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Dissertation

Sulfolobus acidocaldarius – establishing a novel host for cutting-edge biotechnology at its extremes

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Abstract

We live in exciting times! The importance of the once overly dominant production host *Escherichia coli* is dwindling, while cell culture and more exotic microbial hosts are taking over the playing field. Within this Thesis I developed fundamental methods – analytic procedures and process technology alike – with the capability to catalyze the adoption of the extreme thermo-acidophilic Archaeon *Sulfolobus acidocaldarius* in future commercially relevant bioprocesses.

S. acidocaldarius grows optimally at 75 °C and pH 3. It is amenable to genetic manipulation and characterized by several very unique metabolic pathways, making it a promising candidate for bioconversion processes (e.g. for upcycling of biogenic waste streams or for treatment of process wastewaters), and for the production of unique biological material (e.g. tetraether lipids used in medical applications for the formation of drug delivery vehicles). A further promising field of application is bioleaching for the economic mining of low grade ores in large scale. In general, due to its harsh growth conditions the organism is predestined for processes where sterility is difficult to achieve and/or to maintain. Difficult to sterilize batches and continuous processes which run over extended periods of time are important examples.

Important prerequisites for the use of *S. acidocaldarius* in industrial applications, like characterization of the host´s biology, development of genetic tools and clinical safety, can be considered to be met. Nevertheless, some aspects still need to be investigated in much more detail and a selection of remaining challenges were tackled within this Thesis. Firstly, for the investigation of the potential of *S. acidocaldarius* in the field of waste-to-value applications I performed extensive growth experiments on wheat straw and beech wood hydrolysates with wild type strains and genetically modified *S. acidocaldarius* mutants to utilize non-food substrate streams for the production of value-added products. Secondly, to push into pharmaceutical applications and the market of high value products I developed a defined cultivation medium adhering to Quality by Design guidelines. The medium was optimized in regards of salt and trace element composition. Complex protein hydrolysates, which can be inhomogeneous in their composition and are prone to formation of inhibiting substances under growth conditions, were replaced by a combination of sodium glutamate and citric acid. Thirdly, for the assessment of cell viability, a critical parameter during process development and control, fluorescence based assays were developed. Finally, I determined critical process parameters during the cultivation of *S. acidocaldarius* for the direct manipulation of the organisms´ membrane composition. Thereby it is possible to generate a tailor-made pattern of industrially relevant lipids that might be recoverable to an increased yield via a greatly facilitated downstream procedure.

Zusammenfassung

Aufregende Zeiten brechen an! Die Bedeutung des einst so dominanten Produktionsorganismus *Escherichia coli* schwindet, während Zellkulturen und deutlich exotischere Mikroorganismen auf den Plan treten. Die vorliegende Arbeit beschreibt grundlegende analytische und prozesstechnologische Methoden, die das Potential haben, als Katalysator für den Einsatz des extrem thermo-acidophilen Archaeons *Sulfolobus acidocaldarius* in zukünftigen kommerziellen Bioprozessen zu dienen.

S. acidocaldarius wächst optimal bei 75 °C und einem pH Wert von 3. Der Organismus ist zugänglich für genetische Manipulation und zeichnet sich durch mehrere einzigartige Stoffwechselwege aus. Diese Eigenschaften machen ihn zu einem vielversprechenden Kandidaten für Biokonversionsprozesse (z.B. im Upcycling von biogenen Abfallströmen oder in der Abwasserbehandlung), sowie für die Produktion von einzigartigen biologischen Materialien (z.B. von Tetraetherlipiden welche in pharmazeutischen Anwendungen zur Bildung von Vesikeln zur gezielten Arzneimittel-verabreichung verwendet werden). Ein weiteres vielversprechendes Anwendungsgebiet ist die biologische Erzlaugung („Bioleaching") zur effizienten Gewinnung von Metallen aus niedrig konzentrierten Erzen. Aufgrund seiner harschen Wachstumsbedingungen ist der Organismus im Allgemeinen bestens für den Einsatz in Prozessen gerüstet, in denen sterile Bedingungen kaum herzustellen beziehungsweise schwierig aufrechtzuerhalten sind. Aufwändig zu sterilisierende Anlagen und Rohstoffe sowie kontinuierliche Prozesse, welche über ausgedehnte Zeitspannen laufen, sind hierfür wichtige Beispiele.

