but is also available from *Bacillus licheniformis* (Subtilisin Carlsberg) and *Bacillus amyloliquefaciens* (Subtilsin Novo) two closely related *Bacillus* strains. Subtilisin is mainly used in detergents and usually tons of pure enzymes are produced per year (Rao et al. 1998). Another commercially produced product of *B. subtilis* is Bacitracin which is a peptide antibiotic discovered in 1945 (Johnson et al. 1945). Today tons of this polypeptide are produced as feed supplement or for usage in antibiotic ointments.

Because *B. subtilis* is able to secrete large amounts of proteins into the surrounding medium and generally lacks pathogenicity it is also a popular host for recombinant DNA expression. Exogenous polypeptides are introduced into *B. subtilis* and benefit from its excellent expression and secretion abilities (Rao et al. 1998).

By nature *B. subtilis* synthesizes a wide range of secondary metabolites of which many are antibiotics (Stein 2005) and biosurfactants (Soberón-Chávez and Jacques 2011). Years of experience with *B. subtilis* as an industrial used strain, its excellent characterization on genetic, proteomic and physiological level, its strong expression and secretion of peptide containing compounds and its natural ability to produce biosurfactants underline its suitability as a microbial surfactant producer for future biotechnological applications.

1.3.2 Biosurfactants from *Bacillus subtilis*

B. subtilis produces various secondary metabolites. A great number of these are lipopeptides which are highly investigated. The lipopeptides of *B. subtilis* are divided in three major classes: Surfactins, Iturins and Fengycins. All of these compounds exhibit a peptide ring as hydrophilic head and a fatty acid chain as hydrophobic tail.

The Surfactins were first identified in 1968 (Arima et al. 1968). There are approximately 20 different congeners known (Bonmartin et al. 2003) which are always composed of a heptapeptide ring and an interlinked β-hydroxy fatty acid. The peptide ring follows the chiral sequence LLDLLDL with a D-Leu in position 3 and 6 and an L-Asp in position 4. The positions 2, 4 and 7 can be filled with various aliphatic amino acids like Val, Leu, Ile and in rare cases Ala (Tab. 1.3, Bonmatin et al. 1995; Itokawa et al. 1994; Peypoux et al. 1991; Peypoux et al. 1994). A microbial strain producing Surfactin does always secrete different forms of this lipopeptide. What kind of forms are produced (varying amino acids or β-hydroxy fatty acids) depends on the microbial strain and applied culture conditions. The addition of specific amino acids to the culture medium can influence the amino acid sequence in the peptide moiety (Liu et al. 2012; Peypoux and Michel 1992). Iturins distinguish themselves by the incorporation of characteristic amino acids like Tyrosin and Prolin and additionally by the utilization of β-amino acid fatty acids as hydrophobic part of the compound. The chiral sequence is LDDLLDL and the peptide ring is mostly started with L-Asp-D-Tyr-D-Asn with Iturin C as sole exception (Soberón-Chávez and Jacques 2011). The Iturins can be divided in Iturin, Bacillomycin and Mycosubtilin. Each subclass exhibits 1 to 3 different versions (Tab. 1.3). In contrast to Surfactins and Iturins Fengycins are composed of a decapeptide ring with an internal lactone ring and a β-hydroxy fatty acid which is linked to the amino acid residue of a Glutamic acid (Soberón-Chávez and Jacques 2011). Noteworthy is the incorporation of Ornithine a non-proteinogenic amino acid. There are two different forms described: Fengycin A and B (Tab. 1.3).

Worth mentioning is the production of Surfactins, Iturins and Fengycins by other microorganisms which do not belong to the species *B. subtilis*. Closely related strains like *Bacillus amyloliquefaciens*, *Bacillus licheniformis* and other comparable strains are also able to produce various lipopeptides which sometimes are named after their parental strain (e.g. Lichenysin from *Bacillus licheniformis*). All described lipopeptides not originating from *B. subtilis* are summarized in Tab. 1.4.

Tab. 1.3 Lipopeptides produced by *Bacillus subtilis* strains Summarized are all different lipopeptides produced by *B. subtilis*. Presented are the primary structure of the peptide moiety and possible fatty acid chains linked to the peptide rings. Beneath the table is shown which studies elucidated the lipopeptides composition. The table is based on Soberón-Chávez and Jacques 2011.

Name	Primary structure of the peptide moiety	Fatty acid chains
Surfactins		
Surfactin	L-Glu-L-XS_2-D-Leu-L-XS_4-L-Asp-D-Leu-L-XS_7 XS_2 = Val, Leu or Ile XS_4 = Ala, Val, Leu or Ile XS_7 = Val, Leu or Ile	*i*C_{13}, *a*C_{13}, *i*C_{14}, *n*C_{14}, *i*C_{15}, *a*C_{15}
Iturins		
Iturin		
Iturin A	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*C_{14}, *i*C_{15}, *a*C_{15}
Iturin C	L-Asp-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	*n*C_{14}, *i*C_{15}, *a*C_{15}
Bacillomycin		
Bacillomycin D	L-Asn-D-Tyr-D-Asn-L-Pro-L-Glu-D-Ser-L-Thr	*n*C_{14}, *i*C_{15}, *a*C_{15}
Bacillomycin F	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Thr	*n*C_{16}, *i*C_{17}, *a*C_{17}
Bacillomycin L	L-Asn-D-Tyr-D-Asn-L-Ser-L-Glu-D-Ser-L-Thr	*n*C_{14}, *i*C_{15}, *a*C_{15}
Mycosubtilin	L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn	*n*C_{16}, *i*C_{16}, *a*C_{17}
Fengycins		
Fengycin A	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Ala-L-Pro-L-Gln-L-Tyr-L-Ile	*a*C_{15}, *i*C_{16}, *n*C_{16}
Fengycin B	L-Glu-D-Orn-D-Tyr-D-a Thr-L-Glu-D-Val-L-Pro-L-Gln-L-Tyr-L-Ile	*a*C_{15}, *i*C_{16}, *n*C_{16}, C_{17}

Surfactins: Peypoux et al. 1999

Iturin A: Peypoux et al. 1978

Iturin C and Mycosubtilin: Peypoux et al. 1986

Bacillomycin D:Peypoux et al. 1998

Bacillomycin F: Peypoux et al. 1985

Bacillomycin L: Volpon et al. 2007

Fengycins: Schneider et al. 1999

Tab. 1.4 Lipopeptides produced by closely related *Bacillus* strains or other microorganisms Summarized are different lipopeptides similar to Surfactin, Iturin and Fengycin produced by various *Bacillus* strains regarding their name and origin.

Lipopeptide class	Strain	Lipopeptide name	Study
Surfactins	*B. amyloliquefaciens*	Bamylocin A	Lee et al. 2007
	B. mesentericus	Esperin	Thomas and Ito 1969
	B. licheniformis	Lichenysin	Horowitz et al. 1990
	B. pumilus	Pumilacidin	Morikawa et al. 1992
	Marine *Bacillus* strain	Halobacillin	Trischman et al. 1994
	B. coagulans	Surfactin derivate	Huszcza and Burczyk 2006
	B. mycoides	Surfactin derivate	Athukorala et al. 2009
Iturins	*B. cereus*	Mycocerein	Wakayama et al. 1984
	B. amyloliquefaciens	iturinic compound	Yu et al. 2002
	B. pumilus	iturinic compound	Cho et al. 2009
	Paenibacillus koreensis	iturinic compound	Chung et al. 2000
Fengycins	*B. cereus*	Plipastatin A	Nishikiori et al. 1986
		Plipastatin B	Nishikiori et al. 1986
	B. thuringiensis	Fengycin	Kim et al. 2004
	B. amyloliquefaciens	Fengycin	Koumoutsi et al. 2004
Kurstakins	*B. thuringiensis*	Kurstakin	Hathout et al. 2000

1.3.3 Nonribosomal peptide synthesis

The synthesis of secondary metabolites which incorporate a peptide moiety like lipopeptides usually involves a multi-enzyme complex called nonribosomal peptide synthetase (NRPS). This means the amino acids sequence is not given by a mRNA code which is read by ribosomes. Instead a large enzyme complex exhibiting a modular composition (Sieber and Marahiel 2005) is responsible for the recognition of certain amino acids and their linkage. Interestingly, each module is necessary for the incorporation of one amino acid into the growing polypeptide chain. NRPSs are usually encoded by large operons which consist of several consecutive open reading frames (ORFs) coding for the various subunit enzymes.

In detail every module of the NRPS consists of at least three main catalytic subunits to ensure the assembly of amino acids (Fig. 1.1). First, the amino acid is recognized and activated through adenylation by the so called A-domain. Subsequently the activated amino acid is transferred to a

peptidyl-carrier protein (PCP). This subunit guarantees that the activated amino acid is able to travel between two catalytic centers. In the following a condensation enzyme (C-domain) catalyzes the formation of a peptide bond between two amino acids bound to PCPs of consecutive modules. In most cases additional enzymes (secondary catalytic domains) are involved in this nonribosomal peptide synthesis. For example epimerases which allow the epimerization of L-amino acids into D-amino acids. Furthermore, the presence of a starter condensation domain in the first module of the NRPS is not uncommon to enable the addition of the fatty acid chain to the first amino acid of the growing polypeptide chain (Steller et al. 2004). The origin of the β-hydroxy-fatty acid is still not completely understood but the mainly branched fatty acids seem to originate from the amino acids Val, Leu and Ile and are hydroxylated at the β-position prior to CoA ligation (Youssef et al. 2011). The lipopeptide is finally terminated by a thioesterase domain (TE) as part of the last module. Sometimes this enzyme additionally induces the formation of a macrocyclic end product e.g. a lactone ring. At last the lipopeptide is released through the TE-domain into the surrounding environment.

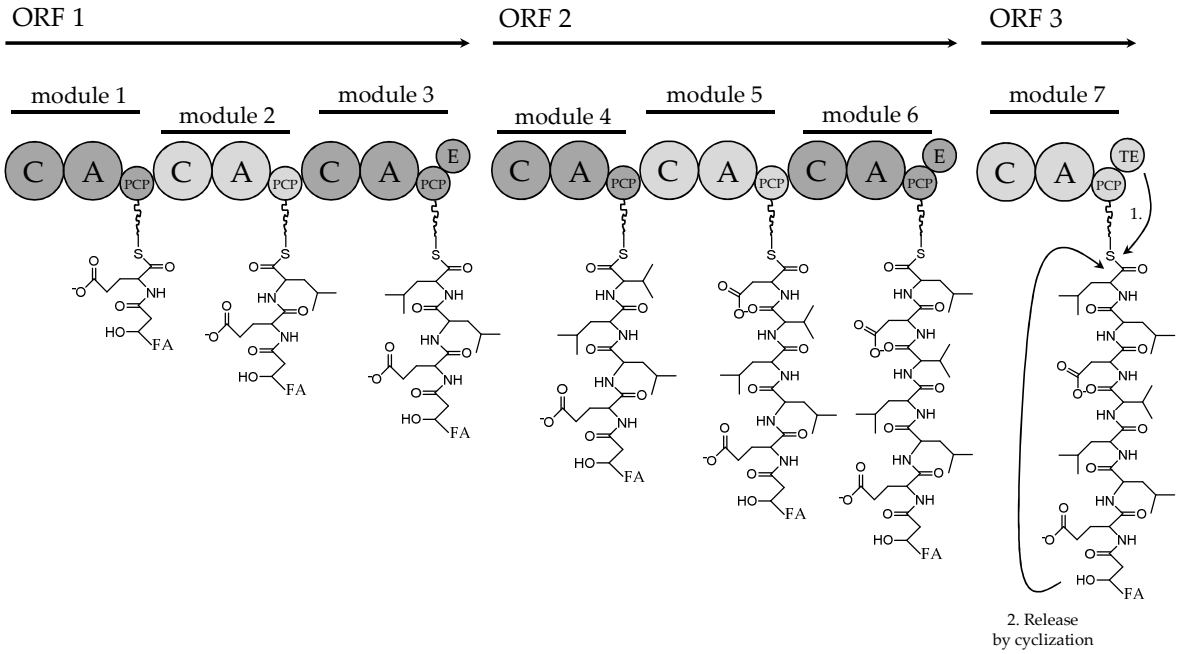


Fig.1.1 Elongation of the peptide chain inside a NRPS Presented is the simplified mechanism of the linkage of amino acids via the three subunits C (condensation-domain), A (activation-domain) and PCP (peptidyl-carrier protein). The first module is responsible for the linkage of the first amino acid L-Glu and the β-hydroxy-fatty acid (the fatty acid is not shown but indicated by "FA"). The PCP subunit is responsible for the binding of each amino acid after it was recognized and activated by subunit A. The C-domain of each module catalyzes the peptide bond of two consecutive amino acids. The E-domains (epimerases) in module 3 and 6 convert L-amino acids into D-amino acids. The final TE-domain (thioesterase) catalyzes the ring formation and thereby the release of the final Surfactin molecule from its NRPS (modified after Sieber and Marahiel 2005).

At this point it is important to emphasize that a NRPS is only functional after post-translational modification of the PCP subunits (Fig. 1.2). The above described mechanism for the elongation of a polypeptide chain determines the transfer of 4'phosphopantheteine residues to all PCP-domains of the NRPS molecule. Here, a phosphopantetheinyl transferase, encoded in *B. subtilis* by *sfp*, attaches a phosphopantetheine at a conserved serine of the PCP by utilization of coenzyme A. This enables the transfer of an activated amino acid to the now terminal thiol group. Subsequently, the multi-enzyme complex shifts from its apoform in its holoform (Mofid et al. 2004). In fact, lots of microorganisms hold large operons encoding NRPSs but are not able to produce the according secondary metabolites because they do not possess the necessary active phosphopantetheinyl transferase (Nakano et al. 1991a).

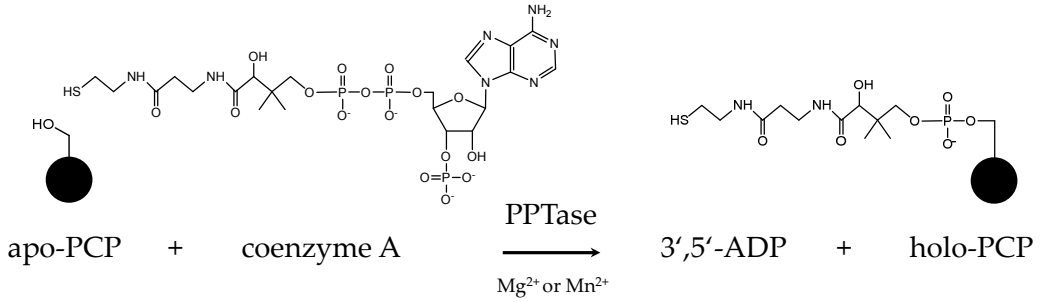


Fig. 1.2 Activation of the PCP-domain in a NRPS Shown is the attachment of a phosphopantetheine residue from coenzyme A to the subunit PCP. The phosphopantetheine transferase (PPTase) catalyzes the reaction using Mg or Mn cations to convert the PCP-domain from its apo in its holoform. The finally attached thiol group is essential for the binding of the activated amino acids. Without the activation of the PCP-domains the NRPS is not active (modified after Mofid et al. 2004).

1.4 SURFACTIN: A PROMISING BIOSURFACTANT

1.4.1 Discovery, characteristics and possible applications of Surfactin

Surfactin was discovered in the late 1960s by a Japanese research group investigating several *B. subtilis* strains isolated from Nattō, a dish made of fermented soy beans (Arima et al. 1968). This first analysis of the newly found lipopeptide already illustrated some of the most interesting facts about Surfactin. For example, it was shown to be composed of a peptide moiety (incorporating the amino acids L-Asp, L-Glu, L-Val, L-Leu and D-Leu) and a fatty acid. Furthermore, the lipopeptides extraordinary strength was demonstrated by showing a reduction of water surface tension from 72 mN/m to 27 mN/m. Additionally, a method describing the precipitation of Surfactin was introduced which is applied for the isolation of Surfactin until today. Last but not least, this first publication investigated the influence of Surfactin on fibrin clot formation showing that Surfactin

inhibits blood clotting and thereby Arima et al. presented a first possible application of the new lipopeptide. During the following years investigations analyzing the structure, characteristics and production of Surfactin have been published making it one of the most intensely studied microbial surfactants.

The structure of the anionic surfactant is divided in two major parts (Fig. 1.3). First the hydrophilic head group which contains the mentioned amino acids. The hydrophilic quality is caused by the unity of amino acids but the amino acids on position 1 and 5, glutamic acid and aspartic acid, are most important (Sen 2010). The second moiety contains the hydrophobic tail group, a 3-hydroxy-13-methylated-tetradecanonic acid. This β-hydroxy-fatty acid forms a peptide bond via the terminal carboxylic group with the amino group of the first amino acid (L-Glu) and an ester bond via the β-hydroxyl group with the carboxylic group of the last amino acid (L-Leu) leading to the formation of a lactone ring (Sen 2010). *Bacillus* mainly produces branched fatty acids which initially originate from the amino acids Val, Leu and Ile (Kaneda 1977). As a result lipid chains incorporated in Surfactin can exhibit different lengths and branches. Described are *iso*, *anteiso* C_{13}, *iso*, *normal* C_{14}, and *iso*, *anteiso* C_{15} (Tab. 1.3, Peypoux et al. 1999). Interestingly, the backbone of Surfactin induces a specific conformation which is thought to cause most of its activities (Sen 2010). As indicated before the structure of Surfactin is relatively variable. The synthesis by the described NRPS sometimes allows the incorporation of similar amino acids on position 2, 4 and 7 inside the peptide ring whereas the other amino acids are highly conserved (Tab. 1.3). In combination with different β-hydroxy-fatty acids this leads to a broad range of slightly distinct Surfactin molecules. *Bacillus* strains which are able to produce Surfactin always create various Surfactin congeners (Vater 1985). What kind of congeners are produced depends on the applied *Bacillus* strain and culture conditions (Liu et al. 2012; Peypoux and Michel 1992).

Surfactin is able to bind both monovalent and divalent cations, but divalent cations like Ca^{2+} are preferred (Thimon et al. 1993). They are thought to stabilize the structure of Surfactin as divalent cations bind between the two acidic amino acids L-Glu and L-Asp which form a shape similar to a "claw" (Peypoux et al. 1999). Interestingly, the addition of Ca^{2+} ions is supposed to function as a template for micelle formation (Osman et al. 1998). Next to ion affinity the influence of Surfactin concentrations on phospholipid membranes was characterized. Bernheimer and Avigad (1970) investigated the influence of Surfactin on erythrocytes and proved the alteration of the membrane integrity. In low concentrations Surfactin is believed to be miscible with the phospholipids of natural membranes (Peypoux et al. 1999) but at high concentrations Surfactin starts to disrupt the membrane by forming patches or channels through which ions can freely pass (Grau et al. 1999; Sheppard et al. 1991). Next to its strong surface activity Surfactin's ability to disrupt natural membranes is its most important characteristic leading to numerous areas of application.

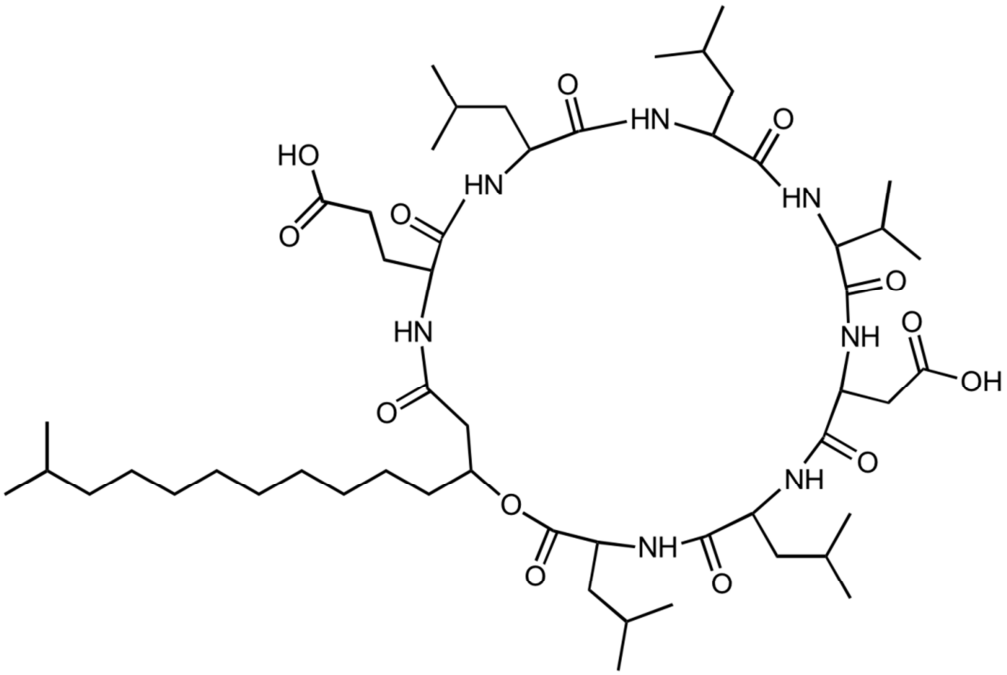


Fig. 1.3: Molecular structure of Surfactin The lipopeptide exhibits a polar part composed of seven amino acids: L-Glu, L-Leu, D-Leu, L-Val, L-Asp, D-Leu and L-Leu. The hydrophobic moiety is presented by a β-hydroxy-fatty acid which is partially integrated into the peptide ring via a peptide and an ester bound to the first and last amino acid of the ring. The presented structure is relatively variable as the amino acids on position 2, 4 and 7 can sometimes be filled with other aliphatic amino acids. Additionally, the length and branching of the β-hydroxy-fatty acid can vary leading to numerous different congeners of the Surfactin molecule.

Surfactin is a very powerful surfactant as a comparison to industrial surfactants reveals. The commercially available surfactant LAS (C_{12}, as sodium salt, 20 °C) exhibits a CMC of 0.50 g/L and lowers the surface tension to 35 mN/m (Kosswig 2000) whereas Surfactin displays a CMC of 0.036 g/L (20 °C, Hirata et al. 2009) and lowers the surface tension to 27 mN/m (Sen 2010). The positive impression of Surfactin is confirmed when taking a closer look to other industrial surfactants which rarely display a CMC lower than 0.05 g/L and mostly do not lower the surface tension of water beneath 30 mN/m (Kosswig 2000). As a result, Surfactin could likewise serve in industrial processes, e.g. in washing detergents or textile industry. However, these applications require a large-scale production of the employed surfactant which is, due to low production rates, at the moment not possible. Therefore investigations concerning applications of Surfactin currently focus on special fields like biomedical applications. This is motivated by several publications analyzing e.g. antitumor, antiviral and antimycoplasma properties (Kameda et al. 1974; Kim et al. 2007; Vollenbroich et al. 1997a; Vollenbroich et al. 1997b). The major bottleneck until today is the hemolytic activity which is also displayed by Surfactin and prolongates its application as therapeutic molecule (Kracht et al. 1999; Sen 2010). However, an employment of Surfactin to inhibit biofilm formation on surgical devices and implants is still desirable (Mireles et al. 2001). Another

Tab. 1.5 Application potentials of Surfactin Listed are various publications focusing on different fields of interests in which Surfactin could be employed.

Application potential	Study
Therapeutic application	
Hemolysis and inhibition of fibrin clot formation	Kikuchi and Hasumi 2002; Kracht et al. 1999
Antitumor and antiproliferative activity against tumor cells	Kameda et al. 1974; Kim et al. 2007
Antimycoplasma properties	Vollenbroich et al. 1997b
Antiviral activity	Vollenbroich et al. 1997a
Inhibition of biofilm formation	Mireles et al. 2001
Antimicrobial activity against multi-drug resistant strains	Fernandes et al. 2007
Suppression of inflammation	Kim et al. 1998
Agricultural application	
Fungicidal activity against *Magnaporthe grisea*	Tendulkar et al. 2007
Induction of plants persistence against phytopathogens	Ongena and Jacques 2008
Induction of plant systemic resistance	Ongena et al. 2007
Insecticidal activity	Assié et al. 2002
Environmental application	
Heavy oil transportation	Ghojavand et al. 2008
Degradation of hydrocarbons	Whang et al. 2008; Whang et al. 2009
Metal remediation	Mulligan 2005
Microbial enhanced oil recovery	Schaller et al. 2004
Cosmetic application	
Extremely weak skin irritation	Yoneda 2001
Suitability as emulsifier and moisturizer	Kanlayavattanakul and Lourith 2009

course is focused by investigations analyzing insecticidal activity (Assié et al. 2002) or Surfactin's ability to induce plant systemic resistance (Ongena et al. 2007). These aspects could be utilized in agriculture. Surfactin seems to exhibit most of the mentioned characteristics discussed in paragraph 1.2.2 which leads to numerous possible applications. The most interesting investigations are summarized in Tab. 1.5. However, emphasized should be the consideration of Surfactin for application in the food sector (Soberón-Chávez and Jacques 2011). Several investigations have discussed the potential of Surfactin (Kralova and Sjöblom 2009; Nitschke and Costa 2007) because

the lipopeptide is a natural component of some fermented Asian food products (Sumi et al. 2000) and some strains isolated from these products are able to produce Surfactin (Cho et al. 2009). As a result of Surfactin's strong surface activity and its numerous potential applications it is desirable to focus on the biotechnological production of this microbial surfactants which is until today the major drawback of its application.

1.4.2 Synthesis of Surfactin: the *srfA* Operon and its regulation

The biosynthesis of Surfactin was discussed for the first time by Kluge et al. (1988) suggesting a nonribosomal synthesis via a NRPS. This presumption was assured by various publications highlighting the different subunits of the Surfactin synthetase which is composed of four subunits (Menkhaus et al. 1993; Ullrich et al. 1991). The operon coding for the Surfactin synthetase is named *srfA* and was identified in 1988 (Nakano et al. 1988). The nearly 27 kb large operon holds 4 major ORFs called *srfA-A*, *srfA-B*, *srfA-C* and *srfA-D* (Galli et al. 1994). The first three ORFs encode modules which are responsible for the incorporation of the seven amino acids into the peptide ring. The last ORF *srfA-D* encodes a thioesterase/acyltransferase (TE/At-domain) which regulates the initiation of the biosynthesis of Surfactin (the linkage of the β-hydroxy-fatty acid and the first amino acid L-Glu, Steller et al. 2004). The modules originating from *srfA-A* guide the incorporation of the first three amino acids, the modules from *srfA-B* are responsible for the incorporation of the next three amino acids and finally *srfA-C* directs the assembly of the last amino acid (Overview: Fig. 1.4 from Soberón-Chávez and Jacques 2011). Each module incorporates three subunits as described in paragraph 1.3.3 (C-domain, A-domain and activated PCP). The last module originating from *sfrA-A* is responsible for the incorporation of a Leu into the peptide ring (position 3). Surfactin features a D-Leu at this position therefore an additional epimerase is incorporated in this module (Fig. 1.2). The same is the case at the last module originating from *srfA-B* (position 6). The module encoded by *srfA-C* causes the incorporation of the last amino acid (L-Leu) but further includes a thioesterase which catalyzes the formation of a lactone bond with the β-hydroxy-fatty acid and causes the release of the synthesized product from the enzymatic template.

Not all *B. subtilis* strains are able to synthesize Surfactin, although an intact version of the *srfA* operon generally exists (Nakano et al. 1991a). The loss of the ability to produce Surfactin is always caused by a mutation in *sfp*, the gene coding for the phosphopanthetine transferase which activates the PCPs inside the NRPS (Hsieh et al. 2004). Interestingly, the *srfA* operon is not abandoned from the genome although it is relatively useless to the microorganisms. This is probably due to *comS* a small gene inside the *srfA-B* ORF (Nakano and Zuber 1989). The gene belongs to a class of communication genes of *B. subtilis* which regulate the strains ability to perform quorum sensing. In

detail *comS* is necessary for natural competence but is not required for the synthesis of Surfactin (Hamoen et al. 1995).

A consequence of the presence of *comS* inside the *srfA* operon is the quorum sensing regulated expression of the operon. Responsible for the transcription initiation is a two-component regulatory system composed of ComP and ComA (Nakano et al. 1991). Both proteins are part of the *Bacillus* quorum sensing system indicated by the syllable "Com" (communication proteins). A brief overview about the expression control of the *srfA* operon is given in Fig. 1.5 (Soberón-Chávez and Jacques 2011). *B. subtilis* constantly secretes a quorum sensing molecule termed ComX through the outer cell membrane. The concentration of ComX outside the cells permanently increases if the cell density constantly rises. At a certain point a critical concentration is reached and the cell membrane enzyme ComP autophosphorylates. The phosphorylated ComP afterwards activates intracellular ComA by phosphorylation. Active ComA (ComA-P) finally acts as transcription factor initiating the expression of the *srfA* operon. Several other quorum sensing molecules (RapC, RapF, RapG and RapH) influence the concentration of active ComA inside the cell which enables the transcription initiation only after a certain concentration of ComA is reached. Eventually ComA proteins will bind to a special sequence in front of the core promotor called dyad symmetry. Two ComA proteins bind next to each other and form a dimer. This subsequently happens at another dyad symmetry some base pairs in front of the first one. The two dimers of ComA finally form a tetramer bending the DNA to form a big loop

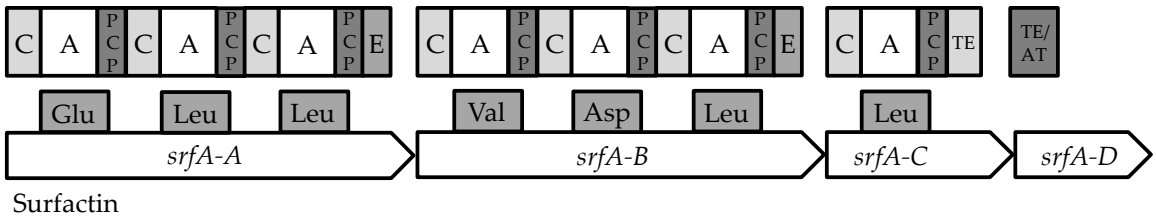


Fig. 1.4 Scheme of the *srfA* operon and modular structure of the Surfactin synthetase Shown is the schematic image of the NRPS structure responsible for the production of Surfactin. The consecutive incorporation of the seven amino acids is indicated with squares above arrows presenting the ORFs *srfA-A*, *srfA-B*, *srfA-C* and *srfA-D*. The boxes above indicate the structure of the module responsible for the incorporation of each amino acid. The modules incorporating D-Leu at position 3 and 6 include an additional epimerase. At the end of ORF *srfA-C* is a thioesterase included responsible for ring closure and release. ORF *srfA-D* encodes a thioeasterase/ acyltransferase which initiates the linkage of the β-hydroxy-fatty acid and L-Glu (modified after Soberón-Chávez and Jacques 2011).

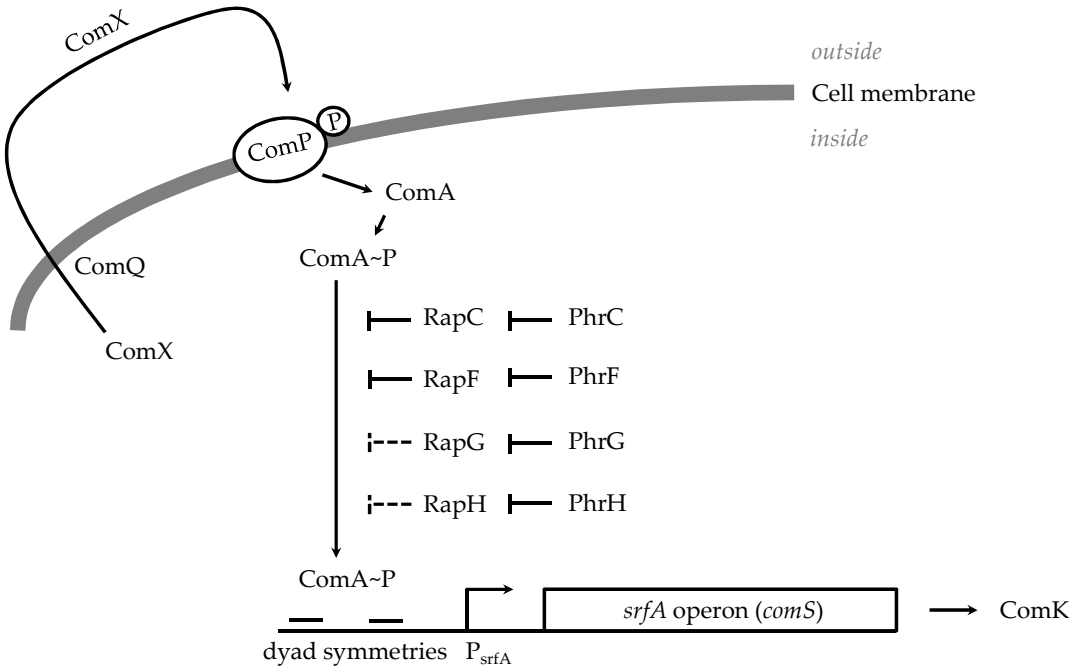


Fig. 1.5 Quorum sensing machinery responsible for the expression of the *srfA* operon Presented is a schematic overview about the most important factors influencing the transcription initiation of the *srfA* operon. *B. subtilis* constantly secretes ComX out of its cell membrane. If a certain concentration is reached (influenced by the cell density) ComP, a transmembrane enzyme, autophosphorylates and activates intracellular ComA. The concentration of active ComA molecules is additionally influenced by several repressors called RapC, RapF, Rap G and RapH. Their presence is also regulated by a complex machinery of quorum sensing molecules. If the concentration of active ComA is high enough dimers will form and bind to dyad symmetries in front of the *srfA* promoter P_{srfA} (modified after Soberón-Chávez and Jacques 2011).

(Nakano and Zuber 1993). This facilitates the binding of the RNA polymerase at the core promoter sequence several base pairs upstream and the transcription of the *srfA* operon starts. In summary, the expression of the *srfA* operon is controlled by the influence of numerous different molecules and is directly depending on the ambient cell density and cultivation history.

1.4.3 Biotechnological processes for the production of Surfactin

1.4.3.1 Microbial production of Surfactin in shake flasks and bioreactor cultivations

The cultivation conditions for the production of Surfactin with *B. subtilis* have been intensely studied since its discovery in the late 1960s (Shaligram and Singhal 2010). Some of the most important publications are listed in Tab. 1.6. Many investigations discussed the employment of an appropriate medium for Surfactin production which led from the usage of complex media (like

nutrient broth (Arima et al. 1968) and "Landy medium" (Sandrin et al. 1990)) to the application of mineral salt media. Often analyzed was the best possible dosage of trace elements like Mn and Fe (Cooper et al. 1981) or the concentration of nitrogen (Davis et al. 1999) in mineral salt medium. Furthermore, was the employment of different carbon sources intensely studied where various sugars (Abdel-Mawgoud et al. 2008) and alternative (mostly cost-saving) substrates (Freitas de Oliveira et al. 2013; Nitschke and Pastore 2004; Ohno et al. 1995) were tested. Most publications preferred the utilization of a mineral salt medium which was almost always at least partly based on the Cooper medium (Cooper et al. 1981) and determined glucose as the optimal carbon source (usually employing 40 g/L). The vast majority of these publications were either conducted in shake flasks or in bioreactors allowing a submerged fermentation. However, some alternative fermentation methods were analyzed like solid state fermentation (Ohno et al. 1995), aqueous two-phase fermentation (Drouin and Cooper 1992) or the application of a membrane bioreactor (Coutte et al. 2010). The employment of many different Surfactin producer strains further aggravates the

Tab. 1.6 Summary of Surfactin yields in various publications Presented are selected investigations with the aim to produce Surfactin. The publications were either performed in shake flasks or via fermentation. The overview displays the employment of a manifold of different *B. subtilis* strains and outlines a variety of different approaches to improve the lipopeptide yield (usage of different carbon sources, mutated strains, different fermentation constructions etc.).