Wesentliche Voraussetzungen für den Einsatz von *S. acidocaldarius* in industriellen Anwendungen, wie etwa die biologische Charakterisierung des Organismus, Entwicklung von molekularbiologischen Werkzeugen, sowie die gesundheitliche Unbedenklichkeit, sind bereits als erfüllt anzusehen. Indes warten noch einige Aspekte darauf im Detail untersucht zu werden und diese Arbeit wird sich eines Teils der noch bestehenden Herausforderungen annehmen. Erstens führte ich Machbarkeitsstudien über die Eignung von Weizenstroh- und Buchenholzhydrolysaten als Nährsubstrate durch, um das Potential von *S. acidocaldarius* im Upcycling von biogenen Abfallströmen zu evaluieren. Zweitens entwickelte ich ein definiertes Kultivierungsmedium zur Produktion von High-End-Chemikalien unter anderem für den Einsatz im pharmazeutischen Bereich, welches, von nationalen und internationalen Gesundheitsbehörden geforderten, Quality by Design Kriterien genügt. Drittens entwickelte ich fluoreszenzbasierte Analysemethoden zur Beurteilung der Zellviabilität, einem entscheidenden Parameter bei Entwicklung und Steuerung von Bioprozessen. Viertens bestimmte ich kritische Prozessparameter währen der Kultivierung von *S. acidocaldarius*, die sich zur gezielten Manipulation der Membranzusammensetzung des Organismus eignen. Dadurch ist es möglich, ein Muster von maßgeschneiderten, industriell relevanten Lipiden mit erhöhten Ausbeuten und deutlich vereinfachter Gewinnung und Aufreinigung zu erhalten.

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Introduction

The Extremes of Biotechnology

Although traditional biotechnology mostly relies on mesophilic bioprocesses, extremophiles are exploited in several fields of the bioprocess industry. One particularly successful application is the utilization of their astonishingly stable enzymes, so called *extremozymes*. For this purpose it is sufficient to get hold of the DNA that codes for the enzyme of interest and no further cultivation or characterization of the natural host organism is necessary. Thanks to the huge amount of available metagenomic data, today even enzymes of not yet cultured extremophiles can be utilized in that way via recombinant expression of the extremozymes in a heterologous host. The extremozymes are deployed in the feed, food and beverage industry, textile and cleaning industry and pulp and paper industry, but also in scientific research and diagnostics. A high demand exists for starch-hydrolyzing (Elleuche and Antranikian, 2013), (hemi)cellulolytic (Beg et al., 2001; Kuhad et al., 2011), pectinolytic (Sharma et al., 2013), chitinolytic (Chavan and Deshpande, 2013), proteolytic (Li et al., 2013), and lipolytic (Hasan et al., 2006) enzymes (Elleuche et al., 2015). Applications for the highly stable enzymes are found at high or low temperature processes, processes at extreme pH values, in organic solvents and under hyper-saline conditions. But also under moderate conditions extremozymes show superior properties compared to conventional enzymes regarding their long time stability, a most crucial factor in applications with immobilized enzymes, where the biocatalysts are used continuously or for multiple consecutive conversion steps.

Besides such applications that utilize only the *genes* of extremophiles and generally produce the extremozymes in a heterologous host organism, a number of applications exist, that are based on the use of the whole extremophilic organism, since the product of interest is generated via a complex metabolic pathway that cannot simply be implemented at will in a heterologous host. This is, for example, the case for the biological production of methane that is carried out with methanogenic Archaea today. Production of biomethane is performed either for the valorization of biotechnological, agricultural or food waste streams (conversion of methyl compounds, formate, acetate or pyruvate), or for the conversion of CO_2 and H_2 into methane known by the catchphrases “biological methanation” and “power-to-gas” (Frigon and Guiot, 2010; Hedderich and Whitman, 2013; Lecker et al., 2017).