Bacillus subtilis strain	Specialty	Yield	Study
IAM 1213	discovery	0.1 g/L	Arima et al. 1968
ATCC 21332	Foam fractionation	0.8 g/L	Cooper et al. 1981
ATCC 51338	UV mutant	1.1 g/L	Roubin et al. 1989
ATCC 21332	Aqueous two phase	0.35 g/L	Drouin and Cooper 1992
ATCC 55033	mutant	4.3 g/L	Carrera et al. 1992
MI 113	Solid state, recombinant	2.0 g/kg	Ohno et al. 1995
C9 (KCTC 8701P)	Shake flask	7.0 g/L	Kim et al. 1997
MTCC 1427	Sucrose	1.0 g/L	Makkar and Cameotra 1998
LB5a	Cassava-processing effluent	3.0 g/L	Nitschke and Pastore 2004
BBk1	Natural isolate	2.9 g/L	Abushady et al. 2005
E8	Ion beam mutant	10.26 g/L	Gong et al. 2009
ATCC 21332	Membrane bioreactor	0.24 g/L	Coutte et al. 2010
LAMI005	Cashew apple juice	0.32 g/L	Freitas de Oliveira et al. 2013

comparability of these studies. Although many investigations focused on the *B. subtilis* strain ATCC 21332 which is supposed to be identical with IAM 1213 (the first described strain by Arima et al. 1968) lots of other studies introduced their own natural isolates (e.g.: Abushady et al. 2005; Freitas de Oliveira et al. 2013; Kim et al. 1997) or presented mutated strains (e.g. by unselected mutagenesis: Gong et al. 2009; Roubin et al. 1989 or by directed mutagenesis: Carrera et al. 1992; Ohno et al. 1995). The combination of several impact factors (medium composition, strain, fermentation approach) as well as differing detection methods make a comparison of production rates almost impossible. Nonetheless, an increase in productivity from originally 0.1 g/L -0.8 g/L (Arima et al. 1968; Cooper et al. 1981) to values approximately between 1 g/L and 3 g/L Surfactin is noticeable. Some rare exception reported the production of 7 g/L and 10.26 g/L Surfactin, respectively (Gong et al. 2009; Kim et al. 1997) but these studies do not reflect the predominant results of other research groups (possibly due to unspecific product detection). However, a large scale production of Surfactin remains in the future due to low product yields. In order to accomplish an industrial scale values should be 10- to 100-fold increased. This might be achieved by simultaneously improving the medium composition, producer strain and fermentation approach.

1.4.3.2 Foam formation: an intricate challenge

The microbial production of biosurfactants is not uncomplicated as the amphiphile molecules attach at the water-air interface during cultivation. This often leads to vigorous foaming which is additionally promoted by stirring and purging inside a bioreactor. The formation of foam is usually avoided at all costs because disregarding could lead to the overflow of the bioreactor content. If a biotechnological process demands handling of foam several different methods can be used to cope with this challenge (Kosaric et al. 2015b). An interesting approach is the partitioning of the surfactants from the culture broth. A great overview about the different possibilities like separation by precipitation, liquid partitioning, membrane and ultrafiltration is given by Sen et al. (2010). However, the vast majority of processes employ either antifoam agents or mechanical devices to destroy upcoming foam. The utilization of antifoam agents prevents the formation of foam comparatively efficient but leads to major difficulties in downstream purification since antifoam agents are hard to remove from surfactants. Additional impellers on the agitator shaft are commonly used to disrupt foam if a mechanical approach is favored. This method requires only little equipment complexity (in comparison to acoustic or ultrasonic methods) but is solely practicable if modest rates of foam are generated (Winterburn and Martin 2012). The application of an additional impeller is therefore by all means insufficient to handle the severe foam formation during production of microbial surfactants.

The most preferably strategies are all those which either avoid foam formation from the beginning or (just the opposite) support foaming to facilitate product enrichment. The first approach focusses on innovative cultivation constructions like membrane bioreactors, two-phase reactors or solid state fermentations which do not allow foam formation. This is a highly desirable strategy but should take construction costs into account as some of these approaches are comparably cost-intensive. The second approach aims on foam fractionation which can be differentiated into cell-free foam fractionation (time–displaced to the actual cultivation) and integrated foam fractionation (fractionation simultaneous to cultivation). Both strategies base on the formation of bubbles which are caused by gas sparged through the medium. The surface active molecules adsorb at the interface and stabilize each other leading to the formation of foam which eventually reduces the liquid fraction if the foam is allowed to leave. The foam carrying the biosurfactant at the liquid/air interface is guided through an upstanding cylinder. The foam moves up the column and medium simultaneously drains down which leads to a "drier" foam at the top. The uprising foam is eventually collected (as "foamate") and contains an enriched amount of surfactants. The enrichment efficiency depends on the column height, column diameter, the air flow rate and the size of the bubbles within the foam (Sen et al. 2010). The cell-free foam fractionation has been intensely studied for microbial surfactants (e.g. Surfactin or rhamnolipids) but yields minor enrichments in comparison to integrated foam fractionation (Winterburn and Martin 2012). As a result most publications applying foam fractionation focused on integrated foam fractionation.

The approach of integrated foam fractionation enables the foam bubbles to leave the bioreactor and causes thereby the enrichment of the biosurfactant and its *in situ* recovery. This was applied for the first time by Cooper et al. (1981). Later studies analyzed different parameters in Surfactin production while applying the same mechanism (Chen et al. 2006a; Davis et al. 2001; Finkelstein et al. 2002; Makkar and Cameotra 2001). In this case the studies of Davis et al. and Chen et al. should be highlighted because these publications investigated Surfactin yields, enrichments and recovery for the first time.

The employment of different feed strategies should not be unmentioned at this point. Since during batch fermentations employing integrated foam fractionation the liquid phase is steadily reduced several attempts have been made to achieve a continuous cultivation e.g. by Davison et al. (2005), Chen et al. (2006b) and Coutte et al. (2013). However, as the applied fermentation processes are still including some obstacles (overgrown membranes, complex constructions etc.) ambitions persist to improve existing fermentation approaches or evolve new strategies to overcome the challenges of foaming culture broths.

1.5 RESEARCH PROPOSAL

Research focusing on the production of microbial surfactants becomes more and more important as sustainable ideas gain attention in production methods and waste management. Microbial surfactants which could be applied in a vast variety of different applications (detergents, textile and food industries, pharmaceuticals, cosmetics, and agriculture) combine those demands for sustainability as they are both produced by renewable resources and harmless to the environment. Surfactin is one of the most studied microbial surfactants as it exhibits extraordinary surface activity. Nevertheless, a commercial production in industrial scale has not yet been established possibly due to low production rates. Studies investigating Surfactin producer strains and production conditions could therefore substantially encourage the ambition of an industrial application.

The aim of this thesis was to both evaluate Surfactin producer strains and culture conditions for the production of Surfactin. The evaluation of different producer strains is crucial to any biotechnological process as the microorganism represents the catalyst of the process. A comparison of different Surfactin producer strains has presumably never been conducted before, hence it was not known if different producer strains vary in productivity. The evaluation of different *B. subtilis* strains regarding various process parameters during fermentation was consequently the first intention of this thesis. Next to this analysis on systematic level an investigation from the genetic point of view was conducted to examine the transformation of a Surfactin producer strain on molecular level. Therefore, it was tested whether the cell-density controlled production of Surfactin, caused by the quorum sensing dependent promoter P_{srfA} in front of the *srfA* operon, could be enhanced after replacement against the strong and constitutive promoter P_{veg} on the genomic DNA of *B. subtilis*.

The second approach of this thesis focused on culture conditions which influence the production of Surfactin by *B. subtilis* in many ways. Different medium components, especially the presence and amount of a certain carbon-source, drastically affect the production of Surfactin. The importance of the initial glucose concentration during cultivation of *B. subtilis* was therefore investigated. Another aspect of culture conditions was focused by the application of different fermentation methods. The production of surfactants is usually accompanied by severe foaming which demands innovative concepts to process the foam. Two entirely different methods were evaluated during this thesis. The first supported the foam formation in order to enhance Surfactin yields and enable *in situ* recovery. The second approach prevented the formation of foam as no gas was purged through the cultivation

broth obtaining an anaerobic fermentation process. Both fermentation approaches were systematically analyzed to determine their effectiveness for comparison.

Four major research projects were planned and conducted during this thesis, these projects were supposed to cover the essentials of microbial surfactant production: identification and characterization of different producer strains, genetic optimization of a producer strain, improvement of the employed medium and examination of established and novel cultivation methods to facilitate the production of surfactants in a biotechnological process. In this manner, an overview of different optimization possibilities is given.

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2. Evaluation of Different Surfactin Producer Strains

This chapter is partially based on the following publication

Evaluation of Different *Bacillus* Strains in Respect of their Ability to Produce Surfactin in a Model Fermentation Process with Integrated Foam Fractionation

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Judit Willenbacher designed all experiments, conducted the fermentations, collected the data, analyzed all results, calculated all values and wrote the manuscript.

Michaela Zwick, **Teresa Mohr** and **Ferdinand Schmid** assisted during all fermentations, conducted analytical experiments and helped analyzing the results.

Chirstoph Syldatk and **Rudolf Hausmann** supervised the project and contributed to scientific discussion.

2.1 INTRODUCTION

Surfactin is produced by *Bacillus subtilis*, a common gram-positive soil bacterium, which is already used in several industrial processes for the production of enzymes and also for the preparation of Nattō, a traditional Japanese dish made of fermented soy beans (Schallmey et al. 2004). In the past, several attempts have been made to develop an economically reasonable fermentation process for the production of Surfactin. The majority focused on using alternative substrates instead of the widely used carbon source glucose (Sheppard and Mulligan 1987; Freitas de Oliveira et al. 2013; Nitschke and Pastore 2004; Ohno et al. 1995). Although the usage of waste products as nutrients for the growth of *Bacillus* seems attractive, those fermentation processes lack high profitability owing to low product yields. Enhanced Surfactin production by using foam fractionation was already shown by Cooper et al. (Cooper et al. 1981), exploiting the natural behavior of surfactants to accumulate at interfaces. By revealing such possibilities for *in situ* product removal and enrichment, Cooper et al. presumably paved the way for new updates on fermentation processes for the production of Surfactin. Based on Coopers study, other research groups started to analyze foam fractionation for product removal, focusing on product recovery and enrichment (Davis et al. 2001; Chen et al. 2006). Up to that point, most fermentation processes were not analyzed in a procedural manner, although this is an important aspect when evaluating the effectiveness of a fermentation process. Thus, next to focusing on product recovery and enrichment, it is also important to analyze the specific productivity.

To establish a reasonable fermentation process for the production of Surfactin, it is not only necessary to evaluate procedural fermentation parameters of preliminary experiments, but it is also crucial to use the most effective microorganism. In most studies *B. subtilis* ATCC 21332 was used to produce Surfactin (Davis et al. 2001; Isa et al. 2007; Sheppard and Mulligan 1987; Cooper et al. 1981; Nitschke and Pastore 2004), as this strain is supposed to be identical with *B. subtilis* IAM 1213, the first described Surfactin producer (Arima et al. 1968). In other studies *B. subtilis* strains were obtained as natural isolates and analyzed regarding their ability to produce the lipopeptide (Chen et al. 2006; Freitas de Oliveira et al. 2013). However, a direct comparison whether a certain *B. subtilis* strain is the most qualified for the production of Surfactin, was never realized.

The intention of the presented study was the identification of Surfactin producer strains and the comparison of these *Bacillus* strains regarding their ability to produce Surfactin in a model fermentation process with integrated foam fractionation. The comparison focuses on widely known Surfactin producers like ATCC 21332, DSM 3256, and DSM 3258, but also on a natural isolate (LM43a50°C), a newly discovered Surfactin producer (DSM 1090) and the *B. subtilis* type strain (DSM 10^T). The study is based on the analysis of parameters like product yield $Y_{P/X}$, substrate yield

$Y_{X/S}$, specific growth rate μ, specific productivity $q_{Surfactin}$, volumetric productivity $q_{Surfactin}$ and the investigation of the product removal by foam fractionation (Surfactin recovery and Surfactin and bacterial enrichment). The intention of the present study is to reveal differences in the Surfactin productivity of various *Bacillus* strains and come to a conclusion if it is possible to give a specific *Bacillus* strain a preference.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

All chemicals used in the current study were purchased from Carl Roth GmbH (Karlsruhe, Germany) and were of analytical grade. The Surfactin standards for high performance liquid chromatography (HPLC) were obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany).

2.2.2 Microorganisms and strain maintenance

Several different *Bacillus* strains were analyzed in the current study regarding their ability to produce Surfactin. All analyzed strains are listed in Tab. 2.2. The six most important strains are described in further detail in Tab. 2.1. Four of these strains were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH): DSM 3256, DSM 3258, DSM 10^T and DSM 1090. Whereas the strain ATCC 21332 was purchased from the ATCC (American Type Culture Collection) and the natural isolate LM43a50°C (deposited at the DSMZ: DSM 28227) was isolated in previous works. All analyzed strains are *B. subtilis* or *Bacillus* sp. wild types. The strains were stored as glycerol stocks, prepared from a culture in Lysogeny Broth (Bertani 1951) from the exponential growth phase, at -80 °C.

Tab. 2.1 *Bacillus* strains employed during fermentation Listed are the employed strains regarding their origin, precise species, synonym and number in other strain collections.

Bacillus strain	species	origin	collection no.*	strain synonym
DSM 10^T	*B. subtilis*	DSMZ	ATCC 6051	*Bacillus natto*
DSM 3256	*B. subtilis*	DSMZ	IAM 1213	*Bacillus natto*
DSM 3258	*B. subtilis*	DSMZ	IAM 1260	
DSM 1090	*B. subtilis*	DSMZ	OUT 8424	*Bacillus natto*
ATCC 21332	*B. subtilis*	ATCC	IAM 1213	
LM43a50°C	*Bacillus sp.*	natural isolate	DSM 28227	

2.2.3 Culture conditions

2.2.3.1 Media

The employed mineral salt medium was based on the fermentation medium of Cooper (Cooper et al. 1981): 4 % glucose, 8.0×10^{-4} M $MgSO_4$, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4$, 4.0×10^{-6} M Na_2EDTA, 1×10^{-6} M $MnSO_4$. Furthermore, the former nitrogen source 0.05 M NH_4NO_3 was replaced with 0.1 M NH_4Cl. The deployed concentration of the phosphate buffer demanded slight changes depending on its usage for inoculum cultures or fermentation processes. For the cultivation in shake flasks the original 0.07 M phosphate buffer (0.03 M KH_2PO_4 and 0.04 M Na_2HPO_4) was used, whereas for the cultivation in benchtop bioreactors a 0.01 M phosphate buffer was employed (4.29×10^{-3} M KH_2PO_4 and 5.71×10^{-3} M Na_2HPO_4).

The sterilization of the medium was performed in separate stages. A 0.222 M glucose solution was prepared and separately autoclaved. The components NH_4Cl, KH_2PO_4, and Na_2HPO_4 were autoclaved together, whereas a separate solution with 8×10^{-3} M $MgSO_4$ was filter-sterilized. The trace elements 7.0×10^{-3} M $CaCl_2$, 4.0×10^{-3} M $FeSO_4$, 4.0×10^{-3} M Na_2EDTA and 1×10^{-3} M $MnSO_4$ were combined in one solution and also filter-sterilized before the complementation of the medium.

2.2.3.2 Preparation of inoculum cultures

For the preparation of the seed culture 500 µL of the glycerol stock solution was inoculated in 100 mL of the described mineral salt medium in a 500 mL baffled shake flask. The shake flask was incubated for 24 h at 120 rpm and 30 °C in a shake incubator chamber (Multitron II, HT Infors, Bottmingen, Switzerland). Furthermore, the seed culture was used to inoculate with a resulting OD_{600} of 0.1 in the aqueous phase at the beginning of the bioreactor cultivation.

2.2.3.3 Cultivation in a 2.5 L benchtop bioreactor

All cultivations were carried out in 2.5 L benchtop bioreactors with a nominal capacity of 1.5 L (Minifors, HT Infors, Bottmingen, Switzerland). The bioreactors were equipped with pH (Mettler-Toledo International Inc., Greifensee, Switzerland) and pO_2 electrodes (Oxyferm, Hamilton Bonaduz AG, Bonaduz, Switzerland), a temperature sensor and Rushton turbines (Fig. 2.1). The airflow was adjusted to 0.07 vvm. The temperature was fixed at 30 °C and the pH was regulated to a value of 7.0 by the addition of 4 M NaOH or 4 M H_3PO_4. At the beginning of the fermentation process the stirrer was adjusted to 300 rpm, afterwards the stirrer was regulated by the online control of the pO_2. The value of dissolved oxygen was not allowed to fall below 10 %, consequentially the stirrer was regulated upwards to reach values between 10 % and 20 %. This

was achieved using a sequence (see appendix) applied in the used process control system (IRIS, HT Infors, Bottmingen, Switzerland). Instead of employing antifoam, the developing foam was channeled through the exhaust cooler into collecting bags, functioning as foam traps (Fig. 2.2). The fermentation process was started with 1.5 L of the described mineral salt medium and the additional volume of the inoculated seed culture. Since the bioreactor cultivation was realized as a batch cultivation, no further medium components were added. During the 30 h of cultivation (the cultivation of DSM 3258 had to be extended to 37,5 h) pH, pO_2, temperature and stirrer speed were consistently monitored. Every two hours a sample was taken from the cultivation broth and the attached foam trap was exchanged for a new one. All fermentations were performed as duplicates.

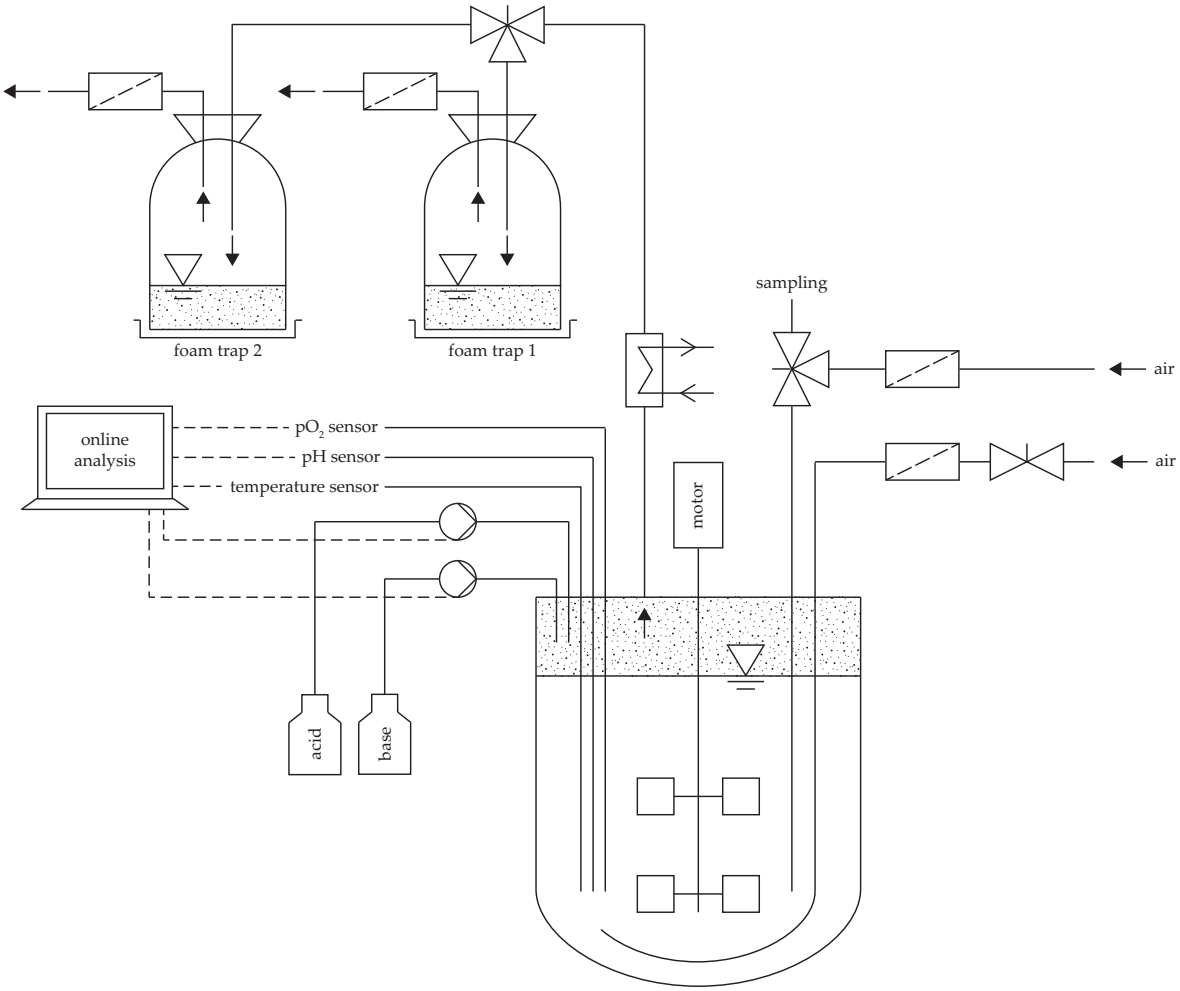

Fig. 2.1 Construction scheme of the employed bioreactor system with integrated foam fractionation The used bioreactor was equipped with pO_2, pH and temperature sensor. The collected data were online analyzed and results were used for automatically regulating the pH. The working volume inside the bioreactor was adjusted to 1.5 L and uprising foam was able to leave the bioreactor via the exhaust cooler. The foam was collected in two foam traps from which solely one was connected to the bioreactor. During sampling the valve junction was altered to allow the foam emission into the next foam trap. In doing so, foam was at all time able to leave the reactor system and excess pressure was prevented.

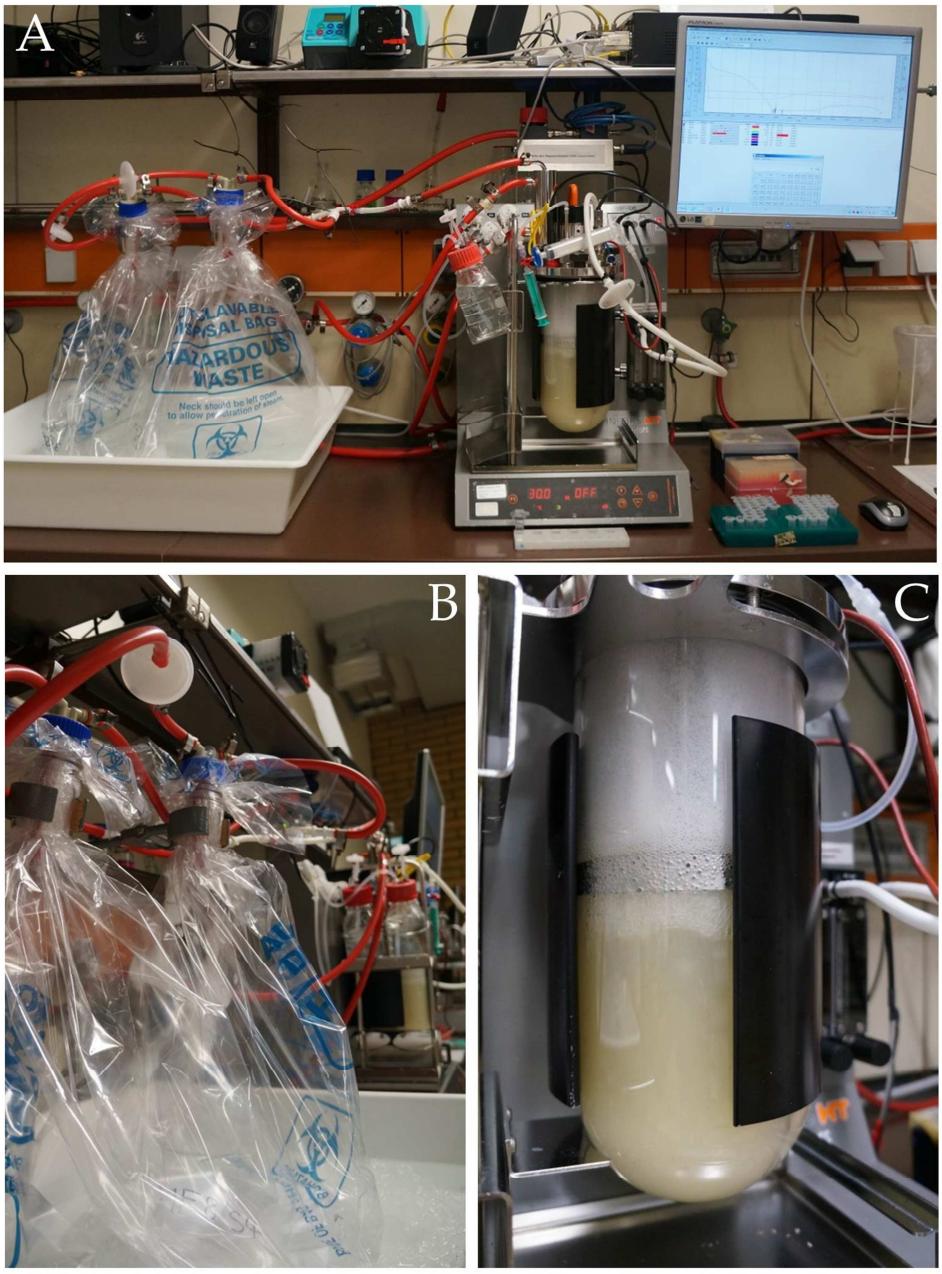

Fig. 2.2 Images of the employed bioreactor system with integrated foam fractionation The bioreactor system was composed of a 2.5 L benchtop bioreactor, two affiliated foam traps and a computer for online analysis of pO_2, pH, temperature, stirrer speed and addition of base and acid (**A**). The foam traps were constructed using plastic bags which were chucked in detached bottlenecks. These setups were fixated with clamps and upheld above a tray holding ice for foam cooling (**B**). During cultivations foam was allowed to rise and leave the bioreactor vessel via the exhaust cooler. As a consequence of foaming the liquid level sank gradually (**C**).

2.2.4 Analytical methods

2.2.4.1 Sampling and sample processing

Sampling took place every two hours. Depending on the occurrence of foam, one (cultivation broth) or two samples (cultivation broth and foam trap) were taken. The offline analysis of the cultivation broth samples included the determination of the OD_{600} (in case of DSM 3258 cell pellets were dried and weighed) and the glucose, ammonium and Surfactin concentration. The foam trap samples were analyzed concerning the foam volume, the cell dry weight (CDW) and the glucose and Surfactin concentration. The CDW of samples was detected gravimetrically after drying the cell pellet until constant weight at 80 °C. The concentration of glucose and ammonium was analyzed using a glucose assay kit (Cat. No. 10 716 251 035, R-Biopharm AG, Darmstadt, Germany) and an ammonium assay kit (1.14752.001, Merck KGaA, Darmstadt, Germany), respectively. The concentrations were determined according to the manufacturer instructions, utilizing a spectrophotometric method (Ultrospec 2100 pro, General Electric Deutschland Holding GmbH, Frankfurt, Germany). The concentration of Surfactin was determined by analyzing the sample supernatant using HPLC (cf. below).

2.2.4.2 Quantification of Surfactin

The concentration of Surfactin was determined using an HPLC-UV/Vis quantification method. The applied Surfactin standard was obtained from Sigma Aldrich and initially used to prepare a 5 mM stock solution. Altogether eleven Surfactin solutions ranging from 0.01 mM to 5 mM Surfactin were prepared in duplicates. The analysis was performed with a standard HPLC device (Agilent 1200 Series, Agilent, Waldbronn, Germany) equipped with a reverse phase column (C18 150 × 4.6 mm, 120 Å, Hyperclone, Phenomenex, California, USA) at 30 °C. An isocratic method was performed with 80 % acetonitrile and 20 % 3.8 mM trifluoroacetic acid for 25 min. The peptide bonds of Surfactin were detected at 210 nm and the following six characteristic Surfactin peaks appeared between retention times of 6 min to 13 min.

2.2.4.3 Data analysis

In order to analyze the fermentation process, several values were calculated to compare the different *Bacillus* strains concerning their productivity of Surfactin. Using the results of CDW, glucose and Surfactin the values of $Y_{X/S}$ [g/g], $Y_{P/X}$ [g/g], μ [h^{-1}], $q_{Surfactin}$ [g/g·h], volumetric $q_{Surfactin}$ [g/L·h], Surfactin recovery [%], Surfactin enrichment and bacterial enrichment were determined.

The value of $Y_{X/S}$ was determined in an integral manner, using the maximal mass of produced CDW (m_{Xmax}) and the corresponding mass of depleted glucose (m_S; Eq. 2.1).

$$Y_{X/S} = \frac{\Delta m_{Xmax}}{\Delta m_S} \quad \textbf{Eq. 2.1}$$

$Y_{P/X}$ was calculated in the same manner as $Y_{X/S}$ using the maximal mass of produced product ($m_{Surfactin\ max}$) and the corresponding CDW over the whole fermentation process (Eq. 2.2).

$$Y_{P/X} = \frac{\Delta m_{Surfactin\,max}}{\Delta m_{Xmax}} \quad \textbf{Eq. 2.2}$$

The specific growth rate μ was determined in two ways. First, by using Eq. 2.3 and second, by calculating the division of the derived, fitted data of CDW (m_X) by the fitted data of CDW.

$$\mu = \frac{ln\frac{m_{X_2}}{m_{X_1}}}{t_2 - t_1} \quad \textbf{Eq. 2.3}$$

The specific productivity $q_{Surfactin}$ was calculated in a differential and an integral manner. The differential values were calculated using Eq. 2.4 and by using the division of the derived, fitted data of Surfactin ($m_{Surfactin}$) by the fitted data of CDW (m_X). For the calculation of integral values the overall produced masses of Surfactin and CDW ($m_{Surfactin\ max}$ and m_{Xmax}) were employed in Eq. 2.4.

$$q_{Surfactin} = \frac{\Delta m_{Surfactin}}{\Delta m_X \cdot \Delta t} \quad \textbf{Eq. 2.4}$$

The volumetric specific productivity $q_{Surfactin}$ (vol. $q_{Surfactin}$) was as well determined in a differential and an integral manner. Differential values were calculated applying Eq. 2.5 and by the division of the derived, fitted data of Surfactin ($m_{Surfactin}$) by the fitted data of the bioreactor volume ($V_{Reactor}$). Integral calculations employed the overall yield of Surfactin ($m_{Surfactin\ max}$) and the average bioreactor volume ($øV_{Reactor}$) in Eq. 2.5.

$$volumetric\ q_{Surfactin} = \frac{\Delta m_{Surfactin}}{V_{Reactor} \cdot \Delta t} \quad \textbf{Eq. 2.5}$$

The Surfactin recovery was determined in an integral manner, summarizing the recovered Surfactin from foam and calculating the division of it by the sum of the summarized Surfactin from foam and the remaining Surfactin in the bioreactor (Eq. 2.6).

$$Surfactin\ recovery = \frac{\Sigma m_{Surfactin\ foam}}{\Sigma m_{Surfactin\ foam} + m_{Surfactin\ reactor}} \cdot 100 \qquad \textbf{Eq. 2.6}$$

The values of Surfactin enrichment were determined in a differential manner. The concentration of Surfactin in foam ($c_{Surfactin\ foam}$) at a specific time was divided by the concentration of Surfactin in the bioreactor ($c_{Surfactin\ reactor}$) at the same time (Eq. 2.7).

$$Surfactin\ enrichment = \frac{c_{Surfactin\ foam}}{c_{Surfactin\ reactor}} \qquad \textbf{Eq. 2.7}$$

The bacterial enrichment was calculated in the same differential fashion as the Surfactin enrichment using the concentration of CDW in foam and bioreactor ($c_{X\ foam}$ and $c_{X\ reactor}$; Eq. 2.8).

$$Bacterial\ enrichment = \frac{c_{X\ foam}}{c_{X\ reactor}} \qquad \textbf{Eq. 2.8}$$

As described before the values of μ, $q_{Surfactin}$ and volumetric $q_{Surfactin}$ were calculated with fitted data of Surfactin and CDW mass as well as bioreactor volume. Some of these fits are shown in Fig. 2.3. The used equation for the logistic fits is shown below:

$$y(x) = y_0 + \frac{a}{1+(\frac{x}{x_0})^b} \qquad \textbf{Eq. 2.9}$$

2.3 RESULTS

2.3.1 Screening of Surfactin producer strains

At the beginning of this study several *Bacillus* strains were analyzed regarding their ability to produce Surfactin in a mineral salt medium based on the study of Cooper et al. (1981). Therefore different *Bacillus* strains which were already stored at the institutes stock collection were tested. Additionally, all described Surfactin producers stored at the DSMZ (and *B. subtilis* ATCC 21332 from the ATCC) as well as all *Bacillus* strains listed under the synonym name *Bacillus natto* at the DSMZ were purchased and analyzed regarding Surfactin productivity. The results of these shake flask cultivations (supernatants were analyzed via HPLC for Surfactin) are listed in Tab. 2.2. Interestingly, two *Bacillus* strains stored at the institute were able to produce Surfactin: *B. subtilis* DSM 10^T and *Bacillus* sp. LM43a50°C. The type strain *B. subtilis* DSM 10^T is described as equal to the strain *B. subtilis* NCIB 3610, which was shown to produce Surfactin in earlier studies (Watrous et al. 2010). Therefore, results of this study confirmed previous investigations. In contrast, strain

LM43a50°C, a natural isolate discovered by a former colleague at the institute during another project, was shown to produce Surfactin for the first time. The described Surfactin producer strains *B. subtilis* ATCC 21332, DSM 3256, DSM 3257 and DSM 3258 delivered however surprising results as one of the strains did not produce Surfactin at all (*B. subtilis* DSM 3257) and another one produced only a very small amount (nearly not detectible, *B. subtilis* DSM 3256). Most surprisingly, one of the ordered *Bacillus natto* strains (*B. subtilis* DSM 1090) did produce Surfactin which was previously not reported. *Bacillus natto* strains were purchase because the first described Surfactin producers were natural isolates from the Japanese dish Nattō (Arima et al. 1968). Strains which originate from this dish are consequently named with the synonym *natto*. In summary this screening shows that the ability to produce Surfactin is variable spread in the genus *Bacillus*. All encountered Surfactin producer strains were applied for further experiments during this study.

Tab. 2.2 Screening of various *Bacillus* strains regarding Surfactin productivity Listed are all *Bacillus* strains which were tested for Surfactin productivity during this thesis. Additionally summarized are the sources of these strains, if Surfactin productivity was described earlier and the outcome of this screening.