The field of waste water treatment also harbors interesting applications for extreme thermophiles as well as halophilic organisms. In particular industrial waste streams often are discharged at high temperatures or come with a high salt burden. Microorganisms that are capable of thriving in such environments are successfully utilized for the elimination of toxic compounds in waste waters that are otherwise not amenable to biological waste water treatment or else would require a preceding cooling or dilution step (Lefebvre and Moletta, 2006; Duncan et al., 2017).

Today, approximately 15% of the global copper production and 5% of the global gold production are based on biological leaching processes that are used for the treatment of low

grade ores (Donati et al., 2016). Since low grade ores will become the dominant resource for most metals in the future (Norgate and Jahanshahi, 2010), the importance of bioleaching is expected to increase steadily. Leaching in open heap systems is usually carried out with moderate thermophiles with a temperature optimum at around 40 to 50 °C, while leaching in controlled reactors is preferentially performed using extreme thermophiles with optimal growth temperatures of 65 °C and higher (Batty and Rorke, 2006). Advantage of the higher temperature is that the most abundant, but under mesophilic conditions not accessible, copper mineral, chalcopyrite, (accounting for more than 70% of earth´s copper) can be utilized under these conditions and overall higher yields are achievable (Panda et al., 2015). Besides copper and gold, cobalt, nickel and zinc are further metals that are produced via bioleaching. Although sulfuric acid is a problematic byproduct of bioleaching, the process is an attractive alternative to the practice of chemical leaching where huge amounts of chemicals, like cyanides, highly toxic both to the environment and human health, are employed (Norgate and Haque, 2012; Jerez, 2017).

Canthaxanthin is a lipid-soluble carotenoid with antioxidant activity used in the cosmetics industry, as food dye and feed additive in fish, crustacean, and poultry farms (Esatbeyoglu and Rimbach, 2017). Although canthaxanthin can also be produced via chemical synthesis, there is an increasing demand for biologically produced canthaxanthin especially in the food and cosmetic industry (Global Market Insights, 2018). Natural canthaxanthin is typically produced using the halophilic Archaeon *Haloferax alexandrines* (Asker and Ohta, 2002) and its market was over 35 million USD in 2017 with an expected annual growth rate of 3%, while the total market of canthaxanthin is expected to reach over 85 million USD by 2024 (Global Market Insights, 2018).

A not yet commercially utilized but potentially interesting product of extremophilic origin is bacteriorhodopsin, a membrane-bound pigment protein produced by the haloarchaeum *Halobacterium salinarum*. Bacteriorhodopsin acts as a light-driven proton pump and contains a retinal molecule that experiences a conformation change upon absorption of a photon. By passing on the conformation change to the surrounding protein, bacteriorhodopsin transfers a proton across the archaeal membrane. Consecutively *H. salinarum* utilizes the forming protein gradient for phototrophic generation of ATP. Bacteriorhodopsin was proposed for the use in numerous optical applications, e.g. holography, artificial retinas, photochromic dyes or spatial light modulators (Coker, 2016).

Over the last years also archaeal lipids sparked strong interest in the biotech industry as vehicles for drug, vaccine and gene delivery due to their outstanding chemical and thermal stability (Quehenberger et al., 2017; van Hoogevest, 2017).

Summarizing, the most important commercial bulk products derived from extremophilic organisms are extremozymes, biomethane, cleaned water and extracted metal. An example for a commercially important high value compounds is the carotenoid canthaxanthin used in the cosmetics, food and feed industry.