Bacillus strain	Source	Described *S* producer	Confirmed *S* productivity
B. subtilis DSM 10^T	DSMZ	(x)	x
Bacillus sp. LM43a50°C	Laboratory stock	-	x
Bacillus sp. A16	Laboratory stock	-	-
Bacillus sp. D17	Laboratory stock	-	-
Bacillus sp. F16	Laboratory stock	-	-
Bacillus sp. F18	Laboratory stock	-	-
Bacillus sp. G18	Laboratory stock	-	-
Bacillus sp. H20	Laboratory stock	-	-
B. subtilis ATCC 21332	ATCC	x	x
B. subtilis DSM 3256	DSMZ	x	x
B. subtilis DSM 3257	DSMZ	x	-
B. subtilis DSM 3258	DSMZ	x	x
B. subtilis DSM 1090	DSMZ	-	x
B. subtilis DSM 5214	DSMZ	-	-
B. subtilis DSM 6198	DSMZ	-	-
B. subtilis DSM 6223	DSMZ	-	-

S = Surfactin; (x) = equivalent strain NCIB 3610 described as Surfactin producer

2.3.1 Correlation between cell dry weight, Surfactin and carbon source during the fermentation processes

The time course of CDW concentration, mass of accumulated Surfactin and glucose concentration is shown in Fig. 2.3 for all cultivations. Both, CDW concentration and mass of Surfactin, could be described by logistic fits (Eq. 2.9). The concentration of CDW during cultivation of DSM 1090 reached a maximum of 6.6 g/L, which resembles the maximal CDW of DSM 3256 with 6.17 g/L. The strains ATCC 21332 and DSM 3258 exceed those values with maximal CDWs of 7.7 g/L and 9.5 g/L. In contrast, DSM 10^T and LM43a50°C only reached values of 2.9 g/L and 3.1 g/L. In summary it becomes apparent that the gained CDW concentrations differed a lot between the analyzed strains, ranging from 2.9 g/L to 9.5 g/L. On the contrary, accumulated Surfactin varied only from 0.53 g to 1.36 g (sole exception DSM 3256). Up to 0.8 g Surfactin were produced by DSM 1090, which was again exceled by ATCC 21332 (1.33 g Surfactin) and DSM 3258 (1.36 g Surfactin). The strains DSM 10^T and LM43a50°C gained values comparable to DSM 1090 with 0.8 g and 0.53 g Surfactin, respectively. On the contrary, DSM 3256 produced just 0.08 g Surfactin. Naturally, strains which grew to high CDW concentrations accumulated larger quantities of Surfactin (ATCC 21332, DSM 3258 and DSM 1090). However, DSM 10^T and LM43a50°C accumulated large quantities of Surfactin despite their low CDW concentration, leading to high values considering the specific productivity $q_{Surfactin}$ (Tab. 2.3). In respect of these findings it is also interesting to analyze the behavior of glucose consumption. Interestingly, the complete consumption of glucose was never reached, even though the increase of CDW stopped. At the beginning of all fermentations the glucose concentration was adjusted to 40 g/L. DSM 1090 metabolized three-quarters of the provided glucose (final concentration: 11.66 g/L). However, DSM 10^T and LM43a50°C only consumed about 10 g/L glucose, retaining 29.2 g/L and 30.96 g/L glucose, respectively. The strains DSM 3256, DSM 3258 and ATCC 21332 reached final concentrations between 15.2 g/L and 22.32 g/L glucose, since they grew to higher CDW concentrations compared to DSM 10^T and LM43a50°C. The lack of any other nutrition, despite glucose, could be a reason for decreasing growth, but in addition to carbon, nitrogen was also always still available. Calculations considering the availability of phosphor showed no limitation as well. Another possibility for the decrease of growth could be the production of byproducts. *B. subtilis* produces several compounds, like acetate, as a consequence of overflow metabolism. Some of the formed byproducts can be toxic, when reaching a certain concentration in the cultivation broth. Studies based on this observation are underway. However, the low consumption of glucose in processes with little CDW growth and the higher consumption of glucose in processes with high CDW growth lead to rather similar values of $Y_{X/S}$ for all analyzed *Bacillus* strains between approximately 0.2 g/g and 0.3 g/g (Tab. 2.3).

The comparable low values for $Y_{X/S}$ are owed to the carbon rich product Surfactin and possible byproducts containing carbon.

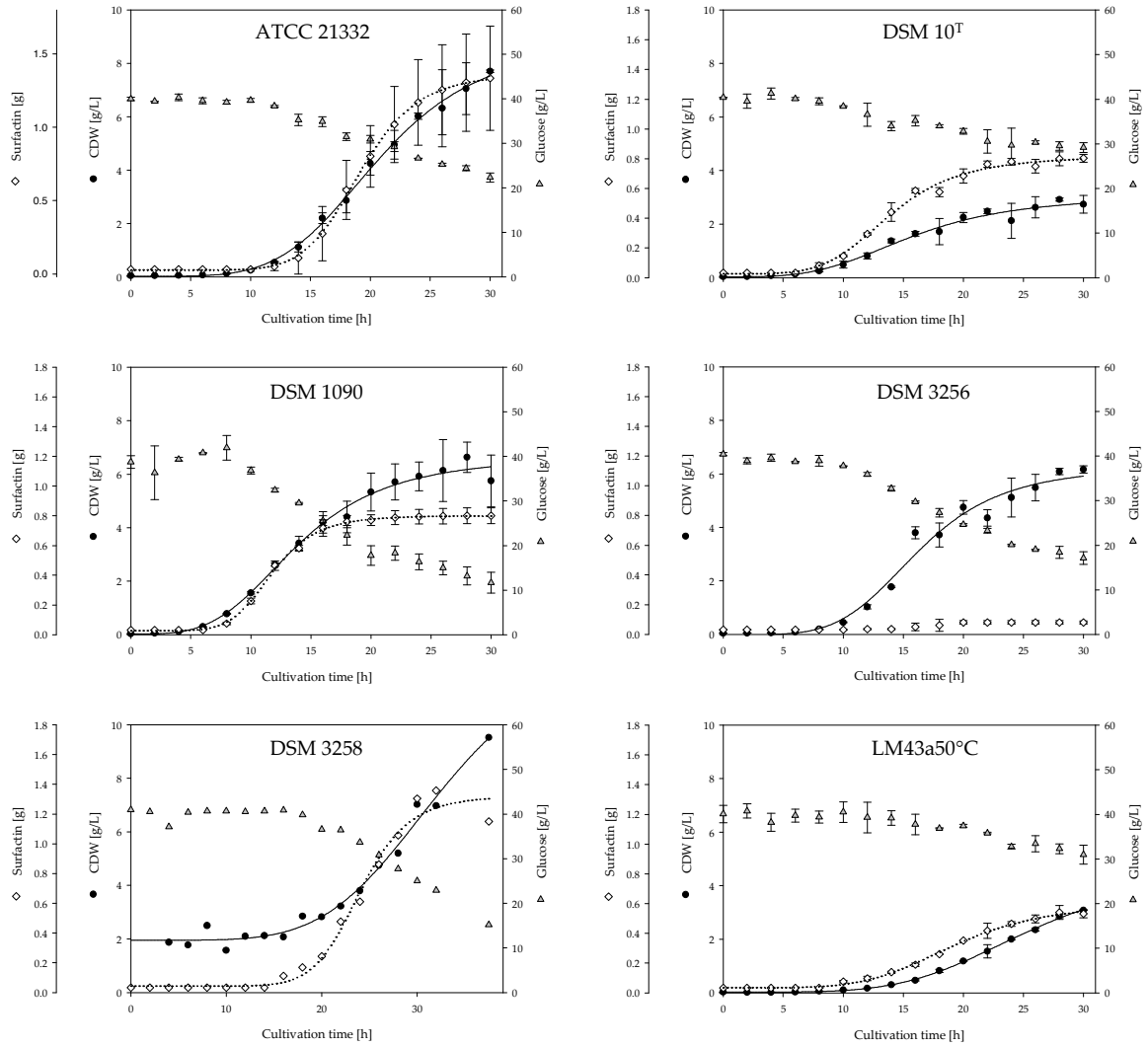

Fig. 2.3 Courses of various fermentations of different Surfactin producer strains Time courses of CDW and Surfactin in comparison to depleted glucose during the fermentation process of ATCC 21332, DSM 10^T, DSM 1090, DSM 3256, DSM 3258 and LM43a50°C. The values for CDW (*dot*), Surfactin (*rhombus*) and glucose (*triangle*) are given as mean values of two fermentations. The *dotted* and *solid lines* represent logistic fits of CDW and mass of Surfactin based on Eq. 2.9.

2.3.2 Analysis of foam fractionation

The analysis of the collected foam traps is presented in Fig. 2.4. The graphs display the values of Surfactin recovery, Surfactin enrichment and bacterial enrichment during the course of one fermentation. Release of foam took place for the first time after 8 h of DSM 1090 cultivation. This resembles the cultivation of DSM 10^T, where foaming started as well after 8 h of cultivation. In

contrast, other strains started foaming not until 14 h (LM43a50°C), 16 h (ATCC 21332) or even just after 18 h of cultivation (DSM 3258). However, DSM 3256 constitutes an exception, as it produces nearly no foam (1 to 3 foam traps). The first foam trap of DSM 1090 displays a high value of Surfactin enrichment (156.96), but subsequent foam traps first display lower values before the Surfactin enrichment reaches values around 70. Such extraordinary high values in the first foam trap could also be observed for DSM 10^T (54.5 % Surfactin recovery, Surfactin enrichment of 157.4) and ATCC 21332 (79.6 % Surfactin recovery, Surfactin enrichment of 55.8), while the other strains

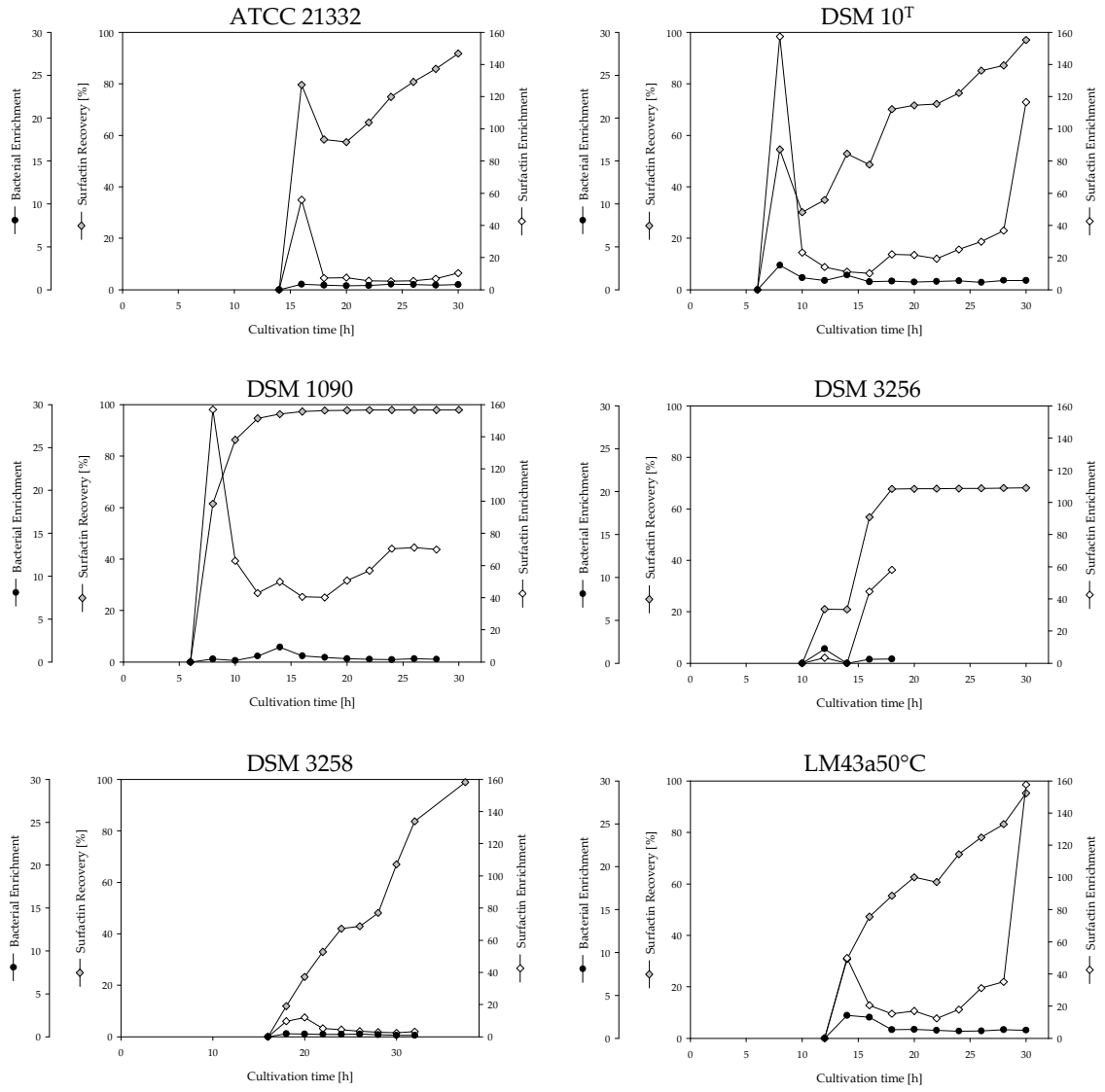

Fig. 2.4 Analysis of foam fractionation Time courses of foam traps during fermentations of *B. subtilis* ATCC 21332, DSM 10^T, DSM 1090, DSM 3256, DSM 3258 and LM43a50°C regarding Surfactin recovery, Surfactin enrichment and bacterial enrichment. The values for bacterial enrichment (*dot*), Surfactin enrichment (*white rhombus*) and Surfactin recovery (*grey rhombus*) are shown as exemplary results of one fermentation.

mainly did not follow this progress. During cultivation almost all strains featured a nearly continuous increase of Surfactin recovery, reaching values from 91.8 % to 98.9 %. The strain DSM 1090 forms an exception by reaching high values of 97 % nearly from the beginning of foaming. The course of Surfactin enrichment varied between the different strains. ATCC 21332 displays nearly no alteration in the behavior of Surfactin enrichment after the first foam trap, equal to DSM 3258, which presents rather low values of Surfactin enrichment during cultivation (reaching a maximum Surfactin enrichment of 12). In contrast, the strains DSM 10^T, DSM 1090 and LM43a50°C exhibit increasing values of Surfactin enrichment, achieving values from 70 to 157.7. The behavior of bacterial enrichment was relatively consistent throughout all strains. DSM 1090 displays one aberration with a value of 1.7, but otherwise exhibits values between 0.16 and 0.7. This resembles the progression of DSM 10^T (aberration of 2.85, ranges otherwise between 0.86 and 1.7) and LM43a50°C (aberration of 2.7, ranges otherwise between 0.84 and 1.0). The strains ATCC 21332 and DSM 3258 exhibit consistently low values of bacterial enrichment throughout the foaming, ranging between 0.52 and 0.65, and 0.16 and 0.34, respectively. However, all strains displayed higher Surfactin enrichment than bacterial enrichment, proving the concept of product enrichment during foam fractionation. Surprisingly, all strains reached excellent values of Surfactin recovery (nearly up to 100 %) and displayed low values of bacterial enrichment (only seldom values above 1.0), confirming that *Bacillus* is particularly suited for foam fractionation.

2.3.3 Growth and production rates

The progression of specific growth μ and specific productivity $q_{Surfactin}$ is presented in Fig. 2.5. The values were calculated in two different approaches. First, in a differential manner using Eq. 2.3 and Eq. 2.4. Second, by applying the derived fitted data of CDW and mass of Surfactin (logistic fit Eq. 2.9). Hereby, it is possible to approach the true values of μ and $q_{Surfactin}$ in the best possible way. The strain DSM 1090 displayed a maximal specific growth rate μ_{max} of 0.77 h^{-1} after 3.5 h of cultivation. Which was the highest value reached for μ_{max}, since for all other strains μ_{max} ranged between 0.07 h^{-1} (DSM 3258) and 0.5 h^{-1} (ATCC 21332). The time of cultivation by which μ_{max} was reached differed a lot between the various strains. While DSM 1090 outreached this point already after 3.5 h, the other strains reached μ_{max} after 7 h (DSM 10^T), 11 h (LM43a50°C), 12.5 h (ATCC 21332) and 18.5 h (DSM 3258) of cultivation. The specific productivity $q_{Surfactin}$ of DSM 1090 gained its maximum of 0.044 g/(g·h) after 10 h of cultivation. Comparable results were achieved by ATCC 21331 and DSM 3258, displaying values of 0.03 g/(g·h) and 0.025 g/(g·h), respectively. In contrast to these strains, DSM 10^T and LM43a50°C revealed higher values for $q_{Surfactin}$ reaching 0.07 g/(g·h) and 0.08 g/(g·h), respectively.

However, DSM 3258 exhibits maximal values comparatively late during cultivation, which correlates with the other results of the foam fractionation, e.g., the late start of foaming. These findings can be explained by the unusual growth behavior of DSM 3258. In contrast to all other analyzed *Bacillus* strains DSM 3258 formed pellets during cultivation which presumably led to slow growth.

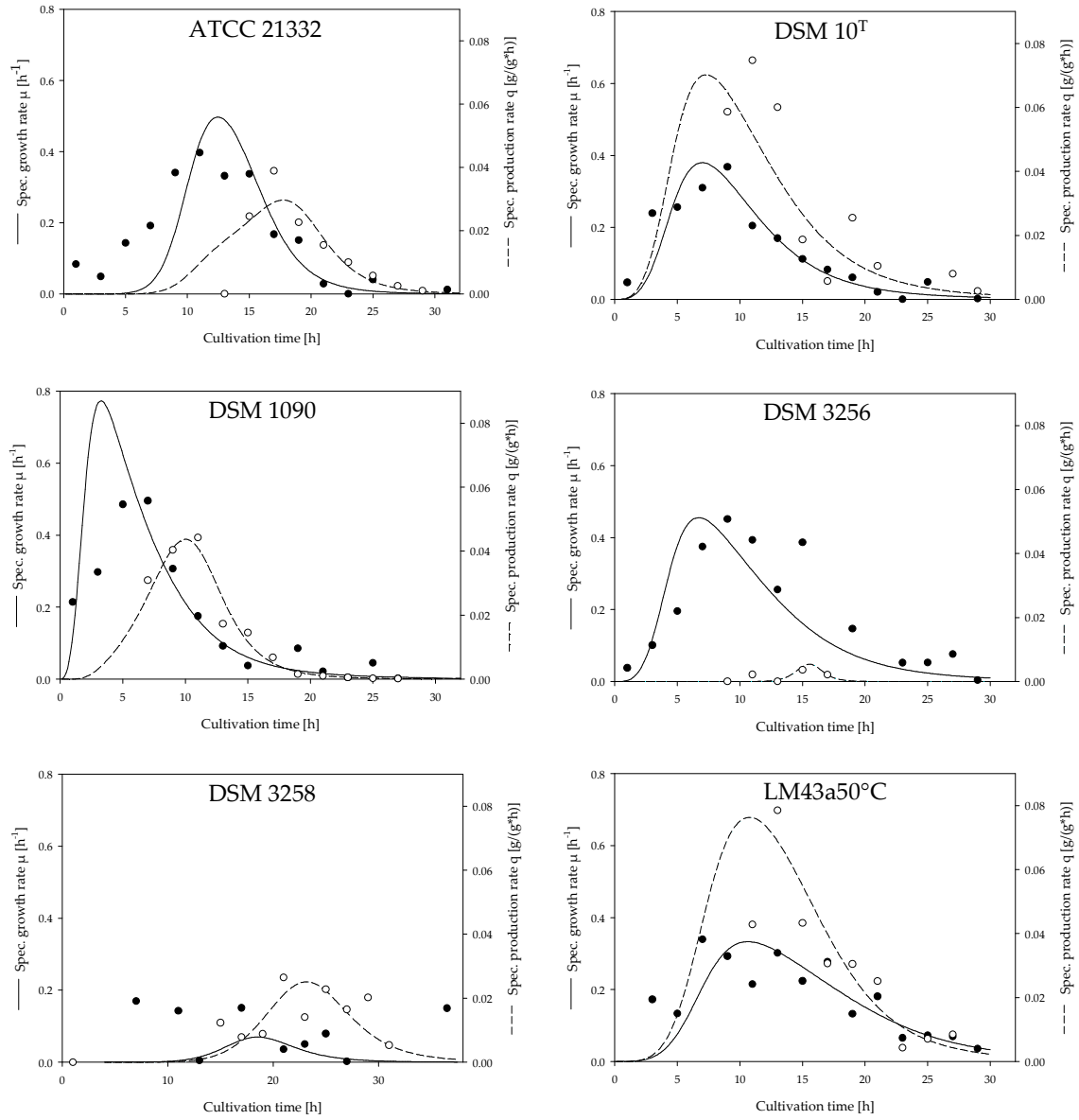

Fig. 2.5 Growths and production rates of various fermentations Progression of specific growth rate μ and specific productivity $q_{Surfactin}$ during the fermentations of *B. subtilis* ATCC 21332, DSM 10^T, DSM 1090, DSM 3256, DSM 3258 and LM43a50°C. The values for μ and $q_{Surfactin}$ are given as differential calculated values (*dots*) and values calculated by using the fitted data of CDW and mass of Surfactin (*lines*). The results for the specific growth rate μ are illustrated by *black dots* and a *solid line*, whereas specific productivity $q_{Surfactin}$ is given by *white dots* and a *dashed line.*

Generally the courses of μ and $q_{Surfactin}$ describe increasing and afterwards decreasing curves, which clearly characterizes a non-exponential growth behavior (which would in chase of μ otherwise be a constant line). The chosen logistic four-parameter model to characterize the courses of CDW and Surfactin mass, as well as bioreactor volume, appears to be well-chosen.

In addition it is crucial to analyze the progression of μ and $q_{Surfactin}$ in comparison to each other. In Fig. 2.5, describing the cultivation of DSM 1090, it is observable that the graphs of μ and $q_{Surfactin}$ are shifted. By including the results calculated from fitted data (lines) and results calculated in a differential manner (dots), it becomes apparent that the time courses for μ and $q_{Surfactin}$ are always shifted, no matter what kind of *Bacillus* strain was cultivated (Fig. 2.5), leading to a lagged product formation.

2.3.4 Comparison of significant procedural values

The most important procedural values for all analyzed *Bacillus* strains are summarized in Tab. 2.3 The listed values are mean values from two fermentations. The values of all calculated parameters are in the same range for all *Bacillus* strains except DSM 3256, which produced nearly no Surfactin. These findings imply that the suitability for Surfactin production in the applied fermentation process is rather similar for all other strains, which leads to the conclusion that there is no most qualified *Bacillus* strain under these analyzed microorganisms. Nevertheless, it is important to highlight the small differences between the strains and name a strain exhibiting mostly high values for all calculated parameters.

The overall $Y_{P/X}$ describes how much product was produced during the whole fermentation process in comparison to produced CDW. The highest level was reached during cultivations of DSM 3258 with a value of 0.22 g/g, whereas DSM 3256 produced nearly no Surfactin, giving an overall $Y_{P/X}$ of 0.01 g/g. The metabolization of glucose is described by the overall $Y_{X/S}$ value, which indicates how much CDW was produced in comparison to incorporated glucose. The highest value of 0.33 g/g was reached by LM43a50°C. In contrast, DSM 3258 and DSM 1090 only reached 0.18 g/g. The value of maximal $q_{Surfactin}$ ranged from 0.02 g/(g·h) (DSM 3258) to 0.08 g/(g·h) (DSM 10^T and LM3a50°C). In contrast to maximal $q_{Surfactin}$, integral $q_{Surfactin}$ summarizes how much Surfactin was produced during the whole fermentation in comparison to total produced CDW and cultivation time. Concerning integral $q_{Surfactin}$, DSM 10^T and DSM 3258 performed best (0.006 g/(g·h)), whereas DSM 3256 reached only 0.0002 g/(g·h). Compared to the specific productivity $q_{Surfactin}$, the volumetric productivity focuses on produced Surfactin in comparison to bioreactor volume. The highest maximal volumetric $q_{Surfactin}$ was reached by ATCC 21332 with a value of 0.11 g/(L·h). In contrast, LM43a50°C gained only 0.03 g/(L·h). The behavior of foam fractionation can be described by Surfactin recovery

and Surfactin and bacterial enrichment. The overall Surfactin recovery displays the percentage of recovered Surfactin over the cultivation time. The highest value was reached by DSM 3258 with 98.6 %, followed by DSM 1090 (98.0 %). DSM 3256 exhibited the lowest value of overall Surfactin recovery with about 63 %. The maximal Surfactin enrichment ranged from 12.7 (DSM 3258) to 161.1 (DSM 1090), whereby most strains displayed values around 80 and 90. The mean bacterial enrichment varied between 0.27 (DSM 3258) and 1.6 (DSM 10^T). Further information about the progression of foaming is given by maximal Surfactin concentrations in foam traps and total recovered Surfactin mass in all foam traps combined. The maximal Surfactin concentration ranged from 1.6 g/L (DSM 3258) to 3.99 g/L (DSM 10^T). Interestingly, most Surfactin was recovered in fermentations of DSM 3258 (averagely 1.05 g). In contrast, fermentations of DSM 3256 gained only 0.05 g Surfactin. Apart from that, the other strains achieved values between 0.4 g and 0.86 g Surfactin.

Tab. 2.3 Comparison of procedural parameters Listed are the summary of calculated processual parameters for the analyzed *Bacillus* strains and comparable data of Chen et al. 2006 and Davis et al. 2001. Represented are the parameters for product and substrate yield as well as data to evaluate the implemented foam fractionation.

	DSM 10^T	DSM 3256	DSM 3258	DSM 1090	ATCC 21332	LM43 a50°C	BBK 006*	ATCC 21332**
$Y_{P/X}$ [g/g]	0.19	0.01	0.22	0.14	0.16	0.12	0.26	0.08
$Y_{X/S}$ [g/g]	0.27	0.27	0.18	0.18	0.30	0.33	0.26	0.13
Max. $q_{Surfactin}$ [g/(g·h)]	0.08	0.01	0.02	0.04	0.04	0.08		
Int. $q_{Surfactin}$ [g/(g·h)]	0.006	0.0002	0.006	0.005	0.005	0.004		
Max. vol. $q_{Surfactin}$ [g/(L·h)]	0.05	0.02	0.07	0.08	0.11	0.03	0.01	0.01
Overall *S* recovery [%]	91.97	62.61	98.58	97.95	69.91	74.48	92.3	95
Max. *S* enrichment	101.9	83.6	12.7	161.2	80.8	88.2	55	62
Mean bact. enrichment	1.60	0.88	0.27	0.52	0.59	1.24	<0.2	0.4
Max. $c_{Surfactin}$ foam [g/L]	3.99	1.76	1.60	1.67	2.39	3.01	2.25	2.1
S in foam [g]	0.74	0.05	1.05	0.78	0.86	0.40	0.20	0.14

S = Surfactin, * = Chen et al., 2006; ** = Davis et al., 2001

As indicated before, DSM 3256 distinguishes itself most from all other strains, because nearly no Surfactin was produced. The small amount of Surfactin led to very low yields in $Y_{P/X}$, specific productivity $q_{Surfactin}$, and recovered Surfactin in foam. These results are surprising, considering that DSM 3256 is supposed to be identical to IAM 1213, the strain that was described by Arima et al. in 1968. However, the loss of productivity might be explained by probably numerous renewals of the stored strain. All other strains displayed rather similar results with some exceptions. LM43a50°C for example gained good results for $Y_{X/S}$, Surfactin enrichment and Surfactin concentration in foam, but reveals deficits in $Y_{P/X}$ and the total mass of Surfactin recovered from foam. Indicating that LM43a50°C is applicable for foam fractionation, because *in situ* product removal is possible, but lacks high production rates. In contrast, DSM 3258 displays lower values in $Y_{X/S}$, Surfactin enrichment and Surfactin concentration in foam, but very high yields in $Y_{P/X}$ and total recovered Surfactin from foam (and additionally very low yields for bacterial enrichment). Although DSM 3258 takes more time for foaming and thus for the production of Surfactin (Fig. 2.4 and Fig. 2.5) and is maybe less suitable for foam fractionation as the other strains (because of low Surfactin enrichment), it exhibits very high product yields. On the other side ATCC 21332 reaches good results in $Y_{X/S}$, total recovered Surfactin from foam and especially in volumetric productivity, whereas values for specific productivity (based on CDW) and $Y_{P/X}$ are comparably low. These results prove that ATCC 21332 is actually an effective Surfactin producer, but if the growth behavior of other strains with higher specific productivity could be improved, other strains could be more suitable for the production of Surfactin. The strain DSM 1090 reached exemplary high values for Surfactin enrichment and recovery making foam fractionation for *in situ* product removal an useful tool to obtain Surfactin. Nevertheless, high values of recovered Surfactin from foam were reached due to its growth behavior, not because of high values in specific productivity $q_{Surfactin}$. In contrast, DSM 10^T displayed good results in Surfactin enrichment, recovery and in specific productivity $q_{Surfactin}$. In total, DSM 10^T reached good or at least average values for most procedural parameters (sole exception bacterial enrichment, which was comparably high), which means that DSM 10^T displayed the best results under the applied conditions. Keeping in mind that growth behavior would have to be improved to obtain even better results and that the variance in all analyzed parameters of the different *Bacillus* strains is not very wide.

2.4 DISCUSSION

Investigations whether Surfactin producers differ from each other regarding procedural parameters during fermentation have not been reported. Therefore six different *Bacillus* strains were characterized in a model fermentation process with integrated foam fractionation.

Additionally these results were compared with conclusions of other studies using similar constructions (Davis et al. 2001; Chen et al. 2006). Both studies used resembling reactor volumes of approximately 1.0 L, employed glucose as sole carbon source and cultivated in batch with integrated foam fractionation. Davis et al. cultivated the widely used strain ATCC 21332, whereas Chen et al. used the *B. subtilis* strain BBK006. The results of their studies are summarized in Tab. 2.3.

The results of Chen et al. regarding $Y_{P/X}$ and $Y_{X/S}$ resemble the range of values calculated in the current study (0.26 g/g and 0.26 g/g), whereas Davis et al. yielded much lower values for $Y_{P/X}$ and $Y_{X/S}$ (0.08 g/g and 0.13 g/g). The values for specific productivity $q_{Surfactin}$ (differential and integral) were not calculated in the compared studies, but values for volumetric productivity were much lower (0.01 g/(L·h)) compared to the fermentation process which was employed in the presented study. The reached values of Surfactin recovery are similar to the results of the current study, reaching over 90 % in both investigations. On the other side, the yields for Surfactin enrichment are comparatively low with 55 and 62 (Chen et al. 2006; Davis et al. 2001), since in the current study most strains gained values over 80. The results for mean bacterial enrichment in contrast are very good (0.2 and 0.4), under the average 1.0 for *B. subtilis* (Parthasarathy et al. 1988). In contrast to these results, some strains investigated in the presented study exhibit mean bacterial enrichments over 1. However, the maximal reached Surfactin concentration in foam traps was around 2 g/L (2.3 g/L and 2.1 g/L), but only 0.20 g and 0.14 g Surfactin was recovered from the foam traps (i.e., only small volumes of foam were obtained in these studies). In contrast, up to 1 g Surfactin was recovered from the foam in one fermentation of DSM 3258. Nevertheless the results of Chen et al. and Davis et al. show that it is possible to compare different studies, if they resemble relatively similar constructions of bioreactors. But to uncover even small differences between *Bacillus* strains in fermentations with integrated foam fractionation, it is necessary to compare them in the exact same fermentation process.

2.5 CONCLUSION

The first experiments of this study revealed two newly described Surfactin producer strains (*Bacillus* sp. LM43a50°C and *B. subt*ilis DSM 1090) and confirmed the productivity of some described Surfactin producer strains (*B. subtilis* DSM 10^T, DSM 3258, ATCC 21332). Surprisingly, some of the stored Surfactin producer strains lost or nearly lost the ability to produce Surfactin (*B. subtilis* DSM 3257 and DSM 3256) which was previously not reported. Subsequently, the current study focused on the comparison of these different *Bacillus* strains, analyzing their ability to produce Surfactin.

The applied fermentation process with integrated foam fractionation proved to be highly suitable for such an analysis, as it is short (30 h to 37.5 h), effective and easy to reproduce. The employed construction to integrate foam fractionation exhibited very good results, allowing *in situ* product enrichment and removal. All analyzed strains, except DSM 3256, showed reproducible, high results for the production of Surfactin with exceedingly high values for absolute Surfactin recovered from foam compared to studies in similar systems. However, there is no most qualified *Bacillus* strain for the production of Surfactin in the employed setup, but strains differ in specific and volumetric productivity. By giving a vast overview over the most important procedural parameters in Tab. 2.3, the current study was set up as a decision guidance for research groups trying to find a Surfactin producer with specific characteristics.

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3. Foam-free Production of Surfactin via Anaerobic Fermentation

This chapter is based on the following publication

Foam-free Production of Surfactin via Anaerobic Fermentation of *Bacillus subtilis* DSM 10^T

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Authors' contribution to this publication

Judit Willenbacher designed all experiments, collected all data, created the graphs and figures, calculated additional data from comparable studies and drafted the manuscript.

Jens-Tilman Rau and **Jonas Rogalla** conducted their Bachelor theses under the supervision of Judit Willenbacher and contributed the data of the fermentations.

Chirstoph Syldatk and **Rudolf Hausmann** supervised the project and contributed to scientific discussion.

3.1 INTRODUCTION

Naturally, amphiphile molecules produced by bacteria in cultivation processes accumulate at gas-liquid interfaces and lead to massive foam formation. The main challenge in cultivating microorganisms producing biosurfactants is to overcome this severe foam production. In the majority of cases foaming is handled by the addition of antifoam agents. Unfortunately, this strategy harbors several disadvantages, as antifoam agents are expensive and very hard to remove in downstream processes. The second most common method to cope with foam formation is to disrupt the foam by shear stress or pressure using foam breakers. However, this method is often insufficient and increases the overall costs for the production of biosurfactants. Another, more elegant, way to manage foaming in biosurfactant production processes is to apply foam fractionation, which was already shown by Cooper et al. in 1981. This technique inverts the disadvantage into an advantage by using the accumulation of biosurfactants in the foam for *in situ* product enrichment and recovery. The Surfactin producer *B. subtilis* is especially suited for the employment of foam fractionation, yielding high values in product recovery and enrichment (Chapter 2). Although this is a possible way to handle foam and to improve product yields, a realization in industrial scale is probably unrealistic in the near future.

Another artful approach is to avoid foaming at all instead of dealing with it. Several attempts have been made to establish foam-free fermentation processes. Ohno et al. for instance employed a solid state fermentation of recombinant *B. subtilis* MI113 (pC112), using soybean curd residue as solid substrate (Ohno et al. 1995), which led to a yield of 2.0 g/kg (Surfactin per wet weight). Another attempt to produce Surfactin in a foam-free fashion implemented a membrane bioreactor (Coutte et al. 2010). A culture of *B. subtilis* ATCC 21332 obtained a maximal Surfactin concentration of 0.242 g/L. However, a significant amount of Surfactin was adsorbed at the membranes and oxygen transfer was reduced significantly. In contrast, Chtioui et al. focused on a rotating disc bioreactor for the production of Surfactin, allowing *B. subtilis* ATCC 21332 to grow free and immobilized in a biofilm at the same time (Chtioui et al. 2012). Aeration was realized above the fluid level, when the overgrown discs arose from the liquid. Maximal Surfactin concentrations of 0.212 g/L were obtained, but oxygen supply was limited and Fengycin concentrations surpassed Surfactin concentrations by far. While all these studies implemented innovative ideas to circumvent foaming, those processes are either difficult to scale up or lack high specificity.

B. subtilis was for a long time believed to be a strict aerobic bacterium. Since 1995 research on the anaerobic growth behavior of *B. subtilis* increased dramatically (Hoffmann et al. 1995; Nakano et al. 1997). By using nitrate as the terminal electron acceptor, *B. subtilis* is able to perform anaerobic respiration via a nitrate reductase encoded by operon *narGHJI* (Ramos et al. 1995). In this manner

nitrate is reduced to nitrite, which thereafter is transformed to ammonium via a nitrite reductase encoded by *nasDEF* (Nakano et al. 1998).