The Extreme Domain of Archaea

History and outer phylogeny: In 1977/78 Carl Woese and coworkers discovered and described the distinctiveness of Archaea and their classification as separate phylogenetic line of decent (Woese and Fox, 1977; Woese, 1987), a finding that would eventually revolutionize the way of how we picture the phylogenetic tree of life: In the year of 1990 Woese, Otto Kandler and Mark Wheelis proposed the today commonly acknowledged reclassification in three domains of life: Bacteria, Archaea[1] and Eukarya. Due to their communality of a lack of a membrane bound nucleus, the domains of Bacteria and Archaea are assigned to the group of prokaryotes. Many early discoveries of Archaea were made in very harsh environments under conditions of elevated temperatures, high pressure, and a limited supply of organic carbon sources. Since these conditions were, during that time, thought to prevail during the dawn of life, the term "Archaea", (from "archaios" ancient greek: *ancient*, *primal*) was chosen based on the assumption these organisms represented the ancestors of all living life forms. Today, we know that, although Archaea and Bacteria share a prokaryotic morphology and similar pathways for central metabolism and energy conversion, information-processing in Archaea and Eukarya is driven by analogous enzymes; though archaeal systems are much simpler. This assumption of Archaea being the ancestors of life as we know it is disproven by this similarity between Archaea and the highly developed Eukarya, forming an exclusive monophyletic clade and due to the fact that Archaea and Bacteria differ substantially in many physiologic and genetic aspects. Key distinctive features between Archaea and Bacteria are: 1) Structure and composition of the ribosomal RNA (mainly 16S-rRNA); 2) Structure of the RNA polymerase and components involved in translation; 3) Lack of peptidoglycan (murein) and instead presence of pseudopeptidoglycan, glycoproteins or S-layer; 4) Due to the different cell wall composition and genetic system Archaea are resistant towards many antibiotics commonly employed against Bacteria (e.g. penicillin, chloramphenicol, kanamycin, rifampicin), while diphtheria toxin, a substance that inhibits protein synthesis in Eukaryotes, is also toxic for Archaea; 5) Composition of archaeal membrane lipids that consist of an isoprenoid core structure linked to glycerol in *sn*-2,3 position via ether bonds compared to in *sn*-1,2 position linked bacterial fatty acid ester lipids; 6) Archaea feature unique metabolic pathways and utilize unusual cofactors and vitamins (Martin, 2004). In addition to the alternatingly shared properties with Bacteria and Eukarya, as much as 50% of the genes found in archaeal genomes are estimated to encode novel proteins with no obvious counterparts in the other domains of life (Allers and Mevarech, 2005).

Inner phylogeny: The overwhelming majority of culturable and well-investigated archaeal species are members of the two phyla Euryarchaeota and Crenarchaeota. Several additional phyla have been proposed, often motivated by the discovery of very few, or even only a

[1] Today "Archaea" is the established term for the description of the phylogenetic entity, although in colloquial language and older scientific literature the (outdated) term "Archaebacteria" can still be encountered occasionally. This dates back to the time before the "Woesian Revolution", when Archaea were still classified as Bacteria, which back then were divided in the two separate domains "Eubacteria" and "Archaebacteria".

single species, that differ significantly from the members of the established phyla. To date Korarchaeota, Nanoarchaeota (Huber et al., 2002), Thaumarchaeota and Lokiarchaeota (Spang et al., 2015) are most prevalent, in addition to the two major phyla. Furthermore, several superphyla ("TACK", "DPANN", "Asgard") emerged that comprise monophyletic clades of various proposed phyla including also lesser known phylogenetic entities. The current state and historical development of the archaeal tree of live are described in an excellent review by Spang et al. (2017).

Ecology: Archaea are renowned for their colonization of extreme habitats, ranging from psychrophile, piezophile, halophile, alkalophile, acidophile to (hyper) thermophile environments. Today it is established that besides these hostile habitats, Archaea also live in mesophilic environments. Due to the fact that oftentimes Archaea occupy unique ecological niches or live in symbiosis with other microorganisms, many species have yet not been cultivated in the lab or can only be grown in co-cultures. This is also the reason why only recently, aided by the rise of environmental metagenomics, the ubiquitous nature of the archaeal domain was revealed (Venter et al., 2004). Still, there is one ecological niche unoccupied by Archaea: so far no pathogenic archaea have been found.

Physiology and Metabolism: Due to their appearance in such diverse habitats, Archaea are exceptionally versatile in their trophic lifestyles and are a source of numerous unique metabolic pathways. Their metabolism can be aerobe, facultative anaerobe or obligate anaerobe. Besides heterotrophs there are facultative and obligate chemolithoautotrophs that use organic substrates or hydrogen gas as electron donor and elemental sulfur as electron acceptor instead of oxygen, and others that aerobically oxidize elemental sulfur. Another common strategy for energy generation is the oxidation of divalent metal ions such as Fe^{2+}. CO_2 fixation is practiced by a number of chemolithoautotrophs via the 3HP/4HB cycle, the dicarboxylate/4HB cycle, the reductive acetyl-CoA pathway or the reductive citric acid cycle. Halobacteria can perform CO_2 fixation via photosynthesis utilizing the purple membrane pigment bacteriorhodopsin in addition to their heterotrophic lifestyle. Some species are known to fixate molecular N_2, to satisfy their nitrogen demand.

Genomics: As mentioned, Archaea share traits with both Bacteria and Eukarya. The same divide is also reflected in the archaeal genome, where genes coding for information processing resemble their eukaryotic counterparts and genes coding for housekeeping functions are similar to bacterial genes. This should not disguise the fact that Archaea on their own are a plentiful source of unique genes. Actually, in an investigation of euryarchaeal genomes 9-15% of inspected protein-coding genes were identified as signature genes, i.e. genes with no known counterparts in the other two domains of life (Graham et al., 2000).

Interestingly, the share of bacterial genes which is estimated to range from 10-30% (Garcia-Vallvé et al., 2000; Koonin et al., 2001) varies significantly between different Archaea and is subject to a constant change via lateral gene transfer. Since informational genes typically represent subunits of larger complexes, they are less prone to lateral gene transfer compared to operational genes that oftentimes encode for independently functional enzymes. Some archaea are more prone to lateral gene transfer than others. Especially the

metabolically diverse groups of methanogens and halophiles, which oftentimes naturally occur in co-culture with Bacteria, were found to incorporate large fractions of bacterial genes (Deppenmeier et al., 2002).

Transcription is carried out with the archaeal RNA polymerase whose core and holoenzyme share remarkable similarity with the eukaryotic RNA polymerase II. The enzymes also share the requirements for efficient promotor recognition, like the TATA-box binding protein and transcription factor B (Blombach and Grohmann, 2017). Nevertheless, the archaeal version of the transcription machinery is much simpler than the eukaryotic counterpart and therefor used as important model system for investigation of transcription processes in eukaryotic systems.

Translation in Archaea is again strikingly similar to Eukarya while the whole process can be seen as mosaic of bacterial and eukaryal features. rRNA and other major components of archaeal ribosomes are very similar to their eukaryotic homologues and also the number of initiation factors (>10 in Archaea and Eukarya) cannot be found in Bacteria which require only 3 initiation factors (Dennis, 1997). Archaea, like Eukarya, use methionine for initiation, while Bacteria rely on N-formylmethionine. Then again, Archaea process polycistronic mRNA, and mRNA recognition often happens at a Shine-Dalgarno sequence in the 5′ uncoding region, features that are characteristic for Bacteria (Bell and Jackson, 1998). Nevertheless another mechanism of archaeal translation initiation on leaderless mRNA is described which resembles the eukaryal initiation process (Benelli et al., 2003).

Genomic structure is very similar in Archaea and Bacteria since they both harbor a single chromosome while they lack a nucleus. Yet again, the archaeal machinery for DNA replication is, although simpler in its composition, more similar to the eukaryotic counterparts (Grabowski and Kelman, 2003) and Euryarchaeota even organize their genome as chromatin with histones, while histones are completely absent in Crenarchaeota (White and Bell, 2002).

DNA repair sparks obvious interest since many archaeal model organisms have to face harsh environmental conditions. Unfortunately, not nearly as much is known about the archaeal DNA repair systems as for the other domains of life (Kelman and White, 2005; Zatopek et al., 2018). Interestingly, the otherwise ubiquitous MutS/MutL repair mismatch repair system is absent from Archaea. Nevertheless, studies of *Sulfolobus acidocaldarius* have shown that Archaea can be as efficient at DNA repair as *Escherichia coli* and mutation rates are comparable with or even lower than those of other microorganisms (Grogan, 2000). Halophiles which are naturally exposed to strong UV radiation are the best studied Archaea in regard of their DNA repair systems of UV-induced DNA damage (Jones and Baxter, 2017). Known mechanisms include photoreactivation (a process that utilizes visible light to revert cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts by directly rearranging bonds with help of a photolyase), nucleotide excision repair (resynthesis of a excised damaged strand by a DNA polymerase), base excision repair (replacement of (oxidatively) altered nucleobases) and homologous recombination (excision and creation of

recognition sites at the broken ends followed by recombinase binding and reconnection of the strands).