The production of biosurfactants under anaerobic conditions was already shown in 1985. The study presents the production of an undefined biosurfactant by *Bacillus licheniformis* in glucose mineral salt medium (Javaheri et al. 1985). The cultivation was performed in shake flasks, in the course of which the decreasing surface tension (from 70 mN/m to 28 mN/m) was measured. Although the characterization of the biosurfactant was only performed by thin layer chromatography and no high pressure liquid chromatography (HPLC) was applied, Javaheri et al. laid the foundation of anaerobic biosurfactant production. Subsequently, Davis et al. investigated the impact of nitrogen, carbon and oxygen conditions on Surfactin production of *B. subtilis* ATCC 21332 (Davis et al. 1999). Interestingly, maximal product yields were obtained under nitrate-limited and oxygen-depleted conditions ($Y_{P/X}$ = 0.075), which gives a further impulse to examine anaerobic Surfactin production. The proof of concept was provided by Zhang et al., who produced Surfactin with *B. subtilis* ATCC

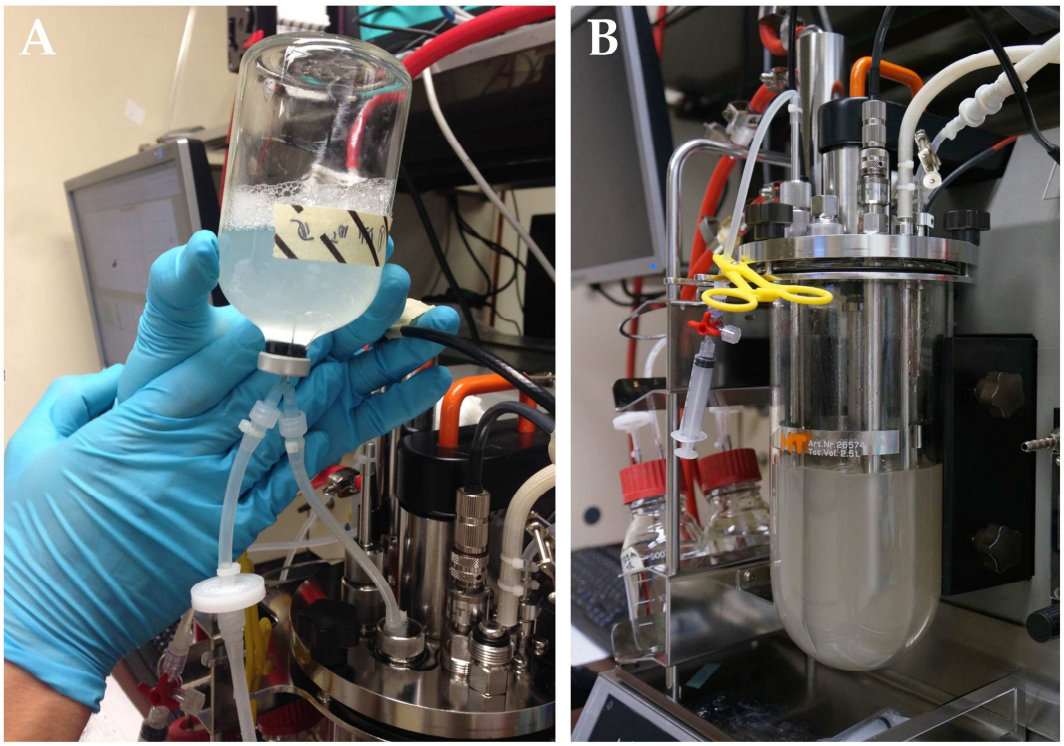

Fig. 3.1 Inoculation and fermentation of *Bacillus subtilis* DSM 10^T in 2.5 L benchtop bioreactor
Direct inoculation of the benchtop fermenter using a serum bottle with preculture. Nitrogen was introduced into the serum bottle via a small filter creating excess pressure inside the bottle. A second tube was used to channel the preculture directly into the inoculum device (**A**). Foam-free cultivation of *B. subtilis* DSM 10^T applying an anaerobic fermentation process (**B**).

21332 strictly anaerobic for the first time (Zhang et al. 2007). The investigation focused on a connected shake flask system, introducing a nitrogen flow to induce vigorous foaming. The foam was channeled through several flasks with distilled water to collect the produced biosurfactant. While these studies demonstrate that anaerobic production of Surfactin is possible, none of them propose a solution to overcome foaming.

The aim of the current study is to combine the relatively new research field of anaerobic biosurfactant production with a foam-free bioprocess strategy (Fig. 3.1 B). Therefore the anaerobic growth behavior of *B. subtilis* DSM 10^T was investigated in a 2.5 L benchtop bioreactor without any gas flow through the liquid phase. Four different glucose concentrations were tested and evaluated regarding their influence on Surfactin production. The processes were analyzed focusing on maximal Surfactin concentrations ($c_{Surfactin}$), growth rates (μ_{max}), product and substrate yields ($Y_{P/X}$, $Y_{X/S}$, $Y_{P/S}$), specific production rates ($q_{Surfactin}$) and specific volumetric production rates (vol. $q_{Surfactin}$).

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All chemicals applied in the current study were of analytical grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). The Surfactin standards for HPLC analysis were obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany).

3.2.2 Microorganism and strain maintenance

The wild type strain *B. subtilis* DSM 10^T was used for all experiments during this study. The microorganism was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and stored as glycerol stocks, prepared from a culture in Lysogeny Broth (Bertani 1951) from the exponential growth phase, at -80 °C.

3.2.3 Culture conditions

3.2.3.1 Media

The employed mineral salt medium was based on the fermentation medium of Cooper (Cooper et al. 1981): 8.0×10^{-4} M $MgSO_4$, 7.0×10^{-6} M $CaCl_2$, 4.0×10^{-6} M $FeSO_4$, 4.0×10^{-6} M Na_2EDTA, 1×10^{-6} M $MnSO_4$. In contrast to the original medium (40 g/L glucose) the concentration of glucose was altered to 2.5 g/L, 5 g/L, 7.5 g/L and 10 g/L, during various cultivations. Furthermore, the former

nitrogen source 0.05 M NH_4NO_3 was replaced with 0.1 M NH_4Cl and 0.1177 M $NaNO_3$. The deployed concentration of the phosphate buffer demanded slight changes depending on its usage for inoculum cultures or fermentation processes. For the cultivation in serum bottles the original 0.07 M phosphate buffer (0.03 M KH_2PO_4 and 0.04 M Na_2HPO_4) was used, whereas for the cultivation in benchtop bioreactors a 0.01 M phosphate buffer was employed (4.29×10^{-3} M KH_2PO_4 and 5.71×10^{-3} M Na_2HPO_4).

The preparation of medium for the cultivation in serum bottles demanded a different approach compared to the preparation of medium for the cultivation in benchtop bioreactors. Four different stock solutions were prepared for the cultivation in serum bottles. One stock solution contained the salt compounds (NH_4Cl, $NaNO_3$, KH_2PO_4, Na_2HPO_4) and was later completed to the final volume of 50 or 100 mL, respectively. The second stock solution included a 5.56-fold glucose solution of the final glucose concentration. In comparison, the third and fourth stock solution contained a 50-fold $MgSO_4$ solution and a 1000-fold solution of the trace elements ($CaCl_2$, $FeSO_4$, Na_2EDTA, $MnSO_4$). All solutions were filled into separate serum bottles and anaerobic conditions were adjusted by 20 alternating cycles of purging with gas (20 vol.-% CO_2 in N_2, 45 s) and evacuating (70 mbar, 45 s). Subsequently the bottles were autoclaved and the salt stock solution was completed under anaerobic conditions to receive the final concentrations of glucose, $MgSO_4$ and trace elements.

For the preparation of the bioreactor medium four stock solutions were prepared in a similar fashion. However, the first stock solution (NH_4Cl, $NaNO_3$, KH_2PO_4, Na_2HPO_4) was autoclaved inside the bioreactor. Whereas the glucose, $MgSO_4$ and trace elements stock solutions were prepared and autoclaved in separate vessels. The medium was completed inside the bioreactor after sterilization and thereafter anaerobic conditions were reached by purging the bioreactor with N_2 (4 Lpm, 1050 rpm, 20 min, Figure 2: valve 1 open).

3.2.3.2 Preparation of inoculum cultures

For the preparation of the first seed culture a loop of *B. subtilis* DSM 10^T from the glycerol stock solution was inoculated in 20 mL of Lysogeny Broth (inside a 100 mL baffled shake flask) and incubated in a shake incubator chamber (Multitron II, HT Infors, Bottmingen, Switzerland) at 30 °C and 120 rpm for 24 h. The second seed culture was inoculated with a resulting OD_{600} of 0.05 under anaerobic conditions in prepared serum bottles with 50 or 100 mL of mineral salt medium, respectively. The serum bottles were incubated in a horizontal position but otherwise in the same manner as the first seed culture. After 24 h of incubation approximately 200 mL of the second seed culture were used to inoculate the aqueous phase of the bioreactor (Fig. 3.1 A). The initial OD_{600}

inside the bioreactor fluctuated between 0.03 and 0.07, depending on bacterial growth of the second seed culture.

3.2.3.3 Cultivation in a 2.5 L benchtop bioreactor

All cultivations were carried out in 2.5 L benchtop bioreactors (Minifors, HT Infors, Bottmingen, Switzerland) with 1.0 L mineral salt medium. The bioreactors were equipped with pH (Mettler-Toledo International Inc., Greifensee, Switzerland) and pO_2 electrodes (Oxyferm, Hamilton Bonaduz AG, Bonaduz, Switzerland), a temperature sensor and Rushton turbines. The temperature was adjusted to 30 °C and the pH was controlled to a value of 7.0 by the addition of 4 M NaOH or 4 M H_3PO_4 (Fig. 3.2). The stirrer was adjusted to 300 rpm the entire time of cultivation. The medium was not exposed to gas flow throughout the whole fermentation process to guarantee an absolutely foam-free cultivation. However, to avoid reflux of air through the exhaust cooler and to allow the

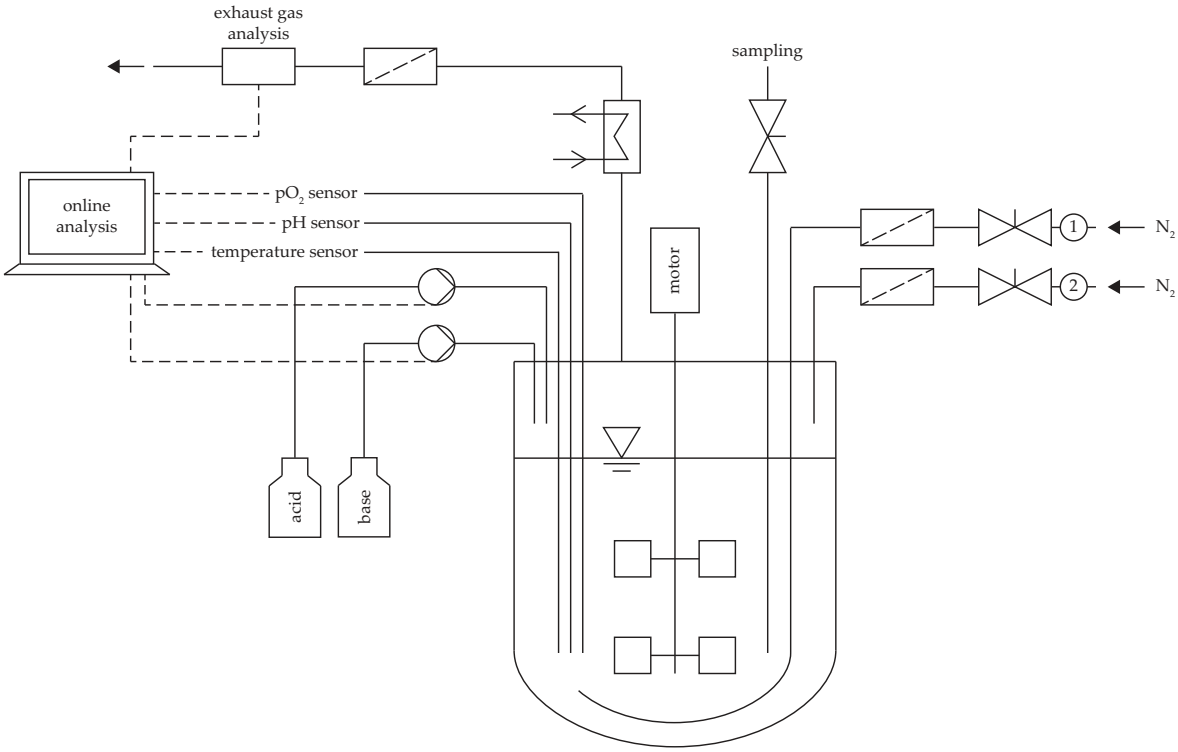

Fig. 3.2 Model of the employed fermentation system A 2.5 L benchtop bioreactor was used for anaerobic cultivation of *B. subtilis* DSM 10^T. The bioreactor was equipped with two Rushton turbines, a temperature sensor, pH and pO_2 electrodes, peristaltic pumps for pH control, an exhaust cooler and attached exhaust gas analysis, which were connected to a computer for online analysis. To adjust anaerobic conditions in the liquid medium and the head space of the bioreactor valve 1 was opened to allow a N_2 flow through the sparger. During fermentation valve 1 was closed and N_2 was allowed to flow through valve 2, enabling a constant gas flow through the head space.

measurement of CO_2 through the exhaust gas analysis, a constant N_2 gas flow through the headspace of the bioreactor with 0.1 Lpm (1.5 L headspace volume) was adjusted (Fig. 3.2: valve 2 open).

The fermentation process was started with 1.0 L of the described mineral salt medium and the additional volume of the inoculated seed culture (200 mL). Since the bioreactor cultivation was realized as a batch cultivation, no further medium components were added. During the cultivation pH, pO_2, CO_2 exhaust, temperature, stirrer speed and addition of acid and base were consistently monitored (Fig. 3.2). Samples were taken from the cultivation broth (4 mL) without allowing any air flow inside the bioreactor. All fermentations were performed as duplicates.

3.2.4 Analytical methods

3.2.4.1 Sampling and sample processing

By day samples were taken every three hours, whereas during nights the intervals were between five and seven hours. The sampling was designed to prevent air from entering the bioreactor system to guarantee anaerobic conditions inside. The offline analysis of the cultivation broth samples included the determination of the OD_{600} and the glucose, nitrate and Surfactin concentration of the supernatant. The concentration of glucose and nitrate was analyzed using a glucose assay kit (Cat. No. 10 716 251 035, R-Biopharm AG, Darmstadt, Germany) and a nitrate assay kit (1.09713.0001, Merck KGaA, Darmstadt, Germany). The concentrations were determined according to the manufacturer instructions, utilizing a spectrophotometric method (Ultrospec 2100 pro, General Electric Deutschland Holding GmbH, Frankfurt, Germany). The concentration of Surfactin was determined by analyzing the sample supernatant using HPLC (Chapter 2).

3.2.4.2 Data analysis

To enable the evaluation of the fermentation processes, several values were calculated to compare the different experiments. Using the results of CDW, glucose and Surfactin mass the values of $Y_{X/S}$ [g/g], $Y_{P/X}$ [g/g], μ [h^{-1}], $q_{Surfactin}$ [g/(g·h)], volumetric $q_{Surfactin}$ [g/(L·h)], were determined as described in Chapter 2 (Eq. 2.1 – Eq. 2.5). Additionally, the product yield $Y_{P/S}$ [g/g] was calculated dividing the maximal produced mass of Surfactin by the corresponding mass of consumed glucose during the entire fermentation process (Eq. 3.1)

$$Y_{P/S} = \frac{\Delta m_{Surfactin\,max}}{\Delta m_S} \qquad \textbf{Eq. 3.1}$$

3.3 RESULTS

3.3.1 Anaerobic growth

Altogether eight fermentations were performed testing four different glucose concentrations as duplicates. The graphs are shown in Fig. 3.3. All figures present the course of the CDW, CO_2, phosphoric acid, nitrate, glucose and Surfactin concentrations with time. The fermentation employing 2.5 g/L glucose endured 55 h. The process was terminated because the levels of CO_2 and CDW were drastically decreasing and the glucose was completely consumed. During the fermentation the CDW continually increased reaching 0.320 g/L at its maximum. The amount of CO_2 (no longer solved in the medium and therefore carried on within the N_2 stream in the headspace) increased simultaneously with the CDW. Meanwhile the glucose concentration consistently decreased until its depletion. In contrast, only 1 g/L nitrate was consumed during this fermentation (during fermentations with 10 g/L glucose about 5 g/L nitrate were used up). The concentration of Surfactin in the fermentation medium started to increase after 24 h of incubation. It reached its maximum at the end of the fermentation yielding 0.09 g/L Surfactin. The amount of added phosphoric acid to adjust the mediums pH level increased significantly after 34 h of cultivation. The demand for pH regulation is caused by *B. subtilis* anaerobic metabolism. In this pathway nitrate is used as terminal electron acceptor. The reduction of nitrate to nitrite via a nitrate reductase and the additional conversion of nitrite to ammonia via a nitrite reductase results in the production of an alkaline end product. In contrast to conventional aerobic cultivations of *B. subtilis*, where the addition of base marks cell growth, the addition of acid represents vivid cell growth under anaerobic conditions. The amount of dissolved oxygen was monitored throughout the fermentation processes but is not shown in the figures, because values were below detection limit.

3.3.2 Comparison of process parameters during anaerobic fermentation with different glucose concentrations

The fermentations of *B. subtilis* DSM 10^T with various glucose concentrations were analyzed regarding product yields and substrate utilization. Tab. 3.1 presents an overview of the most interesting process parameters, such as cultivation time, maximal CDW, maximal Surfactin concentration, maximal growth rate, product yields ($Y_{P/X}$, $Y_{P/S}$, $q_{Surfactin}$, vol. $q_{Surfactin}$) and substrate utilization ($Y_{X/S}$). All illustrated values are mean values of two fermentations. The duration of the fermentation depended on the starting glucose concentration. Fermentations with 2.5 g/L glucose lasted for 55 h, whereas fermentations with 10 g/L glucose averagely endured 161 h. Fermentations

with 5 g/L and 7.5 g/L glucose ran for approximately 100 h. The maximal CDW was reached during fermentations with 7.5 g/L glucose (0.856 g/L). In contrast, only 0.320 g/L CDW were yielded in fermentations with 2.5 g/L glucose. Fermentations with 5 g/L glucose or more reached at least 0.105 g/L Surfactin as maximal concentration. Fermentations with 2.5 g/L glucose earned 0.087 g/L Surfactin. The highest maximal growth rate μ_{max} was reached by fermentations with 7.5 g/L glucose

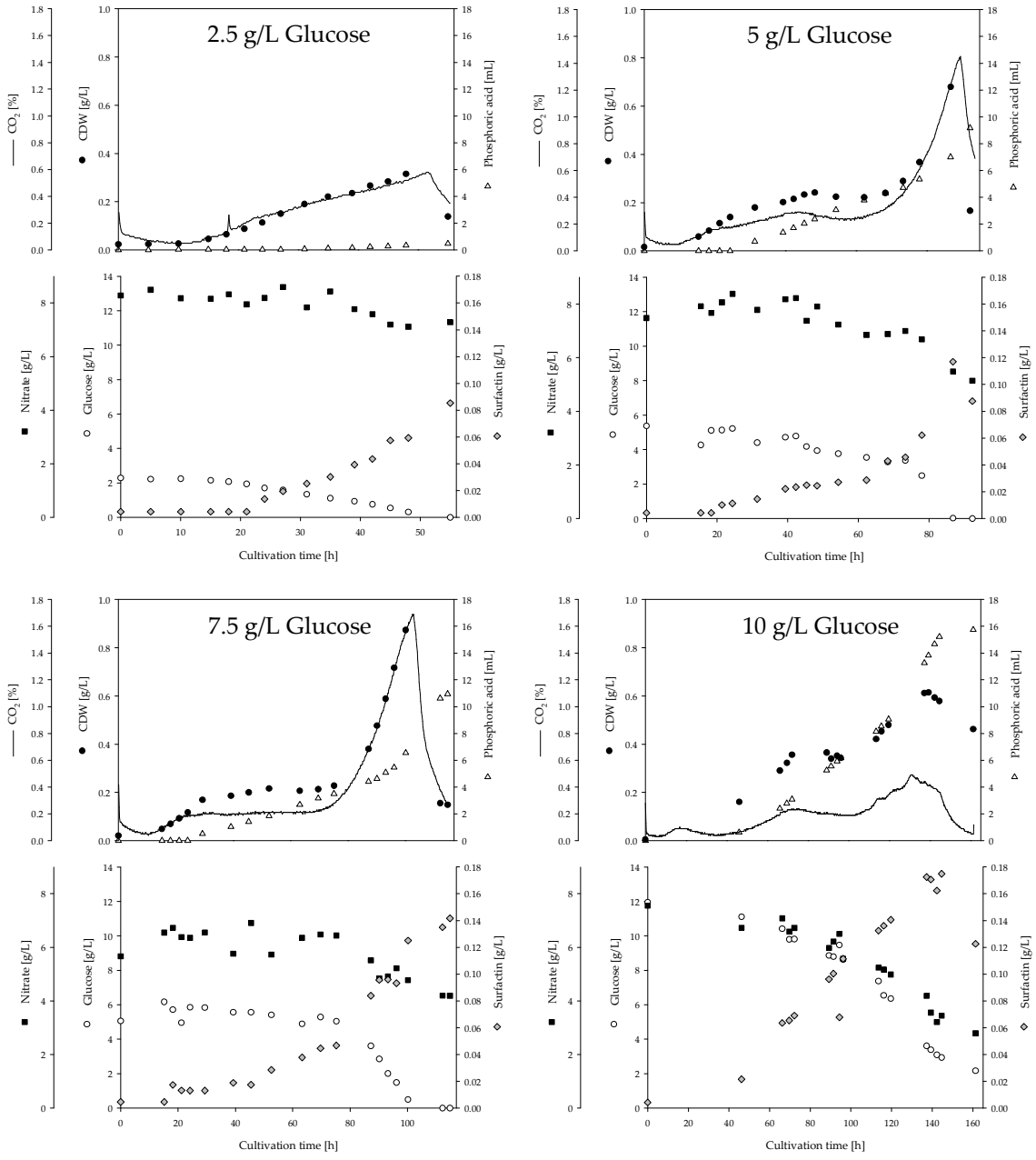

Fig. 3.3 Anaerobic fermentations of *Bacillus subtilis* DSM 10^T employing different glucose concentrations Time courses of CDW [g/L], CO_2 [%], phosphoric acid [mL], nitrate [g/L] and glucose [g/L] in comparison to produced Surfactin [g/L] during the fermentation process of *B. subtilis* DSM 10^T with 2.5 g/L, 5 g/L, 7.5 g/L and 10 g/L glucose. The values for CDW (*black dot*), CO_2 (*line*), phosphoric acid (*grey triangle*), nitrate (*square*), glucose (*white dot*) and Surfactin (*grey rhombus*) are given as examples of one fermentation.

(0.118 h^{-1}), whereas fermentations with 10 g/L glucose only reached maximal growth rates of 0.074 h^{-1}. The values of overall $Y_{P/X}$ differed widely between the fermentations with different glucose concentrations. Fermentations with 5 g/L or 7.5 g/L glucose earned product yields around 0.17 g/g. In contrast, fermentations with 2.5 g/L and 10 g/L reached $Y_{P/X}$ values of 0.278 g/g and 0.259 g/g, respectively. Overall values of $Y_{X/S}$ varied around 0.1 g/g except for fermentations with 10 g/L glucose. These cultivations led to $Y_{X/S}$ values of 0.049 g/g. The results for $Y_{P/S}$ show much higher values for fermentations with low glucose concentrations. Fermentations with 2.5 g/L glucose reached 0.033 g/g instead of 0.011 g/g with 10 g/L glucose in mineral salt medium. Additionally, cultivations using 2.5 g/L glucose yielded high specific production rates of 0.005 g/(g·h). Interestingly, all other fermentations reached only 0.002 g/(g·h). Volumetric specific production rates varied for all fermentations between 0.001 g/(L·h) and 0.002 g/(L·h).

Although cultivations with 2.5 g/L glucose reached only small amounts of CDW and Surfactin, these fermentations are comparably efficient. The cultivation time is much shorter and values for μ_{max}, $Y_{X/S}$ and vol. $q_{Surfactin}$ are comparatively high. Moreover, fermentations with 2.5 g/L glucose reached excellent values for $Y_{P/X}$, $Y_{P/S}$ and specific production rate $q_{Surfactin}$ emphasizing an outstanding conversion of substrate into product. Nevertheless, fermentations with 2.5 g/L glucose yielded only small amounts of Surfactin, due to the short cultivation time. As a consequence it would be interesting to test whether higher overall amounts of Surfactin can be reached by applying a fed-batch process.

Tab. 3.1 Summary of the process parameters during various fermentations Comparison of process parameters during anaerobic fermentation of *B. subtilis* DSM 10^T with different glucose concentrations.

Glucose concentration [g/L]	2.5	5	7.5	10
Cultivation time [h]	55	102	108	161
Max. CDW [g/L]	0.320	0.612	0.856	0.586
Max. $c_{Surfactin}$ [g/L]	0.087	0.105	0.150	0.158
μ_{max} [h^{-1}]	0.105	0.114	0.118	0.074
$Y_{P/X}$ [g/g]	0.278	0.169	0.179	0.259
$Y_{X/S}$ [g/g]	0.120	0.105	0.119	0.049
$Y_{P/S}$ [g/g]	0.033	0.018	0.022	0.011
Int. $q_{Surfactin}$ [g/(g·h)]	0.005	0.002	0.002	0.002
Int. vol. $q_{Surfactin}$ [g/(L·h)]	0.002	0.001	0.002	0.001

All values are mean values of two fermentations

Interestingly, on closer inspections Surfactin concentrations did increase simultaneously to rising initial glucose concentrations possibly due to longer cultivation times. Surprisingly, fermentations employing 10 g/L did also achieve an almost equal value for $Y_{P/X}$ in comparison to fermentations with 2.5 g/L glucose. But this positive result is misleading as overflow metabolism (as a result of the high initial glucose concentration) leads to low values of CDW, μ_{max} and $Y_{X/S}$. This means that the bacterial growth is already strongly restricted under the employment of 10 g/L glucose. As a result data for $Y_{P/S}$ and $q_{Surfactin}$ are comparably low. These findings support the usage of lower initial glucose concentrations for the anaerobic fermentation of *B. subtilis* DSM 10^T for the production of Surfactin to avoid overflow metabolism.

3.4 DISCUSSION

3.4.1 Comparison with other foam-free cultivation systems and aerobic fermentation with foam fractionation

The aim of the current study was to introduce a new approach for a foam-free biosurfactant production process. The results shown in Fig. 3.3 and Tab. 3.1 demonstrate a high efficiency for anaerobic cultivations with low glucose concentrations. Three other fermentation processes are described for the foam-free production of Surfactin. The solid state fermentation analyzed by Ohno et al. is incomparable with aqueous fermentations (Ohno et al. 1995), hence these data are not further discussed in comparison to the current study. However, Chtioui et al. established a rotating disc bioreactor allowing air flow only above the liquid phase. The growth of a *B. subtilis* ATCC 21332 biofilm led to the production of Surfactin and Fengycin (Chtioui et al. 2012). Chtioui et al. provided several results about product yields and substrate utilization. On basis of these findings further process parameters were calculated (see Tab. 3.2) to achieve a more complete comparison with the results of the current study. Coutte et al. introduced a novel membrane bioreactor for the production of biosurfactants (Coutte et al. 2010). The data of the *B. subtilis* ATCC 21332 cultivation were also used for the calculation of additional process parameters (Tab. 3.2). Therefore, Tab. 3.2 compares the data of three different foam-free fermentation processes for the production of Surfactin. To outline the differences between these methods and a traditional aerobic cultivation for the production of Surfactin, these results are additionally collated with a fermentation process applying foam fractionation (results from cultivations of *B. subtilis* DSM 10^T from Chapter 2).

The processes of Chtioui et al. and Coutte et al. each yielded above 0.2 g/L Surfactin. Whereas only 0.087 g/L Surfactin were reached in the current study (with 2.5 g/L glucose in mineral salt

medium). However, the fermentations of Chtioui et al. and Coutte et al. lasted comparatively longer (72 h instead of 55 h). Aerobic fermentations with *B. subtilis* using foam fractionation take much shorter time (30 h) and yield much higher concentrations in foam (3.995 g/L). Values for $Y_{X/S}$ differ only slightly between the foam-free processes (0.120 g/g – 0.189 g/g), but are relatively low compared to cultivations applying foam fractionation (0.268 g/g). The results for volumetric production rates are very similar, too, between the foam-free fermentations (0.002 g/(L·h) – 0.003 g/(L·h)). The foam fractionation fermentation reached a much higher value for vol. $q_{Surfactin}$ in comparison (0.018 g/(L·h)). The product yield in contrast to substrate utilization is given by the parameter $Y_{P/S}$. The values for cultivations of Chtioui et al. and Coutte et al. are both 0.013 g/g. The current study reached a much higher value of 0.033 g/g for $Y_{P/S}$. However, fermentations applying

Tab. 3.2 Summary of the process parameters of different foam-free processes Comparison of different foam-free Surfactin production processes regarding their process parameters and collation with a fermentation process applying foam fractionation.

	Chtioui**	Coutte***	This study	Chapter 2
Surfactin producer	*Bacillus subtilis* ATCC 21332	*Bacillus subtilis* ATCC 21332	*Bacillus subtilis* DSM 10^T	*Bacillus subtilis* DSM 10^T
Fermentation approach	Rotating discs	Membrane bioreactor	Anaerobic, no gas flow	Foam fractionation
Cultivation time [h]	72	72	55	30
Max. $c_{Surfactin}$ [g/L]	0.212*	0.242*	0.087	3.995 (foam)
$Y_{P/X}$ [g/g]	0.068	0.078*	0.278	0.192
$Y_{X/S}$ [g/g]	0.189	0.164*	0.120	0.268
$Y_{P/S}$ [g/g]	0.013	0.013	0.033	0.052
Int. $q_{Surfactin}$ [g/(g·h)]	0.001*	0.001*	0.005	0.006
Int. vol. $q_{Surfactin}$ [g/(L·h)]	0.003*	0.003*	0.002	0.018

** Chtioui et al. 2012, *** Coutte et al. 2010, * the values were calculated during the current study, using data of Chtioui et al. 2012 and Coutte et al. 2010 ($m_{Surfactin}$, CDW, cultivation time and cultivation volume)

foam fractionation still yield higher $Y_{P/S}$ values (0.052 g/g). The specific production rate $q_{Surfactin}$ is five-times higher in anaerobic fermentations using 2.5 g/L glucose (0.005 g/(g·h)) in comparison to other foam-free fermentations (0.001 g/(g·h)). Aerobic processes applying foam fractionation yield rather similar results for $q_{Surfactin}$ (0.006 g(g·h)). Most surprising are the results for $Y_{P/X}$. Fermentations of Chtioui et al. and Coutte et al. reached 0.068 g/g and 0.078 g/g, respectively. In contrast, anaerobic fermentations of the current study employing 2.5 g/L glucose yielded 0.278 g/g. These findings surpass even $Y_{P/X}$ values of aerobic fermentations employing foam fractionation (0.192 g/g).

Interestingly, the results of Chtioui et al. and Coutte et al. show very similar values for efficiency, product yields and substrate utilization although completely different fermentation approaches were applied. This similarity was revealed only after calculating some additional process parameters from the original data of these publications (Tab. 3.2). While rotating disc bioreactors or membrane reactors seem very attractive alternatives to common foam fractionation processes the presented data in Tab. 3.2 expose their low yields in comparison to the results of a classic foam fractionation process. The comparison of the results of Chtioui et al. and Coutte et al. with data of the current study displays a much higher effectiveness of the anaerobic fermentation approach. Although overall less Surfactin was produced, much more Surfactin was produced per CDW. This implies that the bacterial growth is probably lower compared to the rotating discs or membrane bioreactors, but single cells produce more Surfactin under completely anaerobic conditions. These findings explain the much higher values for $Y_{P/X}$, $Y_{P/S}$ and $q_{Surfactin}$. In comparison to an aerobic fermentation process with foam fractionation some process parameters are lower (e.g., vol. $q_{Surfactin}$ and $Y_{X/S}$), but values for $Y_{P/S}$ and $q_{Surfactin}$ are at the same level. Most important is the much higher value for $Y_{P/X}$ under anaerobic conditions. This implies a much better production of Surfactin per CDW not only in comparison to other foam-free processes, but even in comparison to aerobic foam fractionation processes.

3.5 CONCLUSION

The current study demonstrates a new approach to produce Surfactin without any foam formation. Moreover, anaerobic cultivation and foam-free biosurfactant production are combined in one process for the first time. The anaerobic production of Surfactin was shown before, but never analyzed for product yields and substrate utilization. The comparison of different fermentations with various glucose concentrations displayed great efficiency for processes applying low glucose concentrations. Furthermore, the confrontation with other foam-free processes revealed a much higher effectiveness of the anaerobic fermentation process of the current study.

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4. Enhancing the Surfactin production by Investigating the Medium Composition

This chapter is based on the following publication

Enhancement of Surfactin Yield by Improving the Medium Composition and Fermentation Process

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Authors' contribution to this publication

Judit Willenbacher designed all experiments, collected all data, created graphs and figures and drafted the manuscript.

Wladimir Yeremchuk conducted his Master thesis under the supervision of Judit Willenbacher and contributed data of several shake flask experiments and fed-batch fermentations.

Teresa Mohr conducted shake flask experiments to compare the Surfactin productivity of different *Bacillus* strains during employment of different media composition.

Christoph Syldatk and **Rudolf Hausmann** supervised the project and contributed to scientific discussion.

4.1 INTRODUCTION

Mostly the production of secondary metabolites in microorganisms is strongly influenced by the composition of the nutrients in their environment. Therefore, investigating a specific medium, with the aim to produce a certain molecule, usually becomes a highly discussed topic in its field of research. This also applies to the production of Surfactin with *B. subtilis*. Numerous suchlike studies, concerning the optimized medium or employing innovative carbon sources for the production of Surfactin, have been published (Peypoux et al. 1999). However, the typically employed medium still demands further optimization as it does not comply with industrial standards, due to environmentally harmful components and substrate waste.

The first applied medium for the production of Surfactin was introduced together with its discovery in 1968 by Arima et al. Nutrient broth, an undefined medium including peptone and beef or yeast extract, led to a yield of 0.1 g/L Surfactin (Arima et al. 1968). The shake flask experiments of Arima et al. using *B. subtilis* IAM 1213 were the benchmark for Surfactin production until the early 1980s. The Canadian group of Cooper et al. were the first to introduce an enhanced method for the production of Surfactin (Cooper et al. 1981), applying a foam fractionation process in a bioreactor but also introducing the first mineral salt medium for the production of Surfactin. The enhanced method led to a yield of 0.8 g/L Surfactin (Tab. 4.1). Although some research groups used the semisynthetic "Landy medium" (20 g/L glucose, 0.1 % yeast extract; Nakano et al. 1988; Sandrin et al. 1990) the presented mineral salt medium, shortly after its introduction referred to as the "Cooper medium", became the basis for most of the employed media to produce Surfactin until today (Horowitz et al. 1990; Qiu et al. 2014; Yakimov et al. 1995).

During the last 15 years, sustainable resources, especially for use in biotechnological processes, became more and more important. This is based on the aim to combine innovative, microbially produced products with sustainable industrial processes. From this perspective many research groups focused on alternative carbon sources for the production of Surfactin instead of using glucose, as suggested in the Cooper medium. Possible alternative substrates were rice straw and soybean flour, potato process effluent, cashew apple juice, rehydrated whey powder, cassava flour or peat hydrolysate (Cagri-Mehmetoglu et al. 2012; Davison et al. 2005; Freitas de Oliveira et al. 2013; Nitschke and Pastore 2004; Sheppard and Mulligan 1987; Zhu et al. 2013), all retaining low to moderate Surfactin yields (0.29 g/L – 3.0 g/L). However, most studies did not further analyze the improvement of Surfactin production by calculating essential process values like substrate utilization or specific and overall product yields. Other investigations focused on the productivity in respect of different sugars as carbon sources, proving glucose as the most effective (Abushady et al. 2005; Ghribi and Ellouze-Chaabouni 2011). On basis of these findings several studies

investigated the optimal glucose concentration in mineral salt medium for the production of Surfactin or Lichenysin with *B. subtilis* and *B. licheniformis*, respectively (Ghribi and Ellouze-Chaabouni 2011; Qiu et al. 2014; Sen 1997).

Sen (1997) analyzed the influence of glucose, NH_4NO_3, $FeSO_4$ and $MnSO_4$ in the Cooper medium on the production of Surfactin. The study was based on a 2^4 full factorial central composite experimental design, allowing the analysis of 4 different parameters at the same time. The final result revealed 36.5 g/L glucose, 4.5 g/L NH_4NO_3, $4 \cdot 10^{-3}$ g/L $FeSO_4$ and $27.5 \cdot 10^{-2}$ g/L $MnSO_4$ as the optimized medium composition. In contrast, the study of Ghribi and Ellouze-Chaabouni (2011) investigated seven different glucose concentrations from 15 g/L to 45 g/L in mineral salt medium and discovered 40 g/L glucose as the improved carbon source for Surfactin production with *B. subtilis* SPB1. The maximum yield was 0.72 g/L Surfactin. In contrast, a recent study (Qiu et al. 2014) investigated optimized glucose, NH_4NO_3 and buffer concentrations for the production of Lichenysin. Five different glucose concentrations were analyzed from 10 g/L to 50 g/L, where 30 g/L was identified as the improved glucose concentration.