Archaeal model organisms and their genetic tools: For a microbial species to emerge as "model organism" the development of efficient genetic tools for its investigation on a molecular level is of crucial importance. Compared to Bacteria and Eukarya, only a limited number of genetics tools are available for the manipulation of Archaea. Factors that hinder the development of efficient genetic tools are that many archaeal species are resistant against conventional antibiotics utilized for selection in established bacterial genetic systems, caused by fundamental differences in membrane composition and replication machinery. In addition, the environmental requirements of extremophilic archaeal species prevent (thermo-)labile conventional selection markers and antibiotics. Nevertheless, for a few model organisms powerful genetic tools have been developed capable of gene knock-out and insertion of whole genetic cassettes of interest (Leigh et al., 2011; Atomi et al., 2012). On top of their genetic amenability these archaeal model organisms can be readily cultivated in the laboratory with acceptable effort, exhibit reasonable doubling times and allow for biomass titers of at least ~1 g/L which is sufficient for performing investigations on a biomolecular level. Among Euryarchaeota, genetic systems have been developed for halophiles (*Halobacterium salinarum* and *Haloferax volcanii)*, methanogens (*Methanococcus* and *Methanosarcina*), and members of Thermococcales (*Thermococcus kodakarensis* and *Pyrococcus furiosus*). In Crenarchaeota, genetic tools are available for species of the genus *Sulfolobus* (*Sulfolobus islandicus, Sulfolobus solfataricus and S. acidocaldarius*). An overview of important genetic tools for the most important archaeal model organisms is shown in Table 1.

Halophiles: Halophiles were the first Archaea that were routinely transformed with foreign DNA utilizing PEG-mediated transformation (Charlebois et al., 1987; Cline et al., 1989). Genetic systems based on resistances against the antibiotics novobiocin, a gyrase inhibitor, and mevinolin/simvastatin (inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase) are available and applied in *Escherichia coli* shuttle vectors (Holmes and Dyall-Smith, 1990; Holmes et al., 1994). Also, autotrophic selection and counterselection based on uracil/5-FOA has been utilized in a number of halophiles (e.g. *Halobacterium salinarum*, *Haloferax mediterranei*, and *Haloarcula hispanica* (Liu et al., 2011).

Methanogens: In methanogens introduction of DNA is performed using PEG-mediated transformation (Tumbula et al., 1994) and liposome-mediated transformation (Metcalf et al., 1997). A system utilizing the antibiotic puromycin and a resistance based on a puromycin transacetylase gene (Gernhardt et al., 1990) is frequently used. Also, counterselection methods have been developed (Moore and Leigh, 2005) based on 6-azauracil and 8-azahypoxanthine. Fortunately, genetic tools can be transferred between different methanogen species.

Thermococcales: $CaCl_2$-mediated heat shock transformation for introduction of foreign DNA was demonstrated for *Thermococcus kodakarensis* (Sato et al., 2003) and *Pyrococcus furiosus* (Waege et al., 2010), while PEG-mediated transformation is used in *Pyrococcus abyssi* (Lucas et al., 2002). Genetic tools are mostly developed for *T. kodakarensis* and *P. furiosus*, although

for *P. abyssi* shuttle vectors are also described (Lucas et al., 2002). For *T. kodakarensis* (Matsumi et al., 2007) and *P. furiosus* (Waege et al., 2010) the antibiotic simvastatin and an resistence HMG-CoA reductase overexpression cassette are available. Counterselection in *T. kodakarensis* (Sato et al., 2005) and *P. furiosus* (Lipscomb et al., 2011) is possible with the known 5-FOA/pyrF system.

***Sulfolobus*:** Members of the genus *Sulfolobus* represent the only genetically amenable crenarchaeal species to date. In the early days, a number of natural genetic elements (Zillig et al., 1998, 1998; Stedman et al., 2000), including transposons, viruses and cryptic plasmids, were harnessed as transformation tools for *S. solfataricus* and *S. acidocaldarius* (Schleper et al., 1992, 1992; Aagaard et al., 1996; Elferink et al., 1996; Berkner and Lipps, 2008; Wagner et al., 2009). Nowadays, transformation is routinely carried out via electroporation. The development of potent manipulation systems based on selectable markers like *lacS*/β-galactosidase for *S. solfataricus* on lactose minimal medium and later a uracil/5-FOA based system for *pyrEF* deletion mutants of *S. acidocaldarius*, *S. islandicus* (Deng et al., 2009; She et al., 2009; Wagner et al., 2009, 2012) and eventually *S. solfataricus* posed a significant leap forward in the development of the genetic toolbox of the genus *Sulfolobus*. Today, a wide range of *E. coli-Sulfolobus* shuttle vectors and over-expression plasmids are available and described in detail in the literature (Aravalli and Garrett, 1997; Stedman et al., 1999; Jonuscheit et al., 2003; Albers et al., 2006; Aucelli et al., 2006; Berkner and Lipps, 2008). In a recent advancement it was possible to harness the endogenous CRISPR/Cas system of *S. islandicus* for targeted genome editing (Li et al., 2016). This is a next step in the direction of facilitated and accelerated manipulation of the genus *Sulfolobus*.

Table 1: An overview of important genetic tools for the most important archaeal model organisms. Further sources can be found in the text of this Thesis and in two excellent reviews on archaeal genetic tools by Leigh (2011) and Atomi et al. (2012).

		Antibiotics	Selection marker	Transformation method	Relevant reviews and selected publications
Halophiles	*Halobacterium salinarum*	novobiocin, mevinolin/ simvastatin	uracil/5-FOA	PEG-mediated	**Book chapter that reviews the genetic systems for halophiles:** (Berquist et al., 2006)**;** **Strains and plasmids for inducible overexpression in *H. volcanii*:** (Allers et al., 2010)
	Haloferax volcanii	novobiocin, mevinolin/ simvastatin	uracil/5-FOA, leucine, thymidine, tryptophan, histidine, methionine	PEG-mediated	
Methanogens	*Methanococcus*	puromycin	6-azauracil/8-azahypoxanthine, histidine auxotrophy/prototrophy	PEG-mediated	**Review of genetic tools in *Methanococcus*:** (Tumbula and Whitman, 1999)
	Methanosarcina	puromycin	8-aza-2,6-diaminopurine	liposome-mediated	**Overview of genetics and genetic tools for *Methanosarcina*:** (Kohler and Metcalf, 2012)
Thermococcales	*Thermococcus kodakarensis*	simvastatin	uracil/5-FOA, agmatine, 6-methylpurine	heat shock	**Transformation system for *Thermococcus kodakaraensis*:** (Sato et al., 2005)
	Pyrococcus furiosus	simvastatin	uracil/5-FOA, tryptophan auxotrophy	heat shock	**Transformation system for *Pyrococcus* based on a vector conferring simvastatin resistance:** (Waege et al., 2010)
Sulfolobus	*Sulfolobus islandicus*	-	uracil/5-FOA	electroporation	**Overview of genetic tools for the genus *Sulfolobus*:** (Peng et al., 2017)**;** **endogenous CRISPR system as genetic engineering tool for *S. islandicus*:** (Li et al., 2016)**;** **Gene disruption via homologous recombination (*S. solfataricus*):** (Albers and Driessen, 2007)**;** **Genetic toolbox for *S. acidocaldarius*:** (Wagner et al., 2012)
	Sulfolobus solfataricus	-	uracil/5-FOA	electroporation	
	Sulfolobus acidocaldarius	-	uracil/5-FOA	electroporation	

The Poly-Extremophilic Genus *Sulfolobus*

The genus *Sulfolobus* comprises some of the best characterized members of the phylum Crenarchaeota, a group of mostly extreme thermo-acidophilic organisms that represent the second major phylum of the archaeal domain besides the Euryarchaeota. Natural habitat of *Sulfolobus* species are solfataric fields all around the world, including the USA