Although several studies on enhanced glucose concentrations for the production of Surfactin have been conducted, there is no conclusive explanation why high glucose concentrations are required in mineral salt medium. In this sense further experiments were realized with the aim to optimize the Surfactin production. Further obstacles of the previously applied medium were the nitrogen source NH_4Cl, which leads to an unnecessary accumulation of NaCl during pH control with NaOH, and the chelating agent EDTA, which is detrimental to the environment (Oviedo and Rodríguez 2003). As a consequence, experiments were realized to analyze alternative substrates for the substitution of NH_4Cl and EDTA. In summary, the aim of this study was to enhance the Surfactin yield by changing the medium composition and to prove a general production enhancement independent from the applied Surfactin producer strain by changing the medium composition.

Tab. 4.1 Applied media and achieved Surfactin yields in early studies Arima et al. (1968) discovered Surfactin and introduced the first medium for its production. The later following study of Cooper et al. (1981) was the first publication approaching optimizations for the production of Surfactin regarding medium and culture conditions.

B. subtilis strain	Medium	Max. $c_{Surfactin}$	Study
IAM 1213	Nutrient broth	0.1 g/L	Arima et al. 1968
ATCC 21332	Cooper medium	0.8 g/L	Cooper et al. 1981

4.2 MATERIALS AND METHODS

4.2.1 Chemicals, microorganisms and strain maintenance

All applied chemicals and bacterial strains were purchased and handled as described in Chapter 2.

4.2.2 Culture conditions

4.2.2.1 Media

The mainly applied medium during the experiments of the current study was based on the Cooper medium from 1981 (Tab. 4.2: medium A). During research for Chapter 2 and 3 the Cooper medium was only slightly changed regarding the nitrogen source and buffer concentrations (Tab. 4.2: medium B). During research for the current study, next to substitutions for the nitrogen source and chelating agent, it was investigated, whether the glucose concentration could drastically be decreased.

Stock solutions of the various medium components were prepared to conduct different shake flask experiments using various components or concentrations: 1.11 M glucose, 1 M NH_4Cl, 0.5 M $(NH_4)_2SO_4$, 0.3 M KH_2PO_4, 0.4 M Na_2HPO_4, 0.008 M $MgSO_4$, 0.004 M Na_2EDTA, 0.008 M Na_3citrate, 0.007 M $CaCl_2$, 0.004 M $FeSO_4$ and 0.001 M $MnSO_4$. The stock solutions were used to assembly different medium combinations. The following final concentrations were adjusted every time: 0.03 M KH_2PO_4, 0.04 Na_2HPO_4, 0.0008 M $MgSO_4$, 0.007 mM $CaCl_2$, 0.004 mM $FeSO_4$, 0.001 mM $MnSO_4$. This implies that the buffer and trace element composition basically did not change throughout the experiments. In contrast, final concentrations of glucose varied widely (0 g/L, 2 g/L, 4 g/L, 6 g/L, 8 g/L, 10 g/L, 12 g/L, 15 g/L, 20 g/l, 30 g/L, 40 g/L, 50 g/L). Additionally, the substitution of NH_4Cl (0.1 M) by $(NH_4)_2SO_4$ (0.05 M) and the replacement of Na_2EDTA (0.004 mM) with Na_3citrate (0.008 mM) was performed to investigate a novel nitrogen source and chelating agent, respectively.

Fermentations were carried out with the final version of the optimized medium (Tab. 4.2: medium C). Stock solutions for the preparation of the bioreactor medium were prepared as described in Chapter 2, except for the concentration of the glucose stock solution which was about 48 g/L in 250 mL and the usage of 0.05 M $(NH_4)_2SO_4$ and 0.008 mM Na_3citrate. In contrast, to medium B (Tab. 4.2), medium C was limited by the amount of glucose. In order to cultivate for a similar amount of time, compared to cultivations in Chapter 2 (approximately 30 h), glucose was additionally fed to extend the cultivation time (from 20.83 h to 34 h). Therefore, a stock solution of 450 g/L glucose was prepared (23 mL) for inoculation after complete glucose consumption.

Tab. 4.2 Different media for the production of Surfactin with *Bacillus subtilis* The original medium after Cooper et al. (1981) is shown in the first column (A), applying 40 g/L glucose and NH_4NO_3 as source of nitrogen. A slightly changed version of this medium was used throughout most experiments of this thesis (B). Hereby, the nitrogen source NH_4NO_3 was replaced by NH_4Cl. The medium was later further optimized to yield more Surfactin (C), employing less glucose (8 g/L), $(NH_4)_2SO_4$ and Na_3citrate.

	Cooper medium A	Modified after Cooper B	Further optimized C
C	40 g/L glucose	40 g/L glucose	8 g/L glucose
N	50 mM NH_4NO_3	100 mM NH_4Cl	50 mM $(NH_4)_2SO_4$
Mg	0.8 mM $MgSO_4$	0.8 mM $MgSO_4$	0.8 mM $MgSO_4$
Buffer	30 mM KH_2PO_4	30 mM KH_2PO_4	30 mM KH_2PO_4
	40 mM Na_2HPO_4	40 mM Na_2HPO_4	40 mM Na_2HPO_4
Trace elements	0.004 mM Na_2EDTA	0.004 mM Na_2EDTA	0.008 mM Na_3citrate
	0.007mM $CaCl_2$	0.007mM $CaCl_2$	0.007mM $CaCl_2$
	0.004 mM $FeSO_4$	0.004 mM $FeSO_4$	0.004 mM $FeSO_4$
	0.001 mM $MnSO_4$	0.001 mM $MnSO_4$	0.001 mM $MnSO_4$

4.2.2.2 Preparation of inoculum cultures

Inoculum cultures for shake flask experiments were conducted in Lysogeny Broth (Bertani 1951), incubating 20 µL culture of a glycerol stock solution. The precultures were incubated for 24 h at 30 °C and 120 rpm in a shake incubator chamber (Multitron II, HT Infors, Bottmingen, Switzerland). The 20 mL or 100 mL mineral salt medium for main culture (in 100 mL and 500 mL baffled shake flasks, respectively) were inoculated with a resulting OD_{600} between 0.05 and 0.1.

Two consecutive precultures were prepared for cultivations in benchtop bioreactors. The first preculture was inoculated 48 h before starting the bioreactor cultivation. The procedure was identical to precultures for shake flask cultivations. The second preculture was inoculated from the first preculture 24 h before starting the bioreactor cultivation. The 500 mL baffled shake flasks, containing 100 mL of medium C, were inoculated to a resulting OD_{600} of 0.1. The second preculture

was incubated for 24 h at 30 °C and 120 rpm. The benchtop bioreactors were inoculated from the second preculture to a resulting OD_{600} of 0.1.

4.2.2.3 Shake flask cultivations

Cultivations in shake flasks were conducted to investigate different medium compositions and to analyze the Surfactin production of different *Bacillus* strains in medium B and medium C (Tab. 4.2). All shake flask experiments were performed as duplicates. In some cases the flasks were inoculated in a time-displaced way to collect samples of all cultivation phases. The cultivation duration varied between 30 h and 50 h. Samples were taken by day every 2 h to 3 h. The cultivation was stopped if the measured OD_{600} decreased after a significant growth phase.

4.2.2.4 Cultivation in a 2.5 L benchtop bioreactor

Bioreactor cultivations were carried out as described in Chapter 2, using the same benchtop bioreactor system with pH, pO_2 and temperature control (Minifors, HT Infors, Bottmingen, Switzerland). The stirrer speed was adjusted to 300 rpm at the beginning of cultivation and was controlled by an implemented sequence monitoring the level of dissolved oxygen (the stirrer speed was regulated to yield a pO_2 between 10 % and 20 %, see appendix; IRIS, HT Infors, Bottmingen, Switzerland). Since foam fractionation was applied, the foam was channeled through the exhaust cooler and collected in interchangeable bags. In contrast to Chapter 2, fermentations were conducted as fed-batch cultivations. After the depletion of glucose, the level of dissolved oxygen dramatically increased (because the cells suddenly experienced starvation). At this point a glucose stock solution was injected to increase the level of glucose in the bioreactor to the starting glucose concentration of approximately 8 g/L. As a result, the cultivation time was extended for 13.17 h. Samples were taken every 2 h accompanied by the exchange of the foam trap against a new collecting bag (applies only if foam was already leaving the bioreactor). All fermentations were performed as duplicates.

4.2.3 Analytical methods

4.2.3.1 Sampling and sample processing

The samples taken during shake flask experiments were analyzed regarding OD_{600} (later on converted into CDW by division with the correlation factor 3) and Surfactin concentration. The samples taken from the bioreactor were as well analyzed in respect of their OD_{600} (later indicated as CDW) and Surfactin concentration, but furthermore for their glucose concentration. Samples of the foam traps were also analyzed regarding their CDW, Surfactin and glucose concentration. The

employed methods to quantify OD_{600}, glucose, and Surfactin concentration were equivalent to the methods described in Chapter 2.

4.2.3.2 Data analysis

An analysis of different process parameters allowed the evaluation of the applied fermentation process (fed-batch, Tab. 4.2: medium C) with earlier findings from cultivations in the original medium (batch, Tab. 4.2: medium B). Using the results of CDW, mass of glucose and mass of Surfactin, the values of $Y_{X/S}$ [g/g], $Y_{P/X}$ [g/g], $Y_{P/S}$ [g/g], μ [h^{-1}], $q_{Surfactin}$ [g/(g·h)], volumetric $q_{Surfactin}$ [g/(L·h)], Surfactin recovery [%], Surfactin enrichment and bacterial enrichment were determined. The values were calculated as described in Chapter 2 and 3.

4.3 RESULTS

4.3.1 Improvement of the Copper medium to enhance Surfactin yields

The original Cooper medium (Cooper et al. 1981; Tab. 4.2: medium A) presents glucose as carbon source and NH_4NO_3 as nitrogen source. The buffer system is composed of KH_2PO_4 and Na_2HPO_4. Aside from that, the addition of $MgSO_4$ serves as source for sulfur and magnesium. Additionally, the trace elements Fe, Ca and Mn are added together with the chelating agent EDTA. Early experiments contributing to the current thesis were conducted with a slightly modified version of the Cooper medium (Tab. 4.2: medium B). The nitrogen source 50 mM NH_4NO_3 was exchanged against 1 M NH_4Cl mainly because *Bacillus* prefers NH_4 over NO_3 as nitrogen source. Furthermore, the employment of NH_4Cl solely required the analysis of one nitrogen compound. Additionally, the original 70 mM buffer system was replaced by a 10 mM buffer system when *B. subtilis* was cultivated in a benchtop bioreactor allowing pH control. In this way it was possible to monitor the bacterial growth by the online acquisition of NaOH addition. The original glucose concentration was not altered, since earlier studies suggested 40 g/L as the optimal glucose concentration (Ghribi and Ellouze-Chaabouni 2011; Sen 1997).

Although repeatedly reliable results were obtained while employing the slightly changed Cooper medium (Tab. 4.2: medium B) this medium composition was further investigated to avoid unnecessary environmentally harmful components and substrate waste. As a consequence the chelating agent EDTA was replaced by citrate, which is a much more environmentally friendly and more favorable chelating agent. Furthermore, the nitrogen source NH_4Cl was substituted by $(NH_4)_2SO_4$, which prevents the accumulation of NaCl (caused by the addition of NaOH for pH control) inside the bioreactor and increases the amount of sulfur (which is comparably low in the

original Cooper medium). Both substitutions did not affect bacterial growth or Surfactin productivity.

The first shake flask cultivations revealed a higher Surfactin concentration at cultivations employing lower glucose concentrations. Subsequently, a shake flask experiment applying 0 g/L, 2 g/L, 4 g/L, 6 g/L, 8 g/L, 10 g/L, 12 g/L and 15 g/L glucose was conducted encircling the improved glucose concentration for the production of Surfactin with *B. subtilis* DSM 10^T. The results of CDW and Surfactin yields are shown in Fig. 4.1. The obtained CDW during the cultivation was significantly lower in shake flasks employing low glucose concentrations and reached its maximum when 12 g/L glucose was employed (4.6 g/L CDW, Fig. 4.1). In contrast, low glucose concentrations yielded high Surfactin concentrations with maximal values of approximately 0.8 g/L

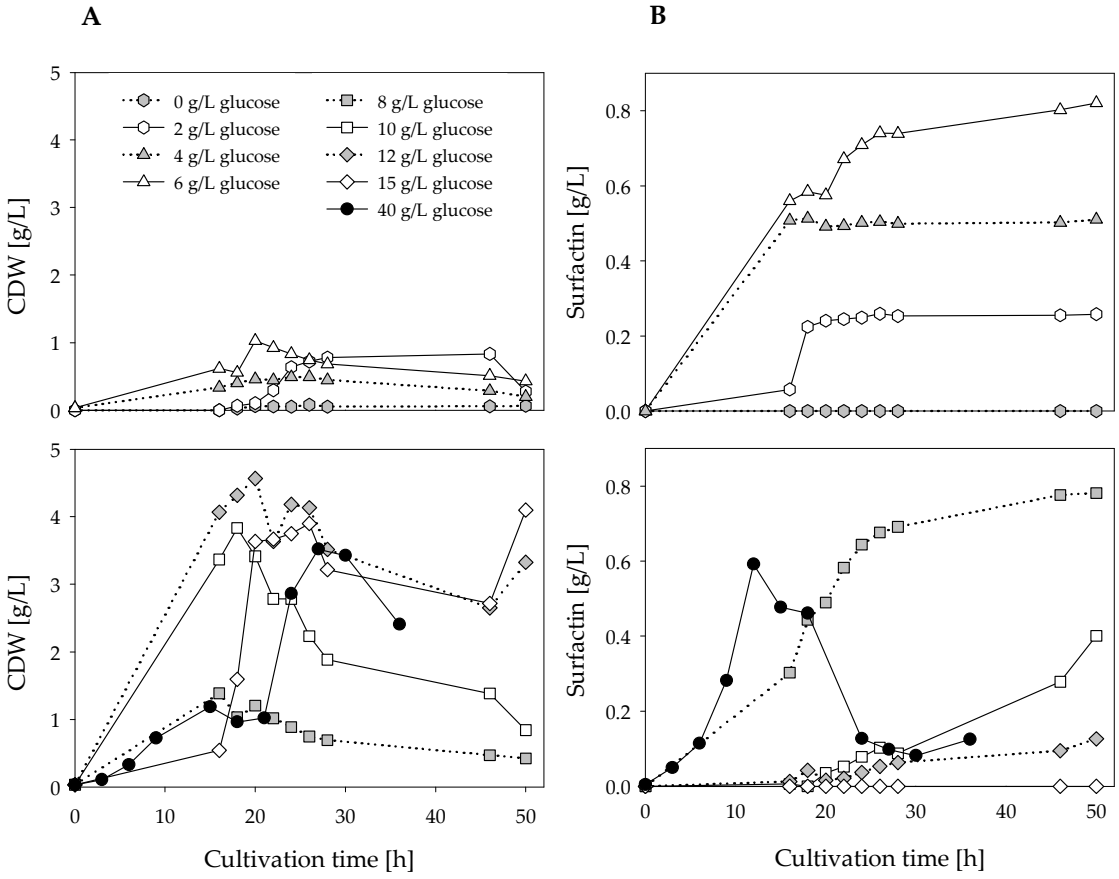

Fig. 4.1 Time course of CDW and Surfactin concentrations of shake flask cultivations of *B. subtilis* DSM 10^T applying various glucose concentrations Cultivations in shake flasks were conducted employing 0 g/L (*grey hexagon*), 2 g/L (*white hexagon*), 4 g/L (*grey triangle*), 6 g/L (*white triangle*), 8 g/L (*grey square*), 10 g/L (*white square*), 12 g/L (*grey rhombus*) and 15 g/L (*white rhombus*) glucose. Data from earlier experiments employing the original 40 g/L are shown as *black dots*. The achieved CDW [g/L] is illustrated in (**A)**, the reached Surfactin concentration [g/L] is displayed in (**B)**. Shake flask experiments were conducted in duplicates and time-displaced flasks to illustrate a continuous course of CDW and Surfactin concentration.

Surfactin during shake flask experiments with 6 g/L and 8 g/L glucose (Fig. 4.1). In contrast, standard cultivations employing 40 g/L glucose reached 3.5 g/L CDW and 0.6 g/L Surfactin (data from a different experiment included in Fig. 4.1). The results of this experiment elucidated a higher productivity for media containing approximately 8 g/L glucose as Surfactin concentrations during cultivations with lower or higher glucose concentrations were less effective.

Fig. 4.2 shows the time course of simultaneous cultivations of *B. subtilis* DSM 10^T in the slightly changed Cooper medium applied in Chapter 2 and 3 (Tab. 4.2: medium B) and in the further optimized medium (Tab. 4.2: medium C). The data reveal a maximal CDW of 2.4 g/L in medium B and 2.2 g/L in medium C. Bacterial growth terminated after 15 h of cultivation in medium C as the further optimized medium is glucose limited. Nevertheless, a significantly higher concentration of Surfactin was produced in the further optimized medium C, yielding a maximum of 1.1 g/L Surfactin. In contrast, cultivations in medium B reached 0.7 g/L as maximal Surfactin concentration.

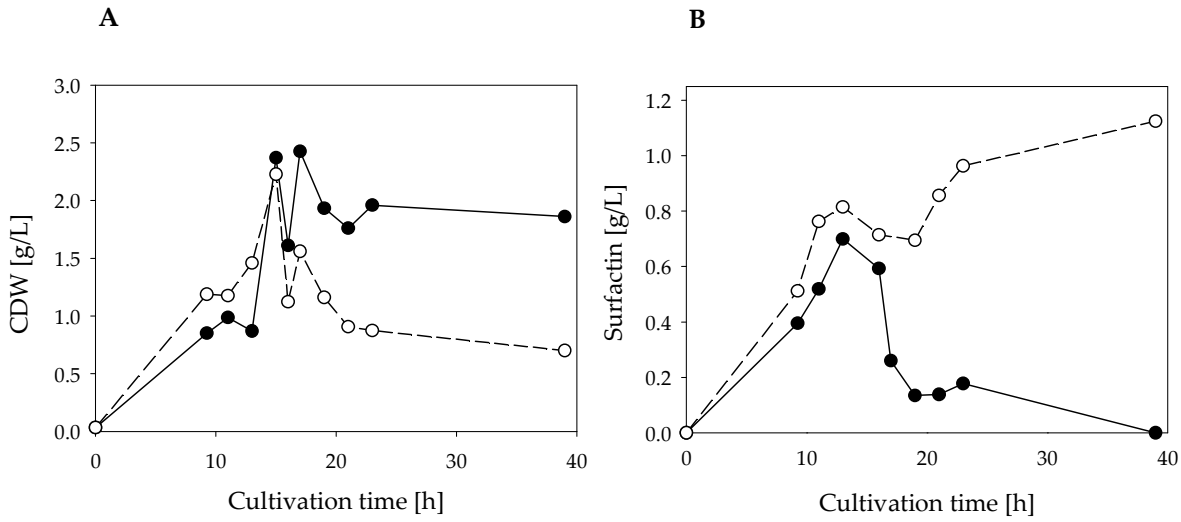

Fig. 4.2 Time course of CDW and Surfactin concentrations of *B. subtilis* DSM 10^T shake flask cultivations in medium B and further optimized medium C The achieved CDW [g/L] is shown in (**A)**, whereas resulting Surfactin concentrations [g/L] are illustrated in (**B)**. The results of cultivation in medium B are given as *black dots*. Data from cultivations in medium C are presented as *white dots*. The cultivations were conducted as duplicates and in time-displaced flasks to illustrate a continuous course of growth and Surfactin production.

4.3.2 Does the optimized Cooper medium enhance Surfactin production in general?

To investigate whether the improved glucose concentration in the enhanced Cooper medium (Tab. 4.2: medium C) depends on the employed *Bacillus* strain DSM 10^T further shake flask experiments were conducted to analyze the Surfactin productivity of several other *Bacillus* strains in the optimized medium C.

The *B. subtilis* strains DSM 10^T, DSM 28227 (LM43a50°C), ATCC 21332, DSM 3256, DSM 1090 and DSM 3258 were analyzed regarding their Surfactin production during cultivation in medium B and medium C (Tab. 4.2). All shake flask experiments were conducted as duplicates. Additionally, two different time-displaced shake flasks were inoculated with the analyzed strains to obtain continuous values during 30 h of incubation (values corresponding to incubation times from 0 h to 6 h and 22 h to 30 h belonged to the same shake flaks, whereas values between 13 h and 19 h belonged to another shake flask). This resulted in the analysis of eight different shake flasks per strain (all shake flasks as duplicates, time-displaced inoculation, two different media). The CDW and Surfactin concentrations of these cultivations are shown in Fig. 4.3. The findings for the production of CDW corresponded to earlier experiments. Some strains grew to much higher CDW values in comparison to others (DSM 28227 and DSM 3256, both around 14 g/L) but all strains (with exception of DSM 3258, which shows a pelleted growth behavior and could therefore not be analyzed regarding CDW in shake flask cultivations) displayed the typical growth behavior in medium B and medium C. The CDW increased during early cultivation in medium C and decreased after the depletion of glucose. The CDW increased until approximately 22 h of cultivation in medium B and stagnated afterwards. Most interestingly, the production of Surfactin seems to be enhanced in all cultivations employing the optimized medium C. Except for the results of DSM 3258, where the Surfactin production increased during cultivation in medium B. However, Surfactin production was generally very low (0.05 - 0.1 g/L) during the cultivations of DSM 3258 and the shown error bars indicate a rather similar production rate during cultivation in medium B and C. In summary, the concentration of Surfactin was doubled to tripled during cultivation of DSM 10^T, DSM 28227, ATCC 21332, DSM 3256 and DSM 1090 employing the enhanced medium C in comparison to the previously applied medium B (sole exception DSM 3258), which proves a general improvement of Surfactin production during cultivation in medium C independent from the applied *Bacillus* strain.

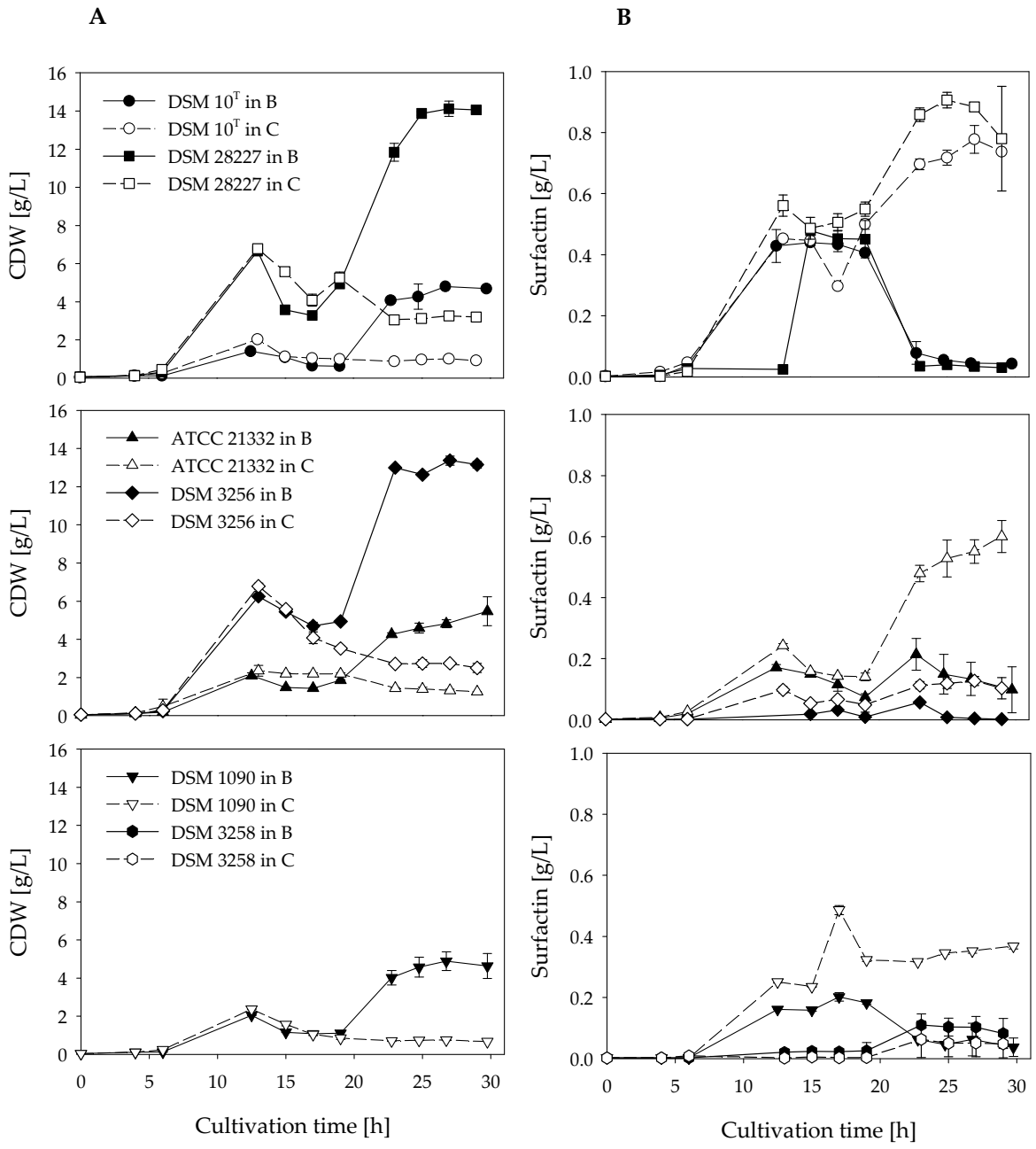

Fig. 4.3 Time course of CDW and Surfactin concentration of various *Bacillus* strains cultivated in medium B and further optimized medium C In summary, the six different Surfactin producers DSM 10^T (*dot*), DSM 28227 (LM43a50°C, *square*), ATCC 21332 (*regular triangle*), DSM 3256 (*rhombus*), DSM 1090 (*triangle upside down*) and DSM 3258 (*hexagon*) were cultivated in medium B (illustrated in *black*) and further optimized medium C (displayed in *white*). The results of achieved CDW [g/L] are shown in (**A)**, whereas Surfactin concentrations [g/L] are illustrated in (**B)**. All experiments were conducted as duplicates and in time-displaced shake flasks to present continuous courses of CDW and Surfactin concentration.

4.4 DISCUSSION

4.4.1 Comparison with other studies

The first systematic approach to analyze an improved composition of the Cooper medium was conducted by Sen in 1997. The study implemented a 2^4 full factorial central composite design, testing four independent medium components, which is an experimental design analyzing the influence of various factors on an experiment without actually testing every intermediate step. Next to glucose NH_4NO_3, $FeSO_4$, and $MnSO_4$ concentrations were altered and analyzed. The enhanced medium composition was found to be 36.5 g/L glucose, 4.5 g/L NH_4NO_3, $4 \cdot 10^{-3}$ g/L $FeSO_4$ and $27.5 \cdot 10^{-2}$ g/L $MnSO_4$, which is - despite the trace elements Fe and Mn - solely a slight change in comparison to the original Cooper medium (Tab. 4.2: medium A). The follow-up study on medium composition by Ghribi and Ellouze-Chaabouni (2011) was based on a mineral salt medium, but not the Cooper medium. In summary, seven different glucose concentrations were tested from 15 g/L to 45 g/L. The most suited glucose concentration was found to be 40 g/L (with 6 g/L urea as nitrogen source and addition of several mineral elements like: Mg, Fe, Ca, Zn, Mn, Cu, and Br). The most recent study including an analysis of medium components was published in 2014 by Qiu et al., investigating improved Lichenysin yields in the Cooper medium. Beside glucose NH_4NO_3 concentrations, the ratio of the Na_2HPO_4 and KH_2PO_4 buffer system was tested. The enhanced medium was composed of 30 g/L glucose, 5 g/L NH_4NO_3 and a ratio of 80 mM Na_2HPO_4 and 60 mM KH_2PO_4, yielding 2.15 g/L Lichenysin. In contrast to the results of the current chapter, these studies determined 30 g/L to 40 g/L as the improved glucose concentration for the production of Surfactin or Lichenysin in mineral salt medium. As it was shown by the results of paragraph 4.3.2 this is probably not caused by the usage of different *Bacillus* strains as nearly all tested Surfactin producer enhanced Surfactin productivity in the optimized medium C with 8 g/L glucose.

As described earlier, Sen (1997) applied a design of experiment approach to analyze the influence of the components concentrations. In this fashion, solely three different concentrations were tested without altering the other three variables (NH_4NO_3, $FeSO_4$ and $MnSO_4$). In the case of glucose, three shake flask experiments were conducted with 0 g/L, 40 g/L and 80 g/L glucose without changing the other medium components. This is a normal and common strategy when a design of experiment is approached, but covers a rather unrealistic range of glucose concentrations. Consequentially, shake flask cultivations containing no carbon source will not yield any Surfactin as cells are not able to grow properly. Furthermore, cultivations employing 80 g/L glucose should also be expected to yield low Surfactin concentrations as excess glucose concentrations negatively affect the growth behavior of *B. subtilis* (Dauner et al. 2001).

Tab. 4.3 Comparison of different approaches to systematically enhance the Surfactin yield by medium optimization The studies of Sen (1997), Ghribi and Ellouze-Chaabouni (2011) and Qiu et al. (2014) attended to find the enhanced medium composition for the production of Surfactin and Lichenysin, respectively. The various approaches regarding glucose concentration are listed in contrast to each other.

Bacillus strain	Glucose conc.	Product yield	Study
B. subtilis DSM 3256	0 g/L	$CMC^{-1} = 2$	Sen, 1997
	40 g/L	$CMC^{-1} = 35$	
	80 g/L	$CMC^{-1} = 5$	
B. subtilis SPB1	15 g/L	0.42 g/L	Ghribi and Ellouze-Chaabouni, 2011
	20 g/L	0.48 g/L	
	25 g/L	0.54 g/L	
	30 g/L	0.60 g/L	
	35 g/L	0.66 g/L	
	40 g/L	0.72 g/L	
	45 g/L	0.69 g/L	
B. licheniformis WXO2-Psrflch	10 g/L	0.60 g/L	Qiu et al., 2014
	20 g/L	0.85 g/L	
	30 g/L	1.25 g/L	
	40 g/L	0.73 g/L	
	50 g/L	0.44 g/L	

The consequence is a much higher yield of Surfactin in cultivations with 40 g/L glucose (Tab. 4.3). Another important reference point is the applied method for the analysis of Surfactin yield. Sen (1997) determined the Surfactin yield via an indirect method measuring the surface tension. The relative Surfactin concentration was defined by serially diluting the culture broth until the critical micelle concentration (CMC) was reached. The number of dilutions which was necessary to start rising the surface tension was designated as CMC^{-1} (Tab. 4.3). Such indirect methods can be used to achieve a certain indication, but do not give specific information about the actual amount of product as the surface tension could be lowered by several other surfactants produced by *Bacillus* (e.g. Iturin or Fengycin). The study of Ghribi and Ellouze-Chaabouni (2011) determined the Surfactin concentrations by approaching an indirect method as well. There, the precipitated and extracted crude product was weighed. This study identified 40 g/L as the most suitable glucose concentration as it yielded 0.72 g/L Surfactin (Tab. 4.3). The determination of the Surfactin yield in

this fashion is rather difficult, as shake flask experiments do not supply much product and during precipitation and extraction with chloroform and methanol lots of product is lost. Moreover, the precipitation with HCl (until reaching pH = 2.0) and following extraction with organic solvents does not necessarily lead to pure product. In contrast, Qiu et al. (2014) applied HPLC to quantify the amount of produced Lichenysin (Tab. 4.3: 1.25 g/L Lichenysin with 30 g/L glucose in mineral salt medium). HPLC is the most accurate detection method, as the product is specifically identified by several peaks at characteristic retention times. Inevitable here is the application of a pure standard (e.g. Surfactin from Sigma-Aldrich). Qiu et al. (2014) were not able to purchase a Lichenysin standard and therefore used Surfactin as a reference. Both Lichenysin and Surfactin produce various isoforms, since different amino acids and fatty acids can be incorporated. It is therefore not very accurate to use Surfactin as HPLC standard for the detection of Lichenysin. However, Lichenysin was not commercially available at the time, hence Qiu et al. (2014) determined the Lichenysin concentration as exactly as possible. Qiu et al. (2014) determined the product yield in the most accurate way in comparison to the other consulted studies. Nevertheless, the study identified 30 g/L glucose as the improved concentration for maximal product yield. The discrepancy between the results of the current study (8 g/L glucose as optimized concentration in the medium) and the study of Qiu et al. (2014; 30 g/L glucose) might be explained by the usage of two different strains (*Bacillus subtilis* and *Bacillus licheniformis*) and could also be referred to different regulation and expression of the *srfA* and *lchA* operons (the upstream region of the two operons seem to be similar but not identical, Sen 2010).

The results of Sen (1997), Ghribi and Ellouze-Chaabouni (2011) and Qiu et al. (2014) conflict the results of this study, which suggest 8 g/L glucose as enhanced concentration. The discrepancy could be explained by the fashion in which the experiments were conducted. All of the above studies focused either on glucose concentrations between 10 g/L and 50 g/L or used unsuitable low or high glucose concentrations (Sen, 1997). None of these studies incorporated experiments with 8 g/L glucose. Another reason could be the manner in which samples were taken. All of the discussed studies analyzed the Surfactin or Lichenysin concentration at the end of cultivation. However, the product yield fluctuates greatly during the cultivation, which means maximal concentrations may have been missed. Additionally, detection methods (especially in the studies of Sen and Ghribi and Ellouze-Chaabouni) for the analysis of Surfactin lack specificity. Multiple applications of the optimized Cooper medium (Tab. 4.2: medium C) during cultivations of *B. subtilis* DSM 10^{T} and additional cultivations of further Surfactin producers in the current study proved a consistent enhancement of Surfactin yield. Therefore the following experiments were conducted in the improved medium C.

4.4.2 Application of the optimized Cooper medium during cultivation in a 2.5 L benchtop bioreactor with integrated foam fractionation

The shake flask cultivations of *B. subtilis* DSM 10^T using the optimized medium (Tab. 4.2: medium C) reached significantly higher values for the production of Surfactin compared to results employing the former medium (Tab. 4.2: medium B). The results of the bioreactor cultivation of *B. subtilis* DSM 10^T applying foam fractionation in medium B (presented in Chapter 2) were already promising concerning Surfactin recovery, enrichment and total mass of Surfactin. After the final optimization of the medium another bioreactor cultivation of *B. subtilis* DSM 10^T with integrated foam fractionation was conducted to compare production rates of Surfactin with results obtained from cultivations presented in Chapter 2.

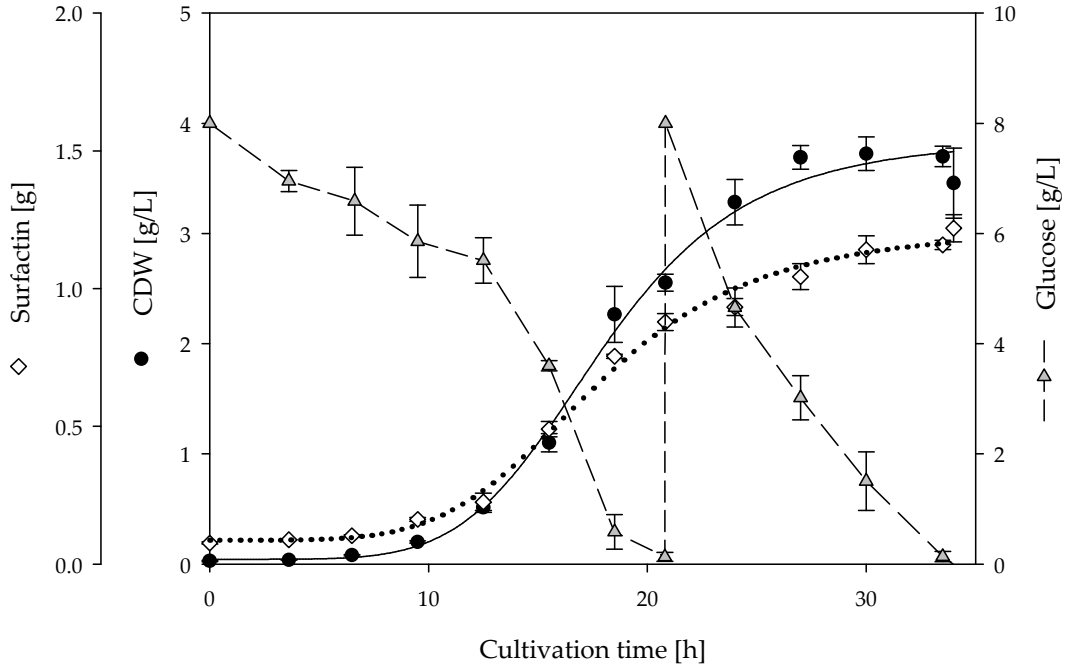

Fig. 4.4 Time course of the fed-batch fermentation of *B. subtilis* DSM 10^T employing medium C
The time courses of CDW (*black dot*, [g/L]), Surfactin (*white rhombus*, [g]) and glucose (*grey triangle*, [g/L]) are displayed as mean values of two fermentations. Glucose was added after its complete consumption (23 mL of 450 g/L glucose, 20.83 h after inoculation). The *dotted* and *solid lines* represent logistic fits of CDW and mass of Surfactin based on Eq. 2.9 (Chapter 2).

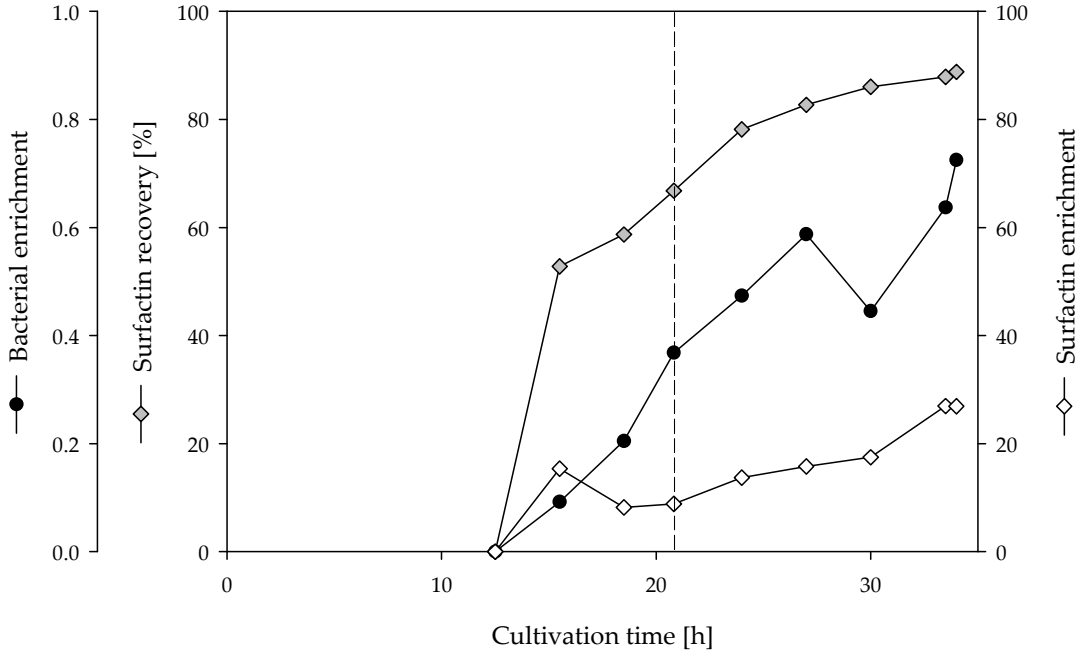

Fig. 4.5 Time course of foam traps during fed-batch fermentation of *B. subtilis* DSM 10^T employing medium C The values of bacterial enrichment (*black dots*), Surfactin recovery (*grey rhombus*) and Surfactin enrichment (*white rhombus*) are displayed as exemplary results of one fermentation. The addition of glucose is indicated by a *dashed line* after 20.83 h of cultivation.

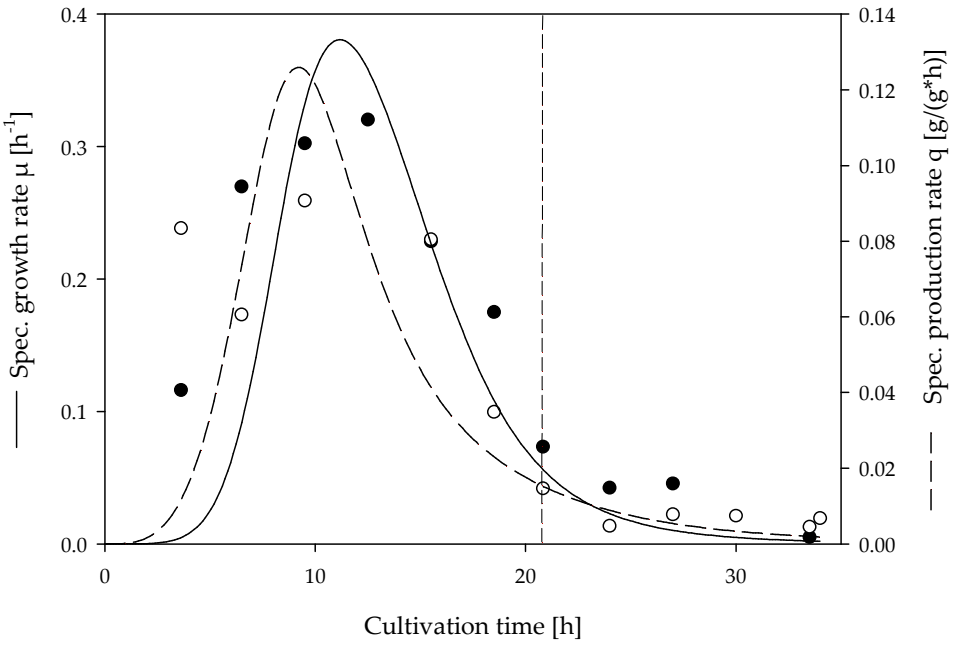

Fig. 4.6 Time course of specific growth rate μ and specific production rate $q_{Surfactin}$ during fed-batch fermentation of *B. subtilis* DSM 10^T in medium C The values for μ and $q_{Surfactin}$ have been calculated in a differential manner (*dots*) and by using the fitted data of CDW and mass of Surfactin (*lines*). The results for μ are given as *black dots* and *sold line*, whereas the results for $q_{Surfactin}$ are presented as *white dots* and *dashed line*. The addition of glucose is indicated by the vertical *dashed line* after 20.83 h of cultivation.

The bioreactor cultivations of Chapter 2 were performed as batch cultivations. As the optimized medium C is glucose limited batch cultivations would stop much earlier compared to cultivations with medium B. Therefore, a fed-batch cultivation was applied for the cultivation of *B. subtilis* DSM 10^T in the optimized medium C. The fermentation plot is shown in Fig. 4.4. Fermentations endured for 34 h and yielded a maximal CDW of 3.8 g/L. The decrease of glucose is visible until its complete depletion after 20.83 h of cultivation. Glucose was added to the culture broth to continue the cultivation (23 mL 450 g/L glucose). The amount of glucose inside the bioreactor decreased again until its consumption after 34 h of cultivation. The increase of CDW and Surfactin followed a logistic growth behavior during cultivations, yielding 1.22 g Surfactin. The analysis of the foam traps is shown in Fig. 4.5 (example of one bioreactor cultivation). The Surfactin recovery increased during cultivations from 52 % to 88 %, whereas Surfactin (15 to 27) and bacterial enrichment (0.1 to 0.7) remained nearly constant. The specific growth rate μ and specific production rate $q_{Surfactin}$ are presented in Fig. 4.6. The growth rate increased for 11 h of cultivation (0.38 h^{-1}) and decreased afterwards. A similar behavior is observed for the production rate, reaching its maximum after 9 h of cultivation (0.12 g/(g·h)). However, the data shown in Chapter 2 describe a lagged product formation, where the production rate reaches its maximum after the growth rate. During the fed-batch cultivation with the optimized medium this is not the case.

An overview about the pre-post comparison of the media B and C (Tab. 4.2) is given in Tab. 4.4. Various process parameters are listed in comparison to emphasize the effects of the different media. The growth behavior of *B. subtilis* DSM 10^T differed only slightly during employment of the optimized medium C. The value of $Y_{X/S}$ decreased in comparison to the fermentation from Chapter 2 ($Y_{X/S}$ = 0.20 g/g in contrast to $Y_{X/S}$ = 0.27 g/g), but values for maximal growth rate μ and cultivation time remained on a similar level. However, the concentration of CDW increased significantly from 2.97 g/L to 3.80 g/L during cultivation with medium C. The analysis of the foam traps identified a considerable decline in Surfactin enrichment, where maximal values of 101.92 decreased to 27.10. The Surfactin recovery decreased as well during employment of medium C in comparison to fermentations applying medium B (83.81 % instead of 91.96 %), although not as drastic as the Surfactin enrichment. In contrast, values for bacterial enrichment improved significantly with a mean value of 0.4 during application of medium C compared to the mean value during fermentations employing medium B (1.60). The production rate increased significantly as values for $Y_{P/X}$ rose from 0.19 g/g (batch, medium B) to 0.26 g/g (fed-batch, medium C). Values for $Y_{P/S}$ increased as well yielding 0.05 g/g instead of 0.03 g/g. The specific production rates $q_{Surfactin}$ and vol. $q_{Surfactin}$ achieved values of 0.009 g/(g·h) and 0.022 g/(L·h) (batch, medium B: $q_{Surfactin}$ = 0.006 g/(g·h), vol. $q_{Surfactin}$ = 0.017 g/(L·h)). The improvement of the production rates becomes even more

Tab. 4.4 Comparison of process parameters during fermentation of *Bacillus subtilis* DSM 10^T employing medium B and C The approach and results of *B. subtilis* DSM 10^T batch fermentation (Chapter 2) is compared to data collected during fed-batch fermentation of *B. subtilis* DSM 10^T employing the further optimized medium C.

	Fed-batch	batch
Applied medium	C	B
Fermentation approach	Foam fractionation	Foam fractionation
Initial glucose conc.	8 g/L	40 g/L
Addition of glucose	23 mL of 450 g/L	-
Final glucose conc.	0 g/L	29.19 g/L
Cultivation time [h]	34	30
Max. CDW [g/L]	3.80	2.97
μ_{max} [h^{-1}]	0.31	0.34
Max. $c_{Surfactin}$ foam [g/L]	3.67	3.99
Foam volume [mL]	435	334
Surfactin in foam [g]	1.02	0.74
Overall Surfactin [g]	1.22	0.81
$Y_{P/X}$ [g/g]	0.26	0.19
$Y_{X/S}$ [g/g]	0.20	0.27
$Y_{P/S}$ [g/g]	0.05	0.03
Int. $q_{Surfactin}$ [g/(g·h)]	0.009	0.006
Int. vol. $q_{Surfactin}$ [g/(L·h)]	0.022	0.017
Overall Surfactin recovery [%]	83.81	91.96
Max. Surfactin enrichment	27.10	101.92
Mean bacterial enrichment	0.41	1.60

significant when analyzing the amount of produced Surfactin. The maximal Surfactin concentration in foam did not increase during cultivation employing medium C (3.67 g/L Surfactin in comparison to 3.99 g/L Surfactin applying medium B), but the total foam volume leaving the bioreactor increased from 334 mL (batch, medium B) to 435 mL (fed-batch, medium C). The collected amount of Surfactin inside the foam traps added up to 1.02 g Surfactin in fermentations

employing medium C in contrast to 0.74 g Surfactin in fermentations applying medium B. In total 1.22 g Surfactin was produced employing the optimized medium C and a fed-batch strategy in comparison to 0.81 g Surfactin during batch fermentations applying medium B. This proves an enhancement of the Surfactin production of approximately 30 % based on the conversion of the fermentation strategy and medium optimization.

4.5 CONCLUSION

As a first conclusion it must be emphasized that Surfactin is not consistently produced throughout the cultivations. This has been taken into account during this study and it became possible to significantly enhance the Surfactin productivity for the strain *B. subtilis* DSM 10^{T}. The substitution of the medium components NH_4Cl and EDTA with $(NH_4)_2SO_4$ and citrate, as well as the alteration of the glucose concentration (from 40 g/L to 8 g/L) improved the production of Surfactin during shake flask experiments. Further shake flask cultivations revealed a general enhancement of Surfactin productivity independent from the employed *Bacillus* strains. The utilization of the improved medium would most likely also lead to better results for other *B. subtilis* strains. Comparable studies did not prefer low glucose concentrations, but failed to analyze concentrations below 10 g/L glucose. The comparison of fermentations employing the optimized medium plus a fed-batch strategy and fermentations applying the original medium in a batch process (Chapter 2) revealed an enhancement of Surfactin production of approximately 30 %. All following studies in the current thesis were therefore conducted using the optimized medium (Tab. 4.2: medium C).

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5. EXCHANGE OF THE NATIVE *SRFA* PROMOTER BY P_{VEG} AND ANALYSIS OF THE EFFECT ON SURFACTIN FORMATION BY *BACILLUS SUBTILIS*

A summary of this chapter is submitted for publication

SUBSTITUTION OF THE NATIVE *SRFA* PROMOTER BY CONSTITUTIVE P_{VEG} IN TWO *B. SUBTILIS* STRAINS AND EVALUATION OF THE EFFECT ON SURFACTIN PRODUCTION

Judit Willenbacher, Teresa Mohr, Marius Henkel, Susanne Gebhard, Thorsten Mascher, Christoph Syldatk and Rudolf Hausmann

The findings of this chapter are submitted for publication.

Authors' contribution to this publication

Judit Willenbacher designed the steps of molecular cloning, performed all experiments for cloning and transformation of *B. subtilis* 3A38, conducted the shake flask experiments of *B. subtilis* 3A38, JWSurf1 and JWSurf2, collected and evaluated the data and wrote the manuscript.

Teresa Mohr conducted the transformation of *B. subtilis* DSM 10^T and shake flask cultivation of *B. subtilis* DSM 10^T and JWSurf3.

Marius Henkel contributed to the strategy of the conducted experiments.

Susanne Gebhard and **Thorsten Mascher** provided the original plasmid pMAD and contributed to scientific discussions.

Christoph Syldatk and **Rudolf Hausmann** supervised the project and contributed to scientific discussions.

5.1 INTRODUCTION

The transcription of the *srfA* operon is regulated by a complex quorum sensing mechanism. That implies that the synthesis of Surfactin is naturally controlled by the amount of signaling molecules in the culture broth. In detail, *B. subtilis* excretes a quorum sensing molecule named ComX, which is recognized outside the cell membrane by a two-component signal transduction system composed of ComP and ComA (Nakano et al. 1991; Soberón-Chávez and Jacques 2011). ComP autophosphorylates if a critical concentration of ComX is reached outside the cell membrane. Thereafter, ComP phosphorylates ComA at the inner side of the cell membrane. In the following, the DNA binding protein ComA forms dimers and binds to specific DNA sequences (dyad symmetries named ComA Box 1 and ComA Box 2) in front of the *srfA* operon. The tetramer formation of two already binding ComA dimers leads to the formation of a DNA loop, initiating the interaction of the RNA polymerase with the DNA (Nakano and Zuber 1993). This complex induction of transcription is based on numerous participating molecules.

Since the Surfactin yields in wild type strains of *B. subtilis* are unsatisfactory for industrial applications, moleculobiological efforts were made to overcome this obstacle. Traditional attempts like overexpression of the coding genes in a heterologous microorganism (e.g. *E. coli*) were quickly discarded, since the transfer of 32 kb gDNA (*srfA* operon and active *sfp*) into a cloning plasmid is rather difficult. Nevertheless, one attempt was conducted using a bacterial artificial chromosome (BAC), yet failing to yield any Surfactin (Lee et al. 2007). Therefore, most genetic studies with the aim to increase the Surfactin yield were conducted in *Bacillus* strains themselves. E.g. genome shuffling was introduced to a *B. amyloliquefaciens* strain, reaching a 3.4-fold increase in Surfactin

Tab. 5.1 Examples for genetic approaches to optimize Surfactin production Listed are two different studies with the aim to improve Surfactin yields in a *Bacillus* strain. The used strains, strategies and results for productivity are summarized.

Strain	Approach	Productivity	Study
B. subtilis			Jung et al., 2012
1012WT		0.021 g/L	
1012WT	Overexpression of *comX* and *phrC*	0.135 g/L	
B. amyloliquefaciens			Zhang et al., 2012
ES-2-4		0.036 g/L	
F2-38	Genome shuffling	0.124 g/L	

yield (Tab. 5.1; Zhao et al. 2012). However, the clarification of the transcription initiation led to various genetic approaches in order to enhance the expression of the Surfactin synthetase in *B. subtilis*. Since the responsible signaling molecules (e.g. ComX and phrC) are well described, Jung et al. analyzed their overexpression in *B. subtilis* 1012WT achieving a 6.4-fold increase in Surfactin production (Tab. 5.1,Jung et al. 2012). Besides these attempts (which still allow expression control by quorum sensing) several studies tried to uncouple the expression of the *srfA* operon from the influence of extracellular signal molecules. This was e.g. performed by Sun et al., replacing the natural promoter P_{srfA} by P_{spac} (Tab. 5.2, Sun et al. 2009). The plasmid pMUTIN4 was employed for integration of the new promoter region in front of the *srfA* operon. But the usage of this type of plasmid does not allow a subsequent cut-out, leaving the fully integrated plasmid inside the genome. However, the induction of the promoter P_{spac} with Isopropyl-β-D-thiogalactopyranosid (IPTG) led to a significant increase in Surfactin yield, proving a positive effect on the regulation system. Another promoter replacement was conducted by Coutte et al. exchanging P_{srfA} against P_{repU} (Tab. 5.2, Coutte et al. 2010). This more elegant markerless promoter substitution introduced P_{repU} in front of *srfA* without any traces. Although several studies proved an enhancement of product yield after replacement of the natural promoter against constitutive P_{repU} (for Mycosutilin and Iturin, Leclère et al. 2005; Tsuge et al. 2001) the results of Coutte et al. did not show an enhancement of Surfactin production. These findings indicated a comparatively strong transcription initiation of the natural promoter P_{srfA}. However, recent studies investigated other promoter substitutions in front of NRPS operons using several different promoters (Tab. 5.2). These markerless constructions implemented inducible P_{xyl}, constitutive P_{43} and native P_{srfA} in front of the *lch* operon (coding for Lichenysin synthetase, Qiu et al. 2014) and constitutive P_{repB} and P_{spac} in front of the *bac* operon (NRPS for the production of the dipeptide Bacilysin, Wu et al. 2014). The promoter replacements positively influenced the expression of the NRPS with P_{xyl} as sole exception, which led to minor product concentrations in comparison to expression with the native promoter.

The summarized studies display positive results for promoter replacements in front of NRPS coding operons in *Bacillus* strains. However, the studies of Qiu et al. (2014) and Wu et al. (2014) showed ambiguous results depending on the employed promoter operon combination. Sun et al. (2009) proved an enhancement in Surfactin yield after promoter exchange, but used a cloning strategy which left the cloning plasmid inside the *Bacillus* genome. Finally, Coutte et al. (2010) introduced an elegant markerless strategy for promoter replacement, but did not succeed in enhancing Surfactin yields. These findings induced further investigation of the genetic optimization of *B. subtilis* for the production of Surfactin. Therefore, the aim of the current study was the markerless construction of *B. subtilis* strains under constitutive expression of the *srfA*

operon introducing the not yet analyzed promoter P_{veg}. This was performed with two different Surfactin producer strains, one strain exhibiting low productivity (3A38) previous to promoter exchange and another strain presenting comparably high product yields (DSM 10^T) before transformation. An enhancement of Surfactin yields was expected after release from quorum sensing control, due to product formation independent from the surrounding cell density.

Tab. 5.2 Comparison of various promoter replacement studies regarding employed promoters and exchange strategies Different studies implementing promoter substitutions in front of operons for lipopeptide and dipeptide production are listed. The employed *Bacillus* strains, operons of interest, used promoters and their characteristics as well as the used exchange strategy are summarized.

Strain	Operon	Promoter	Characteristic	Trace	Study
B. subtilis					Sun et al., 2009
fmbR	*srfA*	P_{srfA}	native		
fmbR-1	*srfA*	P_{spac}	inducible	pMUTIN4	
B. subtilis					Coutte et al., 2010
BBG111	*srfA*	P_{srfA}	native		
BBG113	*srfA*	P_{repU}	constitutive	markerless	
B. subtilis					This study
3A38	*srfA*	P_{srfA}	native		
JWSurf1	*srfA*	P_{veg}	constitutive	markerless	
JWSurf2	*srfA*	P_{veg}	constitutive	markerless	
DSM 10^T	*srfA*	P_{srfA}	native		
JWSurf3	*srfA*	P_{veg}	constitutive	markerless	
B. licheniformis					Qiu et al., 2014
WX-02	*lch*	P_{lch}	native		
WX-02Pxyllch	*lch*	P_{xyl}	inducible	markerless	
WX-02P43lch	*lch*	P_{43}	constitutive	markerless	
WX-02Psrflch	*lch*	P_{srfA}	relocated	markerless	
B. amyloliquefaciens					Wu et al., 2014
FZB42	*bac*	P_{bac}	native		
FZBREP	*bac*	P_{repB}	constitutive	markerless	
FZBSPA	*bac*	P_{spac}	constitutive	markerless	

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

All applied chemicals were of analytical grade and purchased from Carl Roth GmbH (Karlsruhe, Germany). Exceptions are e.g. Surfactin, which was obtained from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany), or restriction enzymes, antibiotics and primers (supplied by companies as indicated in Tab. 5.5, Tab 5.6 and Tab 5.7).

5.2.2 Microorganisms and strain maintenance

Cloning strains like *E.coli* XL1 Blue and DH5α were already stored at the institutes strain collection before experiments for this study were initiated (original companies are indicated in Tab. 5.3). *B. subtilis* DSM 10^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismenn und Zellkutluren GmbH) and *B. subtilis* 3A38 was purchased from the *Bacillus* Genetic Stock Center (BGSC, Columbus, Ohio, USA). The *B. subtilis* strains JWSurf1, JWSurf2 and JWSurf3 were stored as usual in the form of glycerol stocks at -80 °C (preparation described in Chapter 2).

Tab. 5.3 *Bacillus* strains and plasmids utilized in the current study Summary of the bacterial strains including *E. coli* and *B. subtilis* strains and plasmids for cloning and transformations.

Strain	Characteristics	Source or construction
E. coli strains		
XL1 Blue	Cloning strain (tetracycline resistant)	Stratagene
DH5α	Cloning strain	Promega
B. subtilis strains		
3A38	Δ*comI* (parental strain: NCIB 3619)	BGSC
DSM 10^T	Type strain, wild type	DSMZ
JWSurf1	ΔP_{srfA}, fusion of P_{veg} with *srfA* operon	Current study
JWSurf2	ΔP_{srfA}, fusion of P_{veg} with *srfA* operon	Current study
JWSurf3	ΔP_{srfA}, fusion of P_{veg} with *srfA* operon	Current study
plasmids		
pMA-T	pMA-T incorporating sequence of P_{veg}	GeneArt
pMAD	Shuttle vector for markerless constructions, MLS^R	Arnaud et al., 2004
pMAD P_{veg} srfA	pMAD incorporating P_{veg} and flanks	Current study

5.2.3 Culture conditions

5.2.3.1 Media

Lysogeny Broth was used during cloning steps (e.g., for the preparation of overnight cultures) or for inoculation of precultures (Bertani 1951). The optimized mineral salt medium of Chapter 4 was employed for shake flask experiments and was prepared as described in 4.2.2.1 and Tab. 4.2 (medium C). Other specialized media were used during transformation protocols. In case of electrotransformations with *E. coli*, DH5α cells were inoculated and later diluted with SOC medium. The medium contained 3.6 g/L glucose, 0.6 g/L NaCl, 0.2 g/L KCl, 0.6 g/L MgCl, 1.2 g/L $MgSO_4$, 5 g/L yeast extract, and 20 g/L tryptone (Tab. 5.4). The transformation protocol of *B. subtilis* demanded the application of MNGE medium (Tab. 5.4), which was prepared from stock solutions of MN medium (Tab. 5.4), glucose, K-glutamate, Fe(III)ammonium citrate, tryptophan, and $MgSO_4$ (Tab. 5.4). For the initiation of transformation an expression mix was added, containing yeast extract, casamino acids, and tryptophan (Tab. 5.4). All media or media components were autoclaved or filter sterilized (in case of small volumes). For the preparation of agar plates 15 g/L agar was added to the Lysogeny Broth before autoclaving. Sterile antibiotic solutions were added after autoclaving before the plates were poured. All media were stored at room temperature except agar plates containing antibiotics, which were stored at 4 °C.

5.2.3.2 Preparation of inoculum cultures

Inoculum cultures for shake flask experiments were prepared as described in Chapter 2, 3, and 4. For shake flask cultivations precultures of 20 mL Lysogeny Broth in 100 mL baffled shake flasks were inoculated with 10 µL of glycerol stock solutions. The shake flasks were incubated for 24 h at 30 °C and 120 rpm (Multitron II, HT Infors, Bottmingen, Switzerland). The following main culture in 100 mL mineral salt medium in 500 mL baffled shake flasks was inoculated to a resulting OD_{600} of 0.1.

5.2.3.3 Shake flask cultivations

Shake flask experiments were performed for 36 h and 21 h, respectively (30 °C, 120 rpm) in time-displaced inoculated shake flasks to collect continuous data of CDW and Surfactin concentrations. Samples were taken every 3 h. The samples were processed as described in Chapter 2 and 4. To evaluate the data from shake flask cultivations process parameters were calculated from the results of CDW and Surfactin. The differential values of $q_{Surfactin}$ [g/(g·h)] and µ [h^{-1}] were obtained by Eq. 2.4 and Eq. 2.3 from Chapter 2.

Tab. 5.4 Media used in the current study Listed are all applied media during the current study including their components and concentrations. Sole exception is the mineral salt medium used for shake flask and bioreactor cultivations, which was identical to medium C from Chapter 4 (Tab. 4.2).

Medium	Components	Concentration/volume of stock solution
LB	NaCl	10 g/L
	Yeast extract	5 g/L
	Tryptone	10 g/L
SOC	Glucose	3.6 g/L
	NaCl	0.6 g/L
	KCl	0.2 g/L
	MgCl	0.6 g/L
	$MgSO_4$	1.2 g/L
	Yeast extract	5 g/L
	Tryptone	20 g/L
10 x MN	K_2HPO_4	104 g/L
	KH_2PO_4	60 g/L
	Na_3citrate	10 g/L
MNGE (10 mL)	1 x MN	9.2 mL
	Glucose	1 mL of 20 %
	K-glutamate	50 µL of 40 %
	Fe(III)-ammonium citrate	50 µL of 2.2 mg/mL
	Tryptophan	100 µL of 5 mg/mL
	$MgSO_4$	30 µL of 1 M
Expression mix (1.05 mL)	Yeast extract	500 µL of 5 %
	Casamino acids	250 µL of 10 %
	Tryptophan	50 µL of 5 mg/mL
	H_2O	250 µL

5.2.4 Methods of molecular cloning

5.2.4.1 Extraction of genomic DNA

Genomic DNA of *B. subtilis* DSM 10^T and 3A38 was isolated using the ZR Soil Microbe DNA MiniPrep™ kit (Zymo Research Corporation, Irvine, California, USA). The DNA was extracted according to the manufacturer instructions.

5.2.4.2 Plasmid preparation

For the extraction of plasmid DNA the peqGOLD Miniprep Kit I (C-Line) was employed (VWR International GmbH, Erlangen, Germany). The isolation of plasmid DNA was performed according to the manufacturer instructions.

5.2.4.3 Digestion by restriction enzymes

Restriction enzymes from Fermentas (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA, Tab. 5.5) were used to perform DNA digestions. The recommended protocol of the manufacturer was consulted to design the digestions. Usually, 16 µl of nuclease-free water, 2 µl of the recommended buffer, 1 µl (~1 µg) of substrate DNA, and 1 µl of restriction enzyme was mixed to obtain a total volume of 20 µl. The digestion mix was typically incubated at 37 °C for several hours or overnight.

5.2.4.4 Ligation

The Ligation was performed using the T4 DNA Ligase from Fermentas (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The ligation was conducted according to the manufacturer instructions using 100 ng of linear vector DNA, 50 ng of insert DNA (molar ration 1:3), 2 µL buffer, 1 µL ligase, and 10 µL of nuclease-free water. The ligation mix was incubated at 22 °C for 45 min. Subsequently, the ligase was deactivated for 5 min at 70 °C.

Tab. 5.5 Restriction enzymes used in the current study All employed restriction enzymes during the current study are listed regarding name, sequence, and supplier. Sequences are illustrated from 5′ to 3′ end.

Restriction enzyme	Sequence	Supplier
*Bam*HI	GGATCC	Fermentas
*Eco*RI	GAATTC	Fermentas
*Hind*III	AAGCTT	Fermentas
*Xba*I	TCTAGA	Fermentas

5.2.4.5 Transformation

5.2.4.5.1 Transformation protocols for *E. coli* Transformations of *E.coli* were either performed as electrotransformations (*E. coli* DH5α) or using chemically competent *E. coli* cells (XL1 Blue). For transformations applying the electrocompetent *E. coli* cells DH5α, 25 µl competent cells were mixed with 50 ng of plasmid DNA. The mixture was transferred to a sterile and cooled cuvette and pulsed by a Gene Pulser XCell™ Electroporation System (1.8 kV, Bio-Rad Laboratories Inc., California, USA). The pulsation was followed by the addition of 600 µL SOC medium and incubation at 37 °C (1h). Subsequently, the cells were plated in dilutions on agar plates containing 100 µg/mL ampicillin and incubated at 37°C overnight.

For chemical transformations, 200 µL of *E. coli* XL1 Blue were incubated with 100 ng of plasmid DNA for 20 min on ice, followed by a heat shock at 42 °C for 90 s. Subsequently, the cells were incubated for 1 min on ice and afterwards inoculated with 1 mL SOC medium (Tab. 5.4). The mixture was incubated for 30 min at 37 °C and 500 rpm (ThermoMixer, Eppendorf AG, Hamburg, Germany). After incubation the mixture was plated on agar plates containing 100 µg/mL ampicillin and incubated at 37 °C overnight.

5.2.4.5.2 Transformation protocol for *B. subtilis* At the beginning of transformation, 10 mL of MNGE medium (Tab. 5.4) were inoculated with an overnight culture of *B. subtilis* 3A38 or DSM 10^T to a resulting OD_{600} of 0.1. The cells were cultivated until an OD_{600} of 1.0 was reached. Subsequently, 400 µL of the cells were mixed with 4 µg plasmid DNA and incubated at 37 °C and 200 rpm (ThermoMixer, Eppendorf AG, Hamburg, Germany). After 1 h, 100 µL of expression mix was added to induce transformation (Tab. 5.4). The mixture was plated on agar plates containing macrolide-lincosamide-streptogramin B (MLS) selection (1 µg/mL erythromycin, 25 µg/mL lincomycin) and 100 µg/mL X-Gal.

Tab. 5.6 Antibiotics and other solutions for selection on agar plates Listed are all antibiotics and other solutions used during the current study for selection on agar plates. Summarized are names, final concentrations, and suppliers.

Antibiotic/other selection marker	Final concentration	Supplier
Ampicillin	100 µg/mL	Roth
Erythromycin	1 µg/mL	Roth
Lincomycin	25 µg/mL	Sigma Aldrich
X-Gal	100 µg/mL	Sigma Aldrich

Usually after 3 days blue colonies (Fig. 5.8 A) were selected for overnight cultures (containing MLS selection) to promote the integration of pMAD Pveg srfA into the genome. Therefore, 10 mL Lysogeny Broth with MLS selection in 100 mL baffled shake flasks were inoculated on the following day to a resulting OD_{600} of 0.1. The shake flasks were incubated at 30 °C and 180 rpm (Multitron II, HT Infors, Bottmingen, Switzerland) for 2 h. Afterwards the temperature was increased to 42 °C and the shake flasks were incubated under these conditions for another 6 h. At the end of the cultivation dilutions were plated on agar plates containing MLS selection and 100 µg/mL X-Gal. The plates were incubated at 42 °C overnight. On the following day blue colonies were used for colony PCR to check for the integration of pMAD Pveg srfA into the genome (Tab. 5.7, Fig. 5.6, Fig. 5.7 and Fig. 5.8 B). Positive hits were used on the following day for a second temperature shift. Therefore colonies were picked from the plate and inoculated in 10 mL Lysogeny Broth (without MLS selection) and incubated at 30 °C and 180 rpm. After 6 h the temperature was increased to 42 °C. After additional 3 h the cells were plated in dilutions on agar plates containing 100 µg/mL X-Gal (but no MLS selection). The plates were incubated at 42 °C overnight. On the following day white colonies were used for colony PCR to check for the loss of pMAD Pveg srfA on the genome (Tab. 5.7, Fig. 5.7, Fig. 5.9).

5.2.4.6 Polymer chain reactions (PCR)

5.2.4.6.1 Standard PCR protocol The amplification of DNA was performed via polymeric chain reaction (PCR), using the HotStar HiFidelity Polymerase Kit (Qiagen N.V., Venlo, Netherlands). Usually, 5 µL template DNA was mixed with 2.5 µL 10xbuffer, 0.5 µL of dNTP mix, 1 µL of each primer solution (stock solutions of 100 pmol/µL were prepared earlier), 0.125 µL HotStar taq, and 14.875 µL of nuclease free water. The total volume of 20 µL was transferred into a PCR reaction tube and incubated in a PCR cycler (Mastercycler, Eppendorf AG, Hamburg, Germany). The PCR program usually initiated the reaction at 95 °C for 15 min to activate the HotStar taq polymerase. Afterwards three succeeding steps for denaturation (94 °C, 1 min), annealing (temperature depended on Primer pair, 1 min) and elongation (72 °C, time depended on fragment length, 1 kb = 1 min) were performed and repeated in 30 cycles. At the end of the program the fragment was exposed to 72 °C for 10 min. The melting temperature T_M was calculated with Eq. 5.1. The number of the specific nucleobases in the primer sequence was inserted instead of the placeholders G, C, A, and T. The melting temperatures of both primers were compared and the lower temperature was used to estimate the annealing temperature which should be about 2 °C lower than the melting temperature.

$$T_M = 64 + 41 \cdot \frac{G+C-16.4}{A+G+C+T} \qquad \textbf{Eq. 5.1}$$

5.2.4.6.2 Colony PCR Especially at the end of *B. subtilis* transformation several colony PCRs were conducted to check for the integration or loss of the plasmid into or from the genome. Colony PCRs were very similar to standard PCR protocols, but instead of adding 5 µl template DNA a toothpick was used to transfer a very small part of a colony into the PCR reaction tube. The total volume was adjusted by adding 5 µl nuclease free water. Otherwise the PCR protocol followed the standard PCR protocol described in 5.2.4.6.1.

5.2.4.6.3 Overlap PCR For the construction of the pMAD Pveg srfA insert several overlap PCRs had to be conducted. The main difference compared to the standard protocol of 5.2.4.6.1 was the addition of 10 additional cycles before the original cycler program started. At the beginning the PCR mixture was prepared without the addition of primers but two solutions of template DNA fragments (which should overlap during the PCR program). In the following the PCR program was started with the initial 95 °C for 15 min and was continued by a three step cycle (denaturation, annealing and elongation) using the annealing temperature of the two template DNA fragments. This three step cycle was repeated for 10 times. Afterwards, the PCR reaction tube was removed from the cycler and the primer solutions were added to the PCR mix. Consequently, the reaction tube was placed back into the cycler and the PCR program continued using the annealing temperature of both primers.

5.2.4.7 Gel electrophoresis

Gel electrophoresis was performed in order to validate intermediate and final steps during molecular cloning. Therefore a gel electrophoresis chamber and power supply system was used (Bio-Rad Laboratories Inc., California, USA). For the preparation of 1 % agarose gels 1 g agarose was dissolved in 100 mL of 1xTBE buffer (10.8 g/L tris, 5.5 g/L boracic acid, 0.7 g/L Na_2EDTA) under extreme heat. To visualize the DNA under UV light after gel electrophoresis 5 µL of Roti®-gelstain (Carl Roth GmbH, Karlsruhe, Germany) were added after cooling the liquid agarose solution. The final agarose solution was poured into a mold and left for cooling. The stiff agarose gel was placed into the electrophoresis chamber and covered with 1xTBE buffer. The pockets of the gel were loaded with samples and DNA marker (5 µL of 1 kb or 100 bp DNA ladder, New England Biolabs, Ipswich, Massachusetts, USA). Afterwards the power supply system was adjusted to 80 V and the gel chamber was energized until the DNA fragments were sufficiently separated. The DNA fragments were afterwards observed under UV light.

Tab. 5.7 Primers used in the current study Summary of all employed Primers, illustrating their products, sequences, and suppliers. Sequences are illustrated from 5′ to 3′end.

Product and Primer names	Sequence	Supplier
gDNA *B. subtilis* DSM 10^T		
JM_hxIR_for	AGACGCTCTTCGCAAGGG	Eurofins
JM_srfAA_rev	ATTGTCATACCTCCCCTAATC	Eurofins
hxIR upstream flank		
hxIR 700 forward 2	GGATCCAGTAGGCACTTTGAAGTCAA	Invitrogen
hxIR 700 reverse 2	TCATTTCCACTAAACATTATTTACAG	Invitrogen
srfAA downstream flank		
JM_srfAA_700_for	ATGGAAATAACTTTTTACCC	Invitrogen
srfA 700 reverse 2	GAATTCCTTCAGGCACATCTTTAGAG	Invitrogen
P_{veg}		
Pveg forward 2	TTTGGTTTAAAAATTTTTATTTTTCTG	Invitrogen
JM_Pveg_rev	CCAAATTCGTTTTTGTGCATCCG	Invitrogen
Overlap 1: upstream flank + P_{veg}		
hxIR 700 forward 2	GGATCCAGTAGGCACTTTGAAGTCAA	Invitrogen
JM_Pveg_rev	CCAAATTCGTTTTTGTGCATCCG	Invitrogen
Overlap 2: Overlap 1 + downstream flank		
hxIR 700 forward 2	GGATCCAGTAGGCACTTTGAAGTCAA	Invitrogen
srfA 700 reverse 2	GAATTCCTTCAGGCACATCTTTAGAG	Invitrogen
pMAD genome integration		
1 pMAD check fwd	GAAGTTAGGCTGGTAAGAGC	Invitrogen
1 pMAD check rev	ACAGCCGAAATAGCCCAAAG	Invitrogen
2 pMAD check fwd	GGGTCTTGCGGTCTTTATCC	Invitrogen
2 pMAD check rev	CGATGCATGCCATGGTACCC	Invitrogen
Substitution of P_{srfA} by P_{veg}		
Final check fwd	GAGAGCTTGAGCAGGATATG	Invitrogen
Final check rev	TAGCGGCAAAGGTTTCTTCG	Invitrogen

5.2.4.8 Gel extraction

The MinElute Gel Extraction Kit (VWR International GmbH, Erlangen, Germany) was utilized for the extraction of DNA from agarose gels. The isolation was performed according to the manufacturer instructions.

5.2.4.9 Gene sequencing

To validate steps during or at the end of molecular cloning, DNA fragments were amplified and send in for sequencing to GATC Biotech AG (European Genome and Diagnostics Center, Konstanz, Germany). Therefore, samples were adjusted to 50 ng/µl DNA and the posting was further equipped with the necessary primers.

5.2.4.10 Design of cloning steps and sequence alignment

All designs for cloning (e.g. primer design, design of P_{veg} and pMAD Pveg srfA) were conducted using the software Clone Manager (Scientific & Educational Software, Morrisville, North Carolina, USA).

5.2.5 Construction of the plasmid pMAD Pveg srfA

One of the main goals of the current study was to establish a markerless promoter substitution to maintain the natural organization of the genome of *B. subtilis*, which was realized by choosing the shuttle vector pMAD for transformation of *B. subtilis* (Fig. 5.1). This vector allows an efficient allelic replacement in gram-positive bacteria, introducing two flanks homologue to the *Bacillus* gDNA (Arnaud et al. 2004). The plasmid originated from the fusion of pBR322 (originally used in gram-negative bacteria) and temperature-sensitive pE194^{ts} (originally used in gram-positive bacteria). Therefore, pMAD consists of two origins of replication, one for replication in *E. coli* (ori pBR322) and another for replication in *B. subtilis* (ori pE194^{ts}). The selection in gram-negative bacteria is guaranteed by the gene *bla* coding for a β-lactamase acting against ampicillin and *ermC* coding for a rRNA methylase acting against MLS antibiotics for the selection in gram-positive bacteria. In addition, pMAD holds a multiple cloning side (MCS) and the constitutive promoter P_{clpB} in front of *bgaB* (coding for a β-galactosidase). The concept of the allelic replacement is based on the construction of an adequate insert for pMAD, holding two flanks homologous to the gDNA upstream and downstream of the region which is about to be replaced. Therefore three different DNA fragments had to be designed and amplified. First, an upstream flank (700 bp) homologous to the region in front of the natural promoter P_{srfA} was amplified (using the deposited genome sequ-

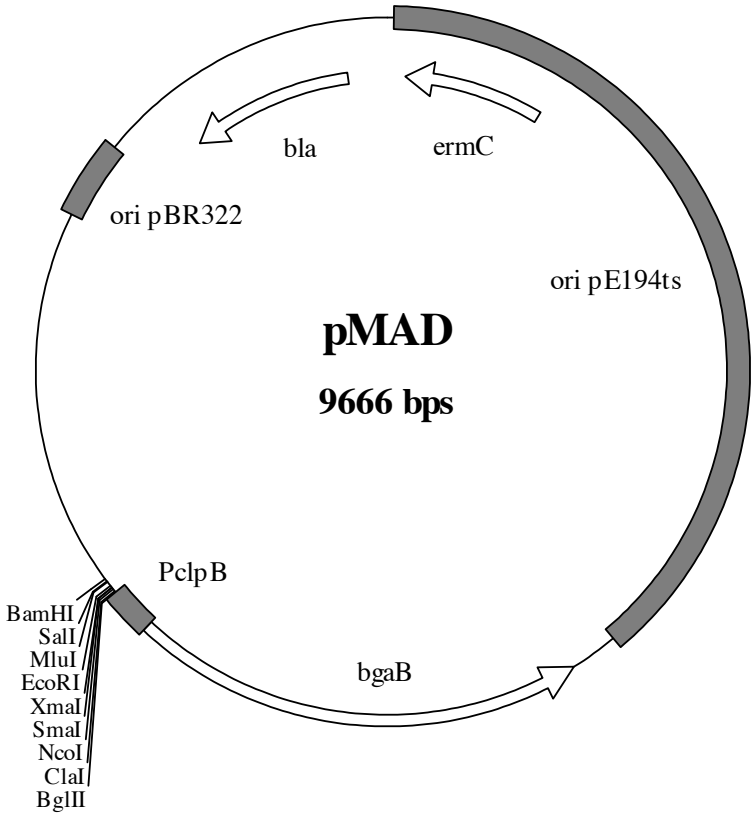

Fig. 5.1 Schematic scheme of the shuttle vector pMAD Presented is the original empty plasmid pMAD used for the construction of pMAD Pveg srfA. The plasmid consists of two origins of replication, two antibiotic resistance genes, the gene *bgaB* coding for a β-galactosidase and a multiple cloning site (MCS).

ence of *Bacillus subtilis* subsp. *subtilis* 6051-HGW from NCBI as reference for primer design etc.). This region includes a small gene called *hxIR* (362 bp) a positive regulator of the *hxIAB* expression HlxIR. Hence, the fragment was called *hxIR* upstream flank. The primers used to amplify this DNA fragment are shown in Tab. 5.7. The second DNA fragment was called *srfAA* downstream flank, as it covered a 700 bp sequence homologous to the beginning of *srfAA* (the total open reading frame is 10,762 bp, the deposited genome sequence of *Bacillus subtilis* subsp. *subtilis* 6051-HGW from NCBI was used as reference for primer design etc.). The primers used to amplify the downstream flank are shown in Tab. 5.7. The results of the PCRs used to amplify the *hxIR* upstream and *srfAA* downstream flank are shown in Fig. 5.3 A. The design of a new promoter region required two important decisions at the beginning concerning the ribosomal binding site (RBS) and the following gap in front of the start codon. Both, RBS and gap, are important for the translation initiation and are not crucial to transcription. In order to leave the translation process as unaffected as possible the RBS and the following gap were left in the natural position. The sequence in front of the RBS was constructed in order to match the sequence of P_{veg}. In doing so, the core promoter regions -35 and -10 were most important. To enable a fusion of the promoter region and the up-

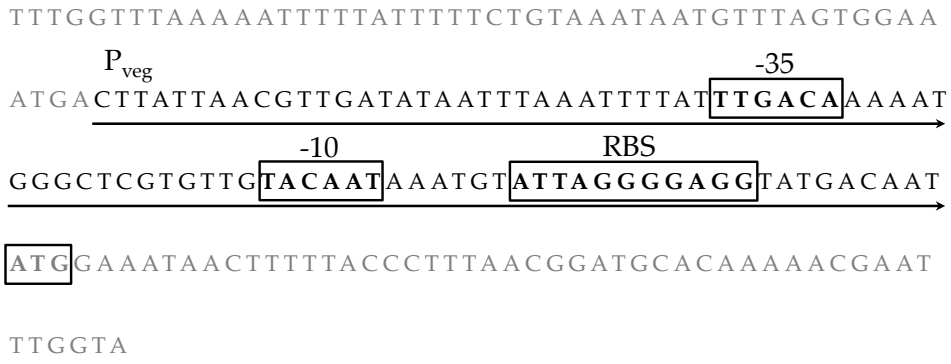

Fig. 5.2 Sequence of P_{veg} and 50 bp flanks The sequence shows the core promoter region (*black*) and 50 bp upstream and downstream fused for later overlap PCRs (*grey*). The most important positions are indicated by *black boxes* showing the -35 and -10 regions as well as RBS and the start codon of the *srfAA* ORF. This DNA fragment was ordered from GeneArt (life technologies).

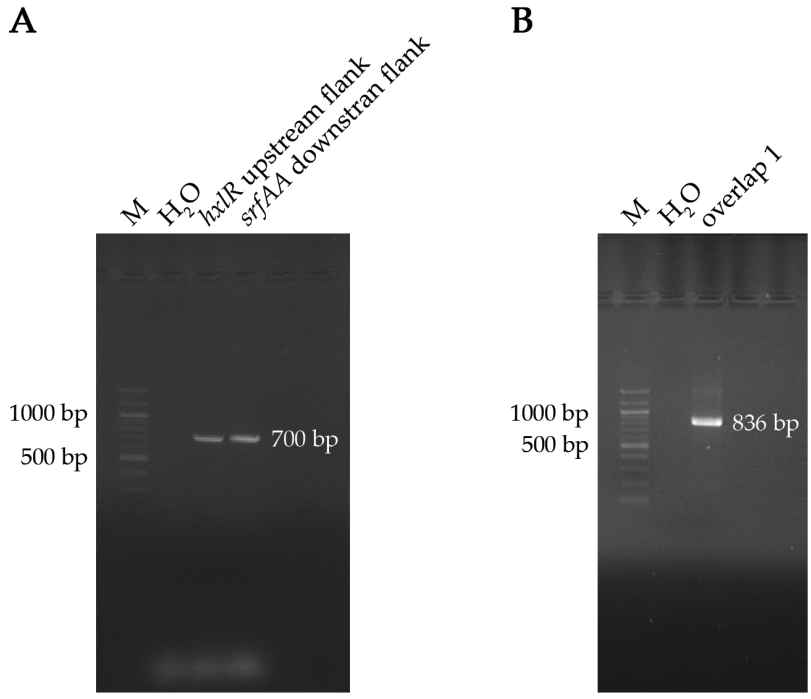

Fig. 5.3 Gel electrophoresis pictures obtained with PCR amplificates of the upstream and downstream flank and the first overlap fragment The amplification of the *hxIR* upstream and *srfAA* downstream flank yielded each 700 bp fragments (**A**). The first overlap PCR (fusion of upstream flank and P_{veg}) amplified a fragment of 836 bp (**B**).

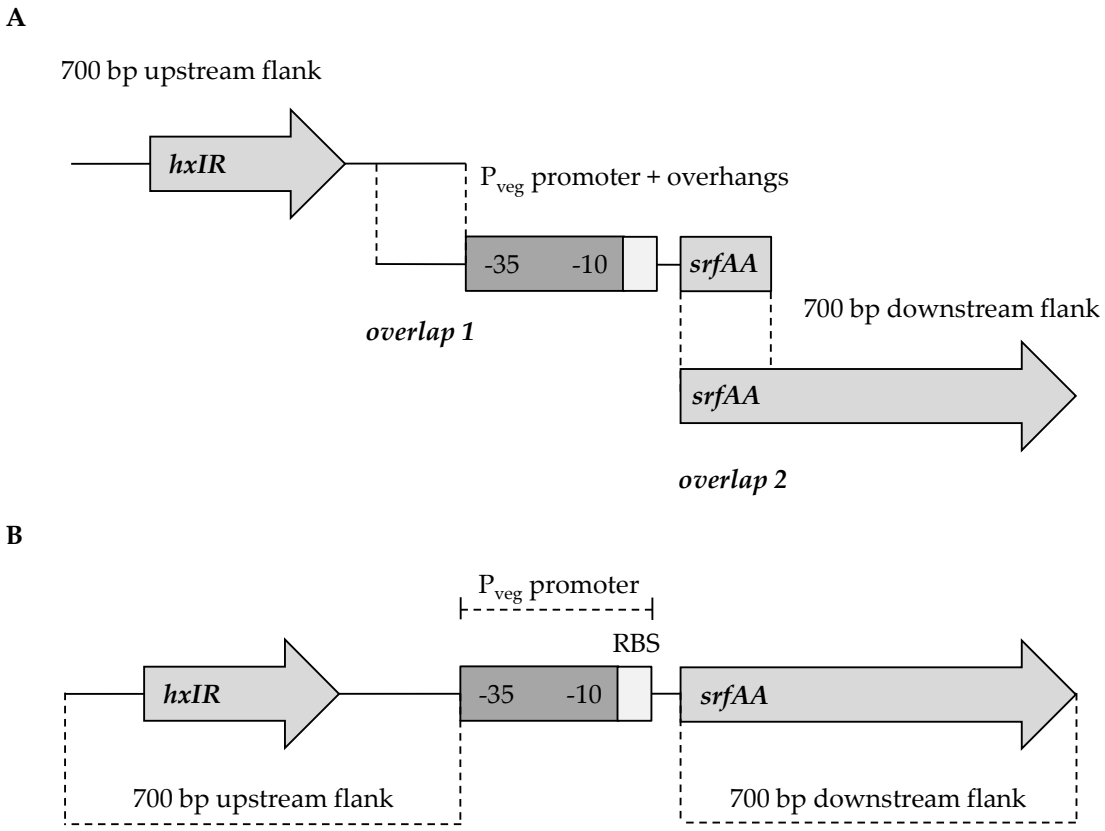

Fig. 5.4 Schematic scheme of the pMAD Pveg srfA insert Presented is a schematic overview of the two overlap PCRs which were necessary for the fusion of the upstream and downstream flank with P_{veg} (**A**) and the resulting insert for pMAD (1,486 bp, **B**).

stream and downstream flank homologous regions had to be included to allow a later overlap PCR. This required the addition of 50 bp homologous to the *hxIR* upstream flank and the *srfAA* downstream flank, respectively. The result of these thoughts and decisions was a 186 bp fragment which was commissioned by GeneArt® (Fig. 5.2, life technologies, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The DNA fragment containing P_{veg} was supplied within the plasmid pMA-T (Tab. 5.3). The sequence composed of P_{veg} and the attached 50 bp flanks was amplified using the primers in Tab. 5.7. In the following, two overlap PCRs were performed to fuse the three DNA fragments (*hxIR* upstream flank, P_{veg}, *srfAA* downstream flank, Fig. 5.4 A). During the first overlap PCR the upstream flank was attached to the new promoter region of P_{veg} (Fig. 5.3 B, primers from Tab. 5.7). In the second overlap PCR the resulting DNA fragment (overlap 1) was fused to the downstream flank (primers see Tab. 5.7). The resulting 1,486 bp fragment was used as insert to create pMAD Pveg srfA from the original pMAD plasmid (Fig. 5.4 B). Therefore, pMAD and the insert were digested using *Bam*HI and *Eco*RI, respectively. The following ligation of the linearized plasmid and the insert resulted in the formation of pMAD Pveg srfA (Fig. 5.5).

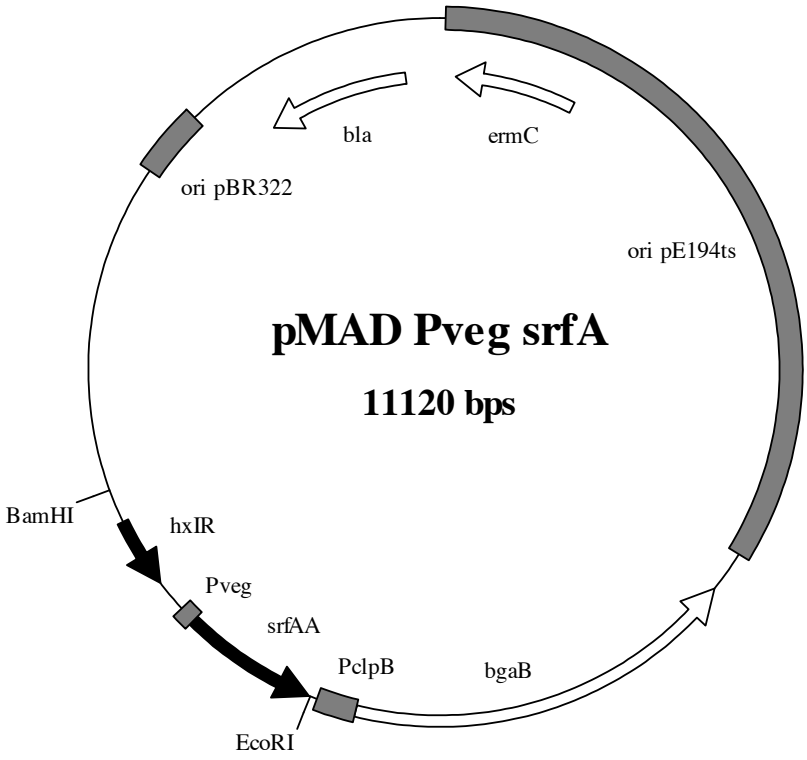

Fig. 5.5 Schematic scheme of pMAD Pveg srfA Plasmid map of the final vector used for transformation of *B. subtilis* 3A38 and DSM 10^T. The original plasmid pMAD includes the constructed insert with two homologous flanks and the promoter P_{veg}. The insert was integrated using the restriction sides of *Bam*HI and *Eco*RI.

5.2.6 Transformation of *Bacillus subtilis* 3A38 and DSM 10^T

The natural competence of *B. subtilis* wild type strains is rather limited. Therefore, *B. subtilis* 3A38 (Konkol et al. 2013) was additionally employed to conduct the transformation with pMAD Pveg srfA next to the transformation of *B. subtilis* DSM 10^T. The mutated strain *B. subtilis* 3A38 (purchased from BGSC) originates from the *B. subtilis* type strain NCIB 3610, which is equivalent to ATCC 6051 and DSM 10^T. *B. subtilis* 3A38 exhibits an enhanced capability for the uptake of exogenous DNA in comparison to its ancestor, because *comI* (encoded on the endogenous plasmid pBS32) is mutated. Usually, the small protein ComI is a single-pass *trans*-membrane protein inhibiting the competence DNA uptake machinery (Konkol et al. 2013).

The initial transformations were conducted as described in 5.2.4.5.2 (first paragraph). The cells were thereafter incubated at 37 °C on agar plates containing MLS selection and X-Gal. After two days blue colonies were picked for overnight culture. The uptake of pMAD Pveg srfA led to the possession of the *bgaB* gene, coding for a β-galactosidase inducing the formation of 5,5'-dibromo-4,4'-dichloro-indigo (an intense blue colorant). This allowed the conclusion that blue colonies consequently absorbed the plasmid. In the following, a first temperature shift was applied,

initiating the integration of pMAD Pveg srfA into the genome by crossover events (Fig. 5.6). The cells were cultivated for 2 h at 30 °C and thereafter 6 h at 42 °C. The temperature sensitive origin of replication (originally from pE194^{ts}) allows the replication of the plasmid at temperatures under 32 °C. In contrast, temperatures above 37 °C inhibit the replication of pMAD encouraging the cells to integrate the plasmid into the gDNA as a result of the consistent selection pressure by erythromycin and lincomycin. After the first temperature shift dilutions of the culture broth were plated on agar plates containing MLS selection and X-Gal and incubated at 42 °C. Blue colonies were obtained from these plates and examined (schematic scheme: Fig. 5.7, blue colonies: Fig. 5.8 A). The construction of the pMAD insert generally allows two different crossover possibilities, leading to two different integration alternatives (Fig. 5.7). If the crossover process is induced by the *hxIR* upstream flank the plasmid will integrate in a fashion that leaves the new promoter P_{veg} behind the original *hxIR* gene (Fig. 5.7: 1. crossover possibility). If the integration is directed by the *srfAA* downstream flank, P_{veg} will be left directly in front of the original *srfAA* ORF (Fig. 5.7: 2. crossover possibility). Both alternatives can conduct the desired substitution of P_{veg} against P_{srfA}. The third alternative, a simultaneous crossover event of the *hxIR* upstream flank and

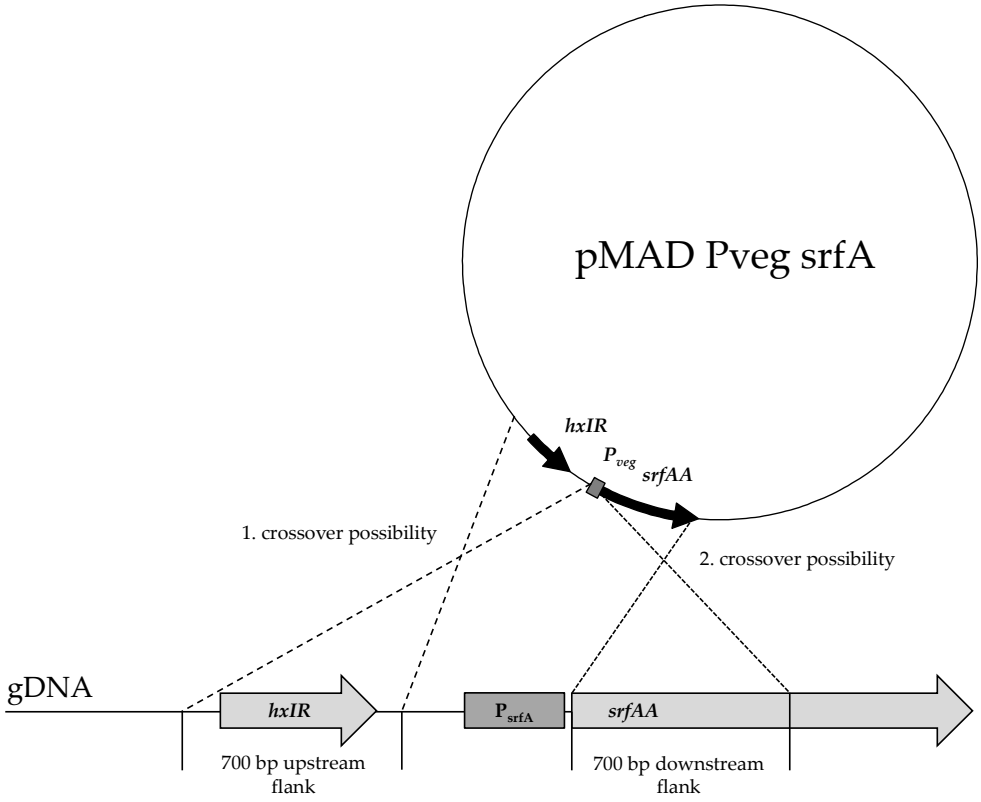

Fig. 5.6 Schematic scheme of the crossover possibilities after the uptake of pMAD Pveg srfA into *B. subtilis* The scheme illustrates the situation after pMAD Pveg srfA uptake and before integration into the gDNA. The two possibilities for crossover events using the *hxIR* flank or *srfAA* flank are presented.

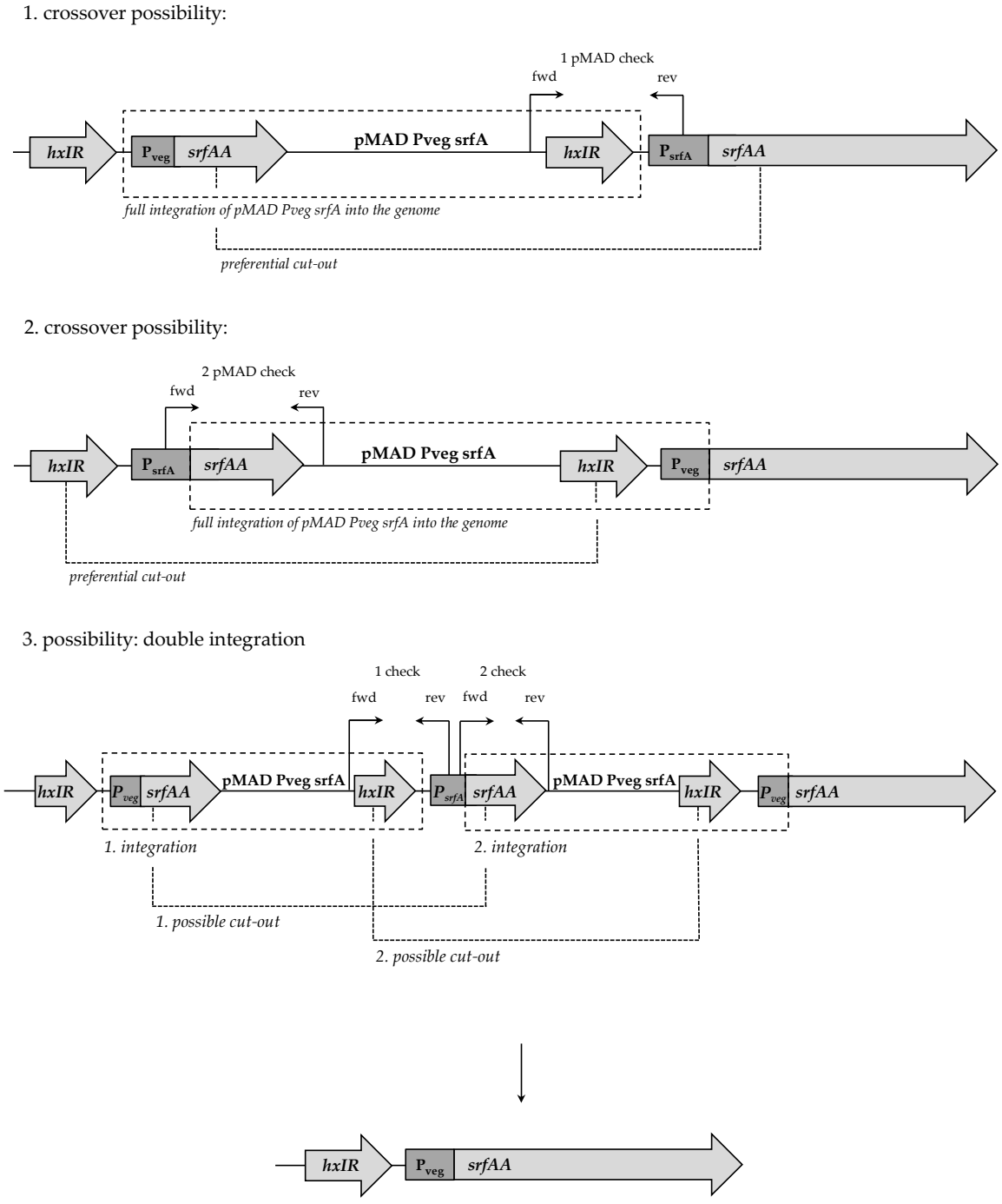

Fig. 5.7 Schematic representation of pMAD Pveg srfA genome integration and desired end product Presented are the three different possibilities for pMAD Pveg srfA integration after single or consecutive cross over events. Genes, part of genes and promoters are indicated as *bold arrows*. Primer binding positions are illustrated as *thin arrows*. The original pMAD Pveg srfA construct is surrounded by a *dashed box*. The preferred region for cut out is indicated by a *dotted line*. The first two crossover possibilities allow the markerless substitution of P_{srfA} by P_{veg} if homologous sequences of *srfAA* and *hxIR* are cut out, respectively. The third possibility occurs after double integration of pMAD Pveg srfA and is not preferable. Solely colonies showing only one band after colony PCR with the primer pairs "1 pMAD check" and "2 pMAD check" are used for further steps to obtain the desired end product (illustrated at the bottom of the scheme).

srfAA downstream flank (Fig. 5.7) is possible but not favorable. As a consequence, a colony PCR was conducted with exemplary blue colonies to analyze if and if so, what kind of crossover event occurred (for primers see Tab. 5.7). Genomic DNA of all selected colonies was therefore amplified using the primers of "1 pMAD check" and "2 pMAD check" (Tab. 5.7, Fig. 5.8 B). If the plasmid did not integrate into the genome no bands were observed. In case the first crossover possibility occurred, reaction tubes containing primers of "1 pMAD check" showed one band. If the second

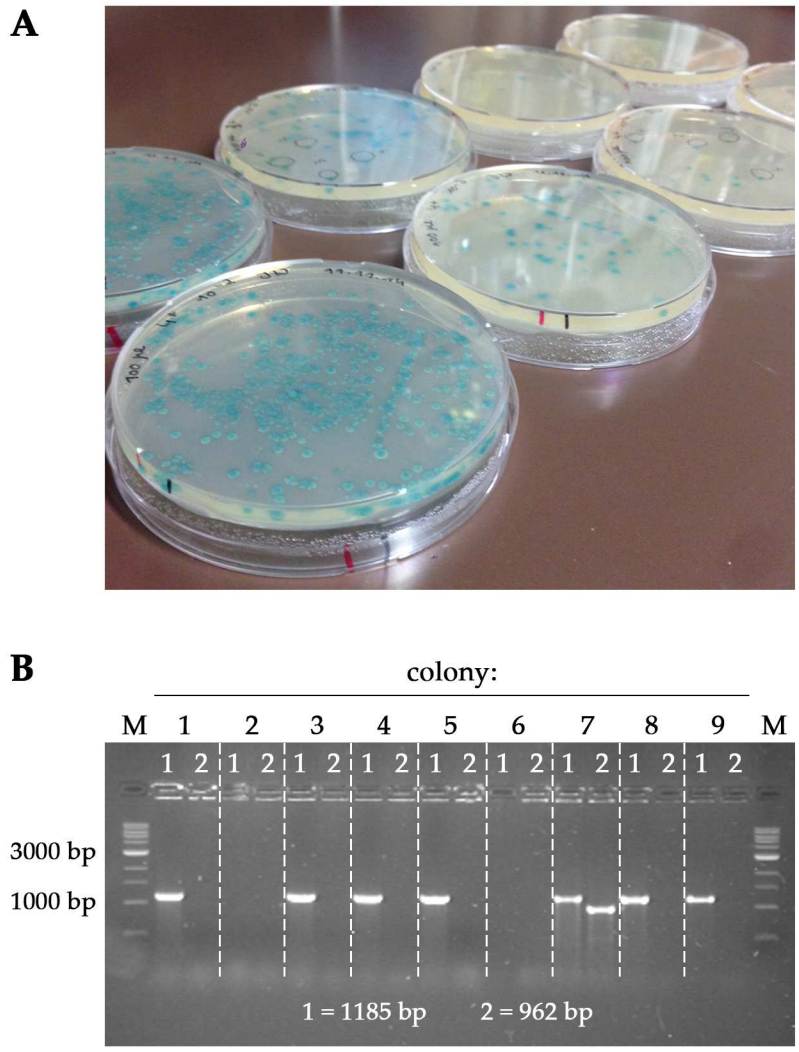

Fig. 5.8 Agar plates showing blue colonies after transformation and a gel electrophoresis picture displaying PCR fragments after colony PCR Illustrated are impressions of the screening process in which pMAD Pveg srfA integrates into the genome of *B. subtilis*. The integration process is monitored via blue colonies and a following colony PCR. Blue colonies after the first temperature shift during *B. subtilis* transformation and after plating on agar plates containing X-Gal are illustrated in (**A**). The integration into the genome is verified via colony PCR. The primer pairs "1 pMAD check" (1) and "2 pMAD check" (2) are used for DNA amplification on each colony. Only colonies showing one band are used for further transformation steps (**B**).

crossover possibility took place a band using the primers of "2 pMAD check" was obtained. If both crossover possibilities occurred two bands were visible. In most cases only one crossover event took place, preferably using the *hxIR* upstream flank (Fig. 5.8 B). Positive clones were used for a second temperature shift. These cultures were incubated for 6 h at 30 °C and afterwards 3 h at 42 °C. Dilutions were again plated, but the agar plates did not contain any antibiotics (only X-Gal). The plates were incubated at 42 °C. On the following day the plates were screened for white colonies. By removing the selection pressure the cells are no longer forced to keep the plasmid inside the gDNA. The main part of the integrated plasmid containing the β-galactosidase got dispensable and was eventually cut out. Clones which lost the β-galactosidase were consequently white. If the plasmid was lost and if so, what kind of cut did occur was analyzed via colony PCR (for primers see Tab. 5.7). In most cases white colonies led to a fragment of 1,100 bp, which was caused by a cut out of pMAD Pveg srfA at the same sides which were used for integration. In three cases a fragment of 778 bp was obtained by colony PCR, corresponding to a successful cut, leaving P_{veg} in front of *srfAA* (Fig. 5.9). The resulting strains from transformation of *B. subtilis* 3A38 were named JWSurf1 andJWSurf2. The transformed strain originating from *B. subtilis* DSM 10^T was named JWSurf3. The gDNA of JWSurf1, JWSurf2 and JWSurf3 was isolated and amplified to yield a DNA fragment exhibiting the promoter region of the *srfAA* ORF. The DNA fragments were sent to sequencing (GATC Biotech AG, Konstanz, Germany). The results confirmed the promoter exchange of P_{srfA} against P_{veg} (the results of sequencing are displayed in the appendix).

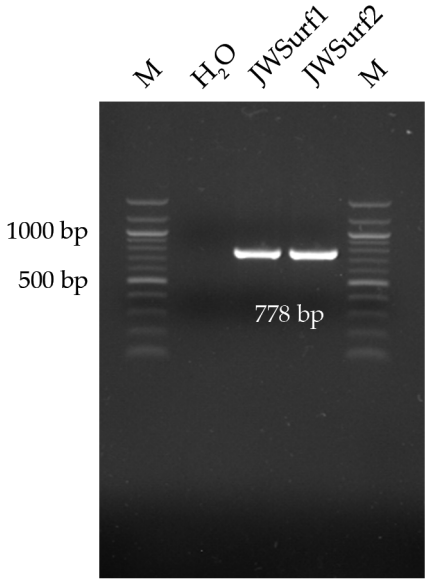

Fig. 5.9 Gel electrophoresis picture of PCR amplificates to finally verify the promoter exchange
After the second temperature shift colony PCRs were conducted amplifying the promoter region of *srfAA* to examine the results of the cut out of pMAD Pveg srfA. The figure illustrates two positive results which yielded a 778 bp fragment in front of *srfAA* (containing P_{veg}) instead of a 1,100 bp fragment (containing original P_{srfA}).

5.3 RESULTS

5.3.1 Surfactin production in shake flasks: ancestral vs. descended strains

The first examinations investigating the Surfactin productivity of the transformed strains *B. subtilis* JWSurf1, JWSurf2 and JWSurf3 were conducted in shake flask cultivations. The strains were cultivated in comparison to their ancestor strains *B. subtilis* 3A38 and DSM 10^T, which are supposed to be identical except mutagenesis of *comI* in *B. subtilis* 3A38 on the endogenous plasmid pBS32. The results of these shake flask cultivations are shown in Fig. 5.10. The cultivations were conducted for 36 h and 21 h, respectively. The optimized mineral salt medium (Tab. 4.2: medium C) was employed during cultivations and shake flasks were inoculated in a time-displaced manner to exhibit a continuous progress of CDW and Surfactin concentration. Interestingly, all strains showed a rather similar progress of cell growth, but displayed very diff-

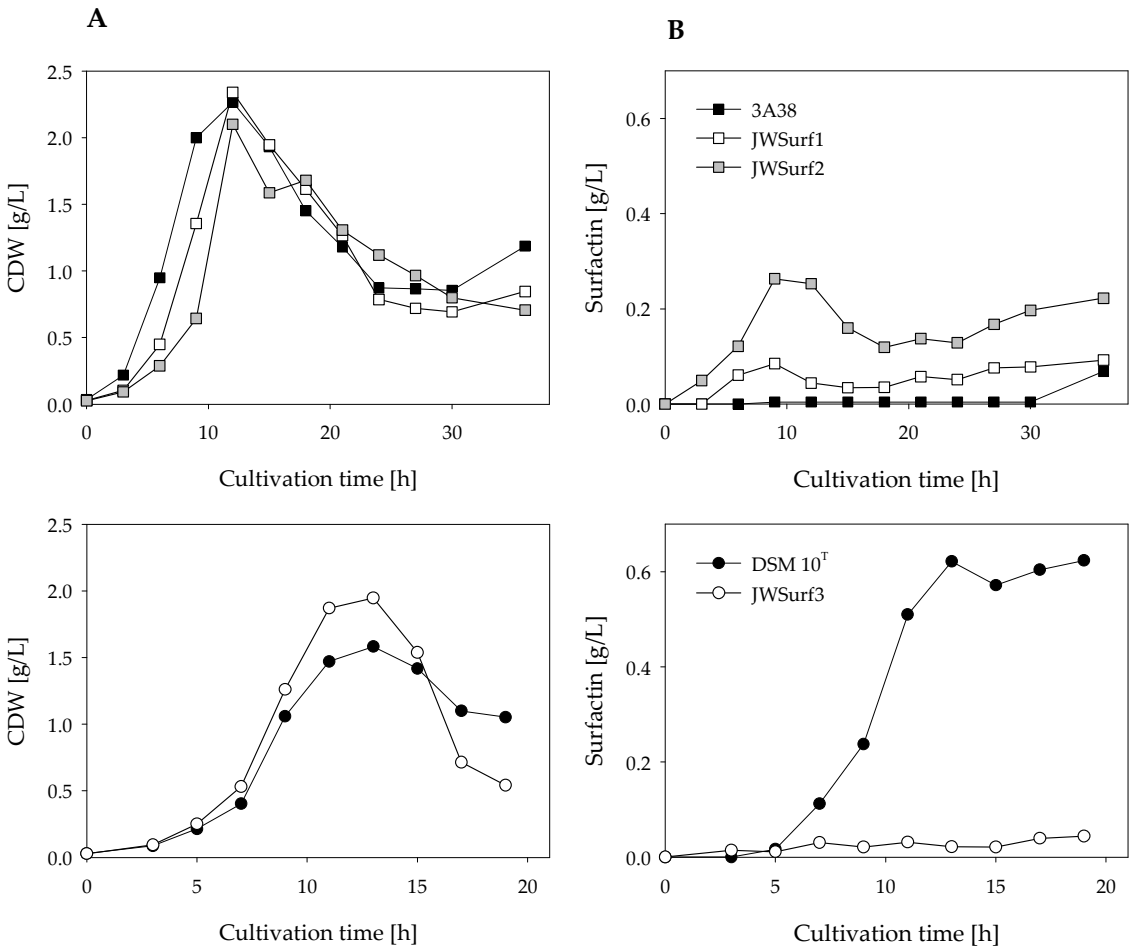

Fig. 5.10 Time course of a shake flask cultivation comparing ancestral and transformed *B. subtilis* strains Illustrated are the CDW (**A**, [g/L]) and Surfactin concentration (**B**, [g/L]) over time. The results of the ancestral strains *B. subtilis* 3A38 and DSM 10^T are indicated as *black square* and *black dot*, respectively, whereas results of *B. subtilis* JWSurf1, JWSurf2 and JWSurf3 are illustrated in *white* and *grey squares* and *white dots*, respectively.

erent Surfactin concentrations. The ancestor strain *B. subtilis* 3A38 produced nearly no Surfactin (0.069 g/L) or at least most of the time Surfactin concentrations beneath the lower detection limit. These results are very surprising as *B. subtilis* DSM 10^T, the almost equivalent strain, produced 0.62 g/L Surfactin. On the contrary, the transformed strains *B. subtilis* JWSurf1, JWSurf2 and JWSurf3 reached Surfactin concentrations between 0.044 g/L and 0.264 g/L. Interestingly, JWSurf1 and JWsurf3 (originating from *B. subtilis* 3A38 and DSM 10^T, respectively) exhibited nearly similar values, whereas JWsurf2 produced slightly more Surfactin. Fig. 5.11 displays the time courses of $q_{Surfactin}$ during shake flask cultivation and values of $q_{Surfactin}$ in comparison to the corresponding specific growth rate μ. The $q_{Surfactin}$ time course of *B. subtilis* DSM 10^T (Fig. 5.11 A) demonstrates a specific progression with local maxima (0.12 g/(g·h)) between 6 h and 13 h of cultivation. In contrast, *B. subtilis* 3A38 demonstrates continuously low values of $q_{Surfactin}$ (0.01 g/(g·h)). The trans-

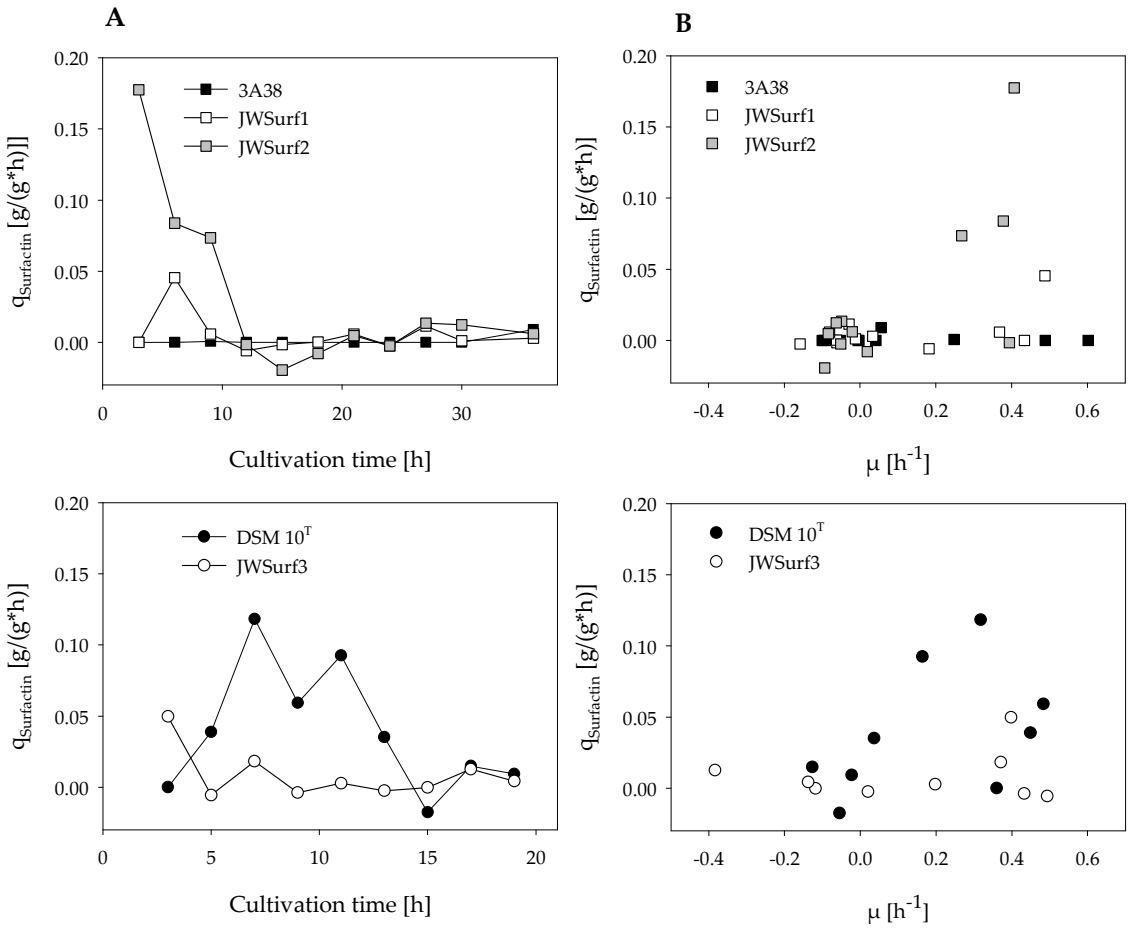

Fig. 5.11 Time course of a shake flask cultivation comparing different *B. subtilis* strains regarding specific growth and production rate Illustrated are the time courses of specific production rate $q_{Surfactin}$ (**A**, [g/(g·h)]) and values of $q_{Surfactin}$ [g/(g·h)] in comparison to the corresponding specific growth rate μ (**B**, [h^{-1}]). The results of *B. subtilis* 3A38 and its descended strains are indicated by *squares*, whereas DSM 10^T and its descended strain are shown by *dots*.

formed strains *B. subtilis* JWSurf1, JWSurf2 and JWsurf3 achieved the highest values of $q_{Surfactin}$ during the beginning of cultivation (3 h – 10 h, 0.05 – 0.18 g/(g·h)) and exhibited low but continuous values of $q_{Surfactin}$ during the following cultivation time (averagely 0.012 g/(g·h)). The values shown in Fig. 5.11 B underline the connection between the formation of product and cell growth. The ancestral strain *B. subtilis* 3A38 presents a continuously low product formation independent from the corresponding cell growth. In contrast, *B. subtilis* DSM 10^T shows a progression in product formation yielding the highest values of $q_{Surfactin}$ during values of 0.1 h^{-1} and 0.5 h^{-1}. The course of $q_{Surfactin}$ presents a local maximum during 0.3 h^{-1}. These findings underline the hypothesis of a cell growth associated product formation. The values of $q_{Surfactin}$ of *B. subtilis* JWSurf1, JWSurf2 and JWSurf3 are mostly increasing simultaneously to the specific growth rate μ. The highest increase is presented by $q_{Surfactin}$ values of JWSurf2 ($q_{Surfactin}$ = 0.18 g/(g·h) at μ = 0.40 h^{-1}), whereas JWSurf1 and JWSurf3 display comparably similar courses ($q_{Surfactin}$ = 0.05 g/(g·h) at μ = 0.4 – 0.5 h^{-1}).

5.4 DISCUSSION

5.4.1 Comparison of the promoter replacement in *B. subtilis* 3A38 and DSM 10^T

The first results illustrate a very differing Surfactin productivity of the ancestral strains *B. subtilis* 3A38 and DSM 10^T. Surprisingly, *B. subtilis* DSM 10^T produces considerably more Surfactin in comparison to the closely related strain *B. subtilis* 3A38. Both strains are supposed to be identical, with the exception of the gene *comI*. Interestingly, the promoter exchange of P_{srfA} against P_{veg} was followed by an increase of Surfactin productivity in *B. subtilis* 3A38 (JWSurf1 and JWSurf2) but lowered the Surfactin productivity in *B. subtilis* DSM 10^T (JWSurf3). Moreover, the strains JWSurf1 and JWSurf3 exhibit very similar values of Surfactin concentrations and $q_{Surfactin}$. This indicates a similar transcription of the *srfA* operon in JWSurf1 and JWSurf3 after introduction of P_{veg}. However, this has to be ascertained by transcription analysis (e.g. by real-time PCR). Anyhow, these findings conflict the previously claimed hypothesis, which suggested a general increase in Surfactin productivity, if the quorum sensing controlled promoter (transcription depending on cell density) is exchanged by a strong and constitutive promoter in front of the *srfA* operon. Until a certain point these considerations are confirmed. The promoter exchange did yield a higher Surfactin productivity in case of *B. subtilis* 3A38. However, this could be the case because the initial Surfactin productivity was comparably low. A strong initial Surfactin productivity (as displayed by *B. subtilis* DSM 10^T) was not further enhanced by the replacement of P_{srfA} against P_{veg}. The studies of Sun et al. (2009) and Coutte et al. (2010) confirm the gained impression after promoter exchange in front of the *srfA* operon in wild type strains producing Surfactin. The study of Sun et al. (2009)

Tab. 5.8 Comparison of various promoter exchange studies regarding productivity and enhancement Various *B. subtilis* strains in which promoters were substituted in front of the *srfA* operon are listed regarding the resulting productivity and enhancement of production.

Strain	Operon	Promoter	Productivity	Enhancement	Study
B. subtilis					Sun et al., 2009
fmbR	*srfA*	P_{srfA}	0.378 g/L		
fmbR-1	*srfA*	P_{spac}	3.866 g/L	10	
B. subtilis					Coutte et al., 2010
BBG111	*srfA*	P_{srfA}	1.504 g/L		
BBG113	*srfA*	P_{repU}	1.213 g/L	-	
B. subtilis					This study
3A38	*srfA*	P_{srfA}	0.069 g/L		
JWSurf1	*srfA*	P_{veg}	0.093 g/L	1.4	
JWSurf2	*srfA*	P_{veg}	0.264 g/L	3.8	
DSM 10^T	*srfA*	P_{srfA}	0.624 g/L		
JWSurf3	*srfA*	P_{veg}	0.044 g/L	-	

employed a Surfactin producer strain initially yielding low Surfactin concentrations (Tab. 5.8). Promoter replacement induced an enhancement of Surfactin yields. The promoter exchange during the study of Coutte et al. (2010) did not achieve an enhancement of Surfactin concentrations. The employed producer strain originally yielded high amounts of Surfactin (Tab. 5.8). If both studies are compared to each other, these findings could be explained by the utilization of different promoters for replacement of P_{srfA} (P_{spac} and P_{repU}, respectively). A possible explanation could be that P_{spac} is a stronger promoter than P_{repU}. But considering the findings of the current study a natural strong promoter activity of P_{srfA} should also be taken into account.

5.4.2 General evaluation of the promoter replacement

The results of the current study indicate a more complex regulation of the Surfactin synthesis then initially thought. Obviously is the synthesis of Surfactin not only depending on promoter activity. Due to its quorum sensing dependence the promoter activity of P_{srfA} was thought to be the bottleneck of the Surfactin synthesis. As a result of the findings of the current study further aspects have to receive attention. For instance is the natural transcription initiation of the *srfA* operon regulated by the intracellular concentration of activated ComA. The more activated ComA is present, the more or the earlier transcription is started. The concentration of activated ComA inside the cell is influenced by several regulatory molecules (RapC, RapF, etc.), which repress the increase

of activated ComA (overview see Soberón-Chavéz and Jacques 2011). As a consequence, the promoter activity could be influenced by the natural ability to produce more or less of these repressors. If *B. subtilis* DSM 10^T produces a lower amount of these regulatory molecules in comparison to *B. subtilis* 3A38 this would consequentially be followed by a higher Surfactin productivity. However, this hypothesis can only be verified by transcription analysis of the responsible genes in *B. subtilis* 3A38 and DSM 10^T. Next to the amount of activated transcription factor the start of transcription is also influenced by several regulating molecules. These are e.g., activated DegU, CodY and AbrB which negatively influence the transcription of the *srfA* operon, due to its part in genetic competence (overview see Hamoen et al. 2003). These regulating factors could also explain differing activity of P_{srfA} in various Surfactin producer strains. Next to the consideration of transcription initiation it is important to realize the overall process of Surfactin synthesis and all its intermediate steps. Different Surfactin producer strains could also vary in their ability to perform translation, assembly the subunits of the Surfactin synthetase, enzyme activity and production of precursor molecules. It becomes apparent that the synthesis of Surfactin is a highly complex process involving several independent steps, which all eventually influence the final concentration of Surfactin. However, subsequent investigations are necessary to fully understand the synthesis of Surfactin and identify the bottleneck of production. Most likely, as indicated by the current study and other investigations, uncoupling from quorum sensing control does not generally yield a significant enhancement of Surfactin concentrations. Hence, promoter activity seems not to be the only limiting factor.

5.5 CONCLUSION

The replacement of the naturally quorum sensing regulated (and herewith cell density dependent) promoter P_{srfA} against the endogenous constitutive promoter P_{veg} was thought to generally enhance Surfactin yields. The markerless promoter replacement was conducted in the two, mostly identical, *B. subtilis* Surfactin producer strains 3A38 and DSM 10^T. The promoter replacement led to an enhancement of Surfactin yields in the producer strain 3A38, initially producing only minor amounts of Surfactin. In contrast, promoter exchange in DSM 10^T (wild type strain producing high Surfactin concentrations) did not achieve an enhancement of Surfactin yields. These findings implicate a much more complex overall process of biosynthesis then earlier thought. Subsequent investigations are necessary to fully understand the differing regulation of P_{srfA} and find a solution to further increase Surfactin yields in naturally strong Surfactin producer strains.

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6. CONCLUSION

Surfactin is one of the most promising microbial surfactants as it exhibits extraordinary surface activity and is produced by the well-established GRAS organism *Bacillus subtilis*. As a consequence, widespread studies were conducted during this thesis to examine the essentials of the microbial production of Surfactin.

At the beginning of this thesis a screening was performed in order to identify one or even more Surfactin producer strains. Six different *Bacillus* strains were discovered and characterized in a model fermentation process applying integrated foam fractionation. At least two of the described strains have not been reported as Surfactin producers before (DSM 1090 and DSM 28227). A comparison of different Surfactin producer strains in this manner was previously not reported. The evaluation revealed only slight differences between the Surfactin producer strains but outlined the characteristic features of every strain which can now be employed according to their distinctive features. In addition, the approach of foam fractionation proved to be a useful tool for the treatment of foam and the study demonstrated an outstanding suitability of *B. subtilis* for such an application.

The following study employed a fermentation process which combined both the abilities of being foam-free and anaerobic. Foam-free or anaerobic cultivations for the production of Surfactin have been conducted before, but the advantages of a combination were previously not reported. In fact, the exclusion of a gas-flow through the medium completely avoided the formation of any foam introducing an innovative foam-free production of Surfactin. Furthermore, this investigation demonstrated a surprisingly high production of Surfactin per CDW under these conditions which highlights the major advantage of this process in comparison to earlier foam-free approaches.

In the following, the work of this thesis was focused on the medium composition. Investigations concerning this topic previously were more or less stagnated as the Cooper medium was applied

relatively unmodified for the production of Surfactin. During this thesis the employment of lower glucose concentrations was investigated, revealing a much higher productivity of the applied *B. subtilis* strain DSM 10^T. Subsequently, the nitrogen source and chelating agent were additionally altered to obtain an improved medium, closer to industrial standards. Furthermore, this investigation proved a general increase in Surfactin productivity as most of the analyzed *Bacillus* strains produced more Surfactin during cultivations employing the optimized medium. Moreover, the establishment of a fed-batch fermentation, where glucose was added after its depletion, led to an increase of Surfactin productivity of 30 %. This study demonstrated the improvement of a well-established medium while avoiding substrate waste.

The creation of an overproducing Surfactin strain could majorly contribute to its application in industrial scale. Therefore *B. subtilis* strains were generated which allowed the constitutive expression of the Surfactin synthetase unaffected from quorum sensing control. It was hypothesized that a promoter replacement of natural P_{srfA} in front of the *srfA* operon against the constitutive promoter P_{veg} would lead to a general increase of Surfactin productivity. The hypothesis was confirmed after promoter exchange in *B. subtilis* 3A38, yielding *B. subtilis* JWSurf1 and JWSurf2 (several fold increase of Surfactin concentrations) but was not verified by promoter replacement in *B. subtilis* DSM 10^T (JWSurf3), which led to a reduction of Surfactin productivity. These findings implicate a much more complex overall process of Surfactin biosynthesis then initially thought. Future investigations are therefore necessary to identify the true limiting factor of Surfactin production.

All of the conducted investigations during this thesis were focused on the microbial production of Surfactin by *B. subtilis*. The biotechnological process of Surfactin production was engaged from its starting point by identifying Surfactin producer strains and approaching a genetic optimization. Furthermore, conditions involving both medium composition and fermentation approach were investigated yielding an increase in productivity. Hence, this thesis provides an overall evaluation of different producer strains and process strategies for the production of Surfactin.

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APPENDIX

A.1 Genomic DNA of *Bacillus subtilis* DSM 10^T in front of *srfA* The sequencing result was obtained by the GATC Biotech AG after PCR using gDNA from *Bacillus subtilis* DSM 10^T and the primers "hxIR 700 forward 2" and "srfA 700 reverse 2" (Tab. 5.7). The start of the *srfA-A* ORF is indicated by a *dashed underline* (the starting ATG is additionally illustrated in *red*). The RBS is indicated in *blue*, the regions -10 and -35 are emphasized in *green*, and the dyad symmetries are illustrated in *orange*. The complete promoter region P_{srfA} is shown by a *dotted underline*. The rho-independent terminator sequence of *hxIR* is indicated by *purple* characters.

TCAGATTTCTTTCGCTTGATTTTATGAGTGAGTATAATGAAAAAAGAGTCTGCAAAAAAGT

AAGTAAGTGATGATACGTATCTAACATACAAAAAGGTTACTAAGGGGGGGATTCTATTGAG

CCGGATGGACGACAAAAGGTTTAATTGTGAGAAGGAATTAACGCTTGCAGTGATTGGCGGT

AAATGGAAAATGCTCATTTTATGGCATTTAGGAAAAGAAGGCACAAAACGGTTCAATGAAT

TAAAAACATTGATTCCTGATATTACGCAGAAGATCCTCGTGAATCAGCTGAGAGAGCTTGA

GCAGGATATGATTGTTCACAGGGAAGTGTATCCAGTTGTCCCGCCGAAGGTTGAATATTCT

CTGACCCCGCACGGAGAAAGCCTCATGCCTATTCTTGAAGCCATGTATGAGTGGGGGAAAG

GCTATATGGAATTGATTGATATCGACAAAAATGTCATGAAAGAATCGTTGTAAGACGCTCT

TCGCAAGGGTGTCTTTTTTTGCCTTTTTTTCGGTTTTTGCGCGGTACACATAGTCATGTAA

AGATTGTAAATTGCATTCAGCAATAAAAAAAGATTGAACGCAGCAGTTTGGTTTAAAAATT

TTTATTTTTCTGTAAATAATGTTTAGTGGAAATGATTGCGGCATCCCGCAAAAAATATTGC

TGTAAATAAACTGGAATCTTTCGGCATCCCGCATGAAACTTTTCACCCATTTTTCGGTGAT

AAAAACATTTTTTTCATTTAAACTGAACGGTAGAAAGATAAAAAATATTGAAAACAATGAA

TAAATAGCCAAAATTGGTTTCTTATTAGGGTGGGGTCTTGCGGTCTTTATCCGCTTATGTT

AAACGCCGCAATGCTGACTGACGGCAGCCTGCTTTAATAGCGGCCATCTGTTTTTTGATTG

GAAGCACTGCTTTTTAAGTGTAGTACTTTGGGCTATTTCGGCTGTTAGTTCATAAGAATTA

AAAGCTGATATGGATAAGAAAGAGAAAATGCGTTGCACATGTTCACTGCTTATAAAGATTA

GGGGAGGTATGACAATATGGAAATAACTTTTTACCCTTTAACGGATGCACAAAAACGAATT

TGGTACACAGAAAAATTTTATCCTCACACGAGCATTTCAAATCTTGCGGGGATTGGTAAGC

TGGTTTCAGCTGATGCGATTGATTATGTGCTTGTTGAGCAGGCGATTCAAGAGTTTATTCG

CAGAAATGACGCCATGCGCCTTCGGTTGCGGCTAGATGAAAACGGGGAGCCTGTTCAATAT

ATTAGCGAGTATCGGCCTGTTGATATAAAACATACTGACACTACTGAAGATCCGAATGCGA

TAGAGTTTATTTCACAATGGAGCCGGGAGGAAACGAAGAAACCTTTGCCGCTATACGATTG

TGATTTGTTCCGTTTTTCCTTGTTCACCATAAAGGAAAATGAAGTGTGGTTTTACGCAAAT

GTTCATCACGTGATTTCTGATGGTATCTCCATGAATATTCTCGGGAATGCGATCATGCACA

TTTATTTAGAATTAGCCAGCGGCTCAGAGACAAAAGAAGGAATCTCGCATTCATTTATCGA

TCATGTTTTATCTGAACAGGAATATGCTCAATCGAAGCGGTTTGAAAAGGACAAGGCGTTT
TGGAACAAACAATTTGAATCGGTGCCTGAACTTGTTTCCTTGAAACGGAATGCATCCGCAG
GGGGAAG

A.2 Genomic DNA of *Bacillus subtilis* 3A38 in front of *srfA* The sequencing result was obtained by the GATC Biotech AG after PCR using gDNA from *Bacillus subtilis* 3A38 and the primers "final check fwd" and "final check rev" (Tab. 5.7). The start of the *srfA-A* ORF is indicated by a *dashed underline* (the starting ATG is additionally illustrated in *red*). The RBS is indicated in *blue,* the regions -10 and -35 are emphasized in *green,* and the dyad symmetries are illustrated in *orange*. The complete promoter region P_{srfA} is shown by a *dotted underline*. The rho-independent terminator sequence of *hxIR* is indicated by *purple* characters.

TATTCTCTGACCCCGCACGGAGAAAGCCTCATGCCTATTCTTGAAGCCATGTATGAGTGGG

GGAAAGGCTATATGGAATTGATTGATATCGACAAAAATGTCATGAAAGAATCGTTGTAAGA

CGCTCTTCGCAAGGGTGTCTTTTTTTGCCTTTTTTTCGGTTTTTGCGCGGTACACATAGTC

ATGTAAAGATTGTAAATTGCATTCAGCAATAAAAAAAGATTGAACGCAGCAGTTTGGTTTA

AAAATTTTTATTTTTCTGTAAATAATGTTTAGTGGAAATGATTGCGGCATCCCGCAAAAAA

TATTGCTGTAAATAAACTGGAATCTTTCGGCATCCCGCATGAAACTTTTCACCCATTTTTC

GGTGATAAAAACATTTTTTTCATTTAAACTGAACGGTAGAAAGATAAAAAATATTGAAAAC

AATGAATAAATAGCCAAAATTGGTTTCTTATTAGGGTGGGGTCTTGCGGTCTTTATCCGCT

TATGTTAAACGCCGCAATGCTGACTGACGGCAGCCTGCTTTAATAGCGGCCATCTGTTTTT

TGATTGGAAGCACTGCTTTTTAAGTGTAGTACTTTGGGCTATTTCGGCTGTTAGTTCATAA

GAATTAAAAGCTGATATGGATAAGAAAGAGAAAATGCGTTGCACATGTTCACTGCTTATAA

AGATTAGGGGAGGTATGACAATATGGAAATAACTTTTTACCCTTTAACGGATGCACAAAAA

CGAATTTGGTACACAGAAAAATTTTATCCTCACACGAGCATTTCAAATCTTGCGGGGATTG

GTAAGCTGGTTTCAGCTGATGCGATTGATTATGTGCTTGTTGAGCAGGCGATTCAAGAGTT

TATTCGCAGAAATGACGCCATGCGCCTTCGGTTGCGGCTAGATGAAAACGGGGAGCCTGTT

CAATATATTAGCGAGTATCGGCCTGTTGATATAAAACATACTGACACTACTGAAGATCCG

A.3 Genomic DNA of *Bacillus subtilis* JWSurf1 in front of *srfA* The sequencing result was obtained by the GATC Biotech AG after PCR using gDNA from *Bacillus subtilis* JWSurf1 and the primers "final check fwd" and "final check rev" (Tab. 5.7). The start of the *srfA-A* ORF is indicated by a *dashed underline* (the starting ATG is additionally illustrated in *red*). The RBS is indicated in *blue*, and the regions -10 and -35 are emphasized in *green*. The complete promoter region P_{veg} is shown by a *dotted underline*. The rho-independent terminator sequence of *hxIR* is indicated by *purple* characters.

GGGAAGTGTATCCAGTTGTCCCGCCGAAGGTTGAATATTCTCTGACCCCGCACGGAGAAAG

CCTCATGCCTATTCTTGAAGCCATGTATGAGTGGGGGAAAGGCTATATGGAATTGATTGAT

ATCGACAAAAATGTCATGAAAGAATCGTTGTAAGACGCTCTTCGCAAGGGTGTCTTTTTTT

GCCTTTTTTTCGGTTTTTGCGCGGTACACATAGTCATGTAAAGATTGTAAATTGCATTCAG

CAATAAAAAAAGATTGAACGCAGCAGTTTGGTTTAAAAATTTTTATTTTTCTGTAAATAAT

GTTTAGTGGAAATGACTTATTAACGTTGATATAATTTAAATTTTATTGACAAAAATGGGC

TCGTGTTGTACAATAAATGTATTAGGGGAGGTATGACAATATGGAAATAACTTTTTACCCT

TTAACGGATGCACAAAAACGAATTTGGTACACAGAAAAATTTTATCCTCACACGAGCATTT

CAAATCTTGCGGGGATTGGTAAGCTGGTTTCAGCTGATGCGATTGATTATGTGCTTGTTGA

GCAGGCGATTCAAGAGTTTATTCGCAGAAATGACGCCATGCGCCTTCGGTTGCGGCTAGAT

GAAAACGGGGAGCCTGTTCAATATATTAGCGAGTATCGGCCTGTTGATATAAAACATACTG

ACACTACTGAAGATCCGAATGCGATAGAG

A.4 Genomic DNA of *Bacillus subtilis* JWSurf2 in front of *srfA* The sequencing result was obtained by the GATC Biotech AG after PCR using gDNA from *Bacillus subtilis* JWSurf2 and the primers "final check fwd" and "final check rev" (Tab. 5.7). The start of the *srfA-A* ORF is indicated by a *dashed underline* (the starting ATG is additionally illustrated in *red*). The RBS is indicated in *blue,* and the regions -10 and -35 are emphasized in *green*. The complete promoter region P_{veg} is shown by a *dotted underline*. The rho-independent terminator sequence of *hxIR* is indicated by *purple* characters.

AGCAGGATATGATTGTTCACAGGGAAGTGTATCCAGTTGTCCCGCCGAAGGTTGAATATTC

TCTGACCCCGCACGGAGAAAGCCTCATGCCTATTCTTGAAGCCATGTATGAGTGGGGGAAA

GGCTATATGGAATTGATTGATATCGACAAAAATGTCATGAAAGAATCGTTGTAAGACGCTC

TTCGCAAGGGTGTCTTTTTTTGCCTTTTTTTCGGTTTTTGCGCGGTACACATAGTCATGTA

AAGATTGTAAATTGCATTCAGCAATAAAAAAAGATTGAACGCAGCAGTTTGGTTTAAAAAT

TTTTATTTTTCTGTAAATAATGTTTAGTGGAAATGACTTATTAACGTTGATATAATTTAAA

TTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTATTAGGGGAGGTATGACAAT

ATGGAAATAACTTTTTACCCTTTAACGGATGCACAAAAACGAATTTGGTACACAGAAAAAT

TTTATCCTCACACGAGCATTTCAAATCTTGCGGGGATTGGTAAGCTGGTTTCAGCTGATGC

GATTGATTATGTGCTTGTTGAGCAGGCGATTCAAGAGTTTATTCGCAGAAATGACGCCATG

CGCCTTCGGTTGCGGCTAGATGAAAACGGGGAGCCTGTTCAATATATTAGCGAGTATCGGC

CTGTTGATATAAAACATACTGACACTACTGAAGATCCGAA

A.5 Genomic DNA of *Bacillus subtilis* JWSurf3 in front of *srfA* The sequencing result was obtained by the GATC Biotech AG after PCR using gDNA from *Bacillus subtilis* JWSurf3 and the primers "final check fwd" and "final check rev" (Tab. 5.7). The start of the *srfA-A* ORF is indicated by a *dashed underline* (the starting ATG is additionally illustrated in *red*). The RBS is indicated in *blue,* and the regions -10 and -35 are emphasized in *green.* The complete promoter region P_{veg} is shown by a *dotted underline*. The rho-independent terminator sequence of *hxIR* is indicated by *purple* characters.

ATTGTTCACAGGGAAGTGTATCCAGTTGTCCCGCCGAAGGTTGAATATTCTCTGACCCCGC

ACGGAGAAAGCCTCATGCCTATTCTTGAAGCCATGTATGAGTGGGGGAAAGGCTATATGGA

ATTGATTGATATCGACAAAAATGTCATGAAAGAATCGTTGTAAGACGCTCTTCGCAAGGGT

GTCTTTTTTTGCCTTTTTTTCGGTTTTTGCGCGGTACACATAGTCATGTAAAGATTGTAAA

TTGCATTCAGCAATAAAAAAAGATTGAGCGCAGCAGTTTGGTTTAAAAATTTTTATTTTTC

TGTAAATAATGTTTAGTGGAAATGACTTATTAACGTTGATATAATTTAAATTTTATTTGAC

AAAAATGGGCTCGTGTTGTACAATAAATGTATTAGGGGAGGTATGACAATATGGAAATAAC

TTTTTACCCTTTAACGGATGCACAAAAACGAATTTGGTACACAGAAAAATTTTATCCTCAC

ACGAGCATTTCAAATCTTGCGGGGATTGGTAAGCTGGTTTCAGCTGATGCGATTGATTATG

TGCTTGTTGAGCAGGCGATTTAAGAGTTTATTCGCAGAAATGACGCCATGCGCCTTCGGTT

GCGGCTAGATGAAAACGGGGAGCCTGTTCAATATATTAGCGAGTATCGGCCTGTTGATATA

AAACATACTGACACTACTGAAGATCCGAATGCGATAGAGTTTATTTCACAATGGAGCCG

A.6 Sequence applied for pO_2 adjustment by the process control system IRIS The illustrated sequence regulated the stirrer speed during aerobic fermentations. The initial stirrer speed was adjusted to 300 rpm. During fermentation values of dissolved oxygen were not allowed to fall below 10 %. The process control system therefore continuously red the pO_2 values and regulated the stirrer speed accordingly.

```
//pO2 Regelung ueber Ruehrer

#0
if (stirrer.sp < 300) {stirrer.sp=300} else {seq=1}

#1
if (po2.v <=18 AND po2.v >= 8) {seq=2} else {seq=5}// pO2 != 8.....18

#2
if(seq_time>=50) {seq=0} // Warteschleife aus seq=0 (pO2 = 8....18)

#3
if(seq_time>=10) {seq=0}

#4
if (seq_time>=20) {seq=0}

#5
if (po2.v < 4 AND stirrer.sp <=1180) {seq=8} else { if (po2.v >38 AND
stirrer.sp >=315) {seq=9} else {seq=12} } // Ueberpruefung auf hohe
Sollwertdifferenz

#12
if (po2.v < 6 AND po2.v >4 AND stirrer.sp <=1190) {seq=10} else  {if
(po2.v >23 AND po2.v <38 AND stirrer.sp >=305 )    {seq=11} else {if
(po2.v >18 AND stirrer.sp >=302) {seq=6} else {seq=7} }}

#6
if (stirrer.sp > 302) {stirrer.sp = stirrer.sp - 2}
seq=2;

#7
if (stirrer.sp < 1198) {stirrer.sp = stirrer.sp + 2}
seq=2;

#8
stirrer.sp = stirrer.sp + 20; // starke Regelung aus seq=4
seq=3;

#9
stirrer.sp = stirrer.sp - 15 ; // starke Regelung aus seq=4
seq=3 ;

#10
stirrer.sp = stirrer.sp + 10;
seq=4;

#11
stirrer.sp = stirrer.sp - 5;
seq=4;
```

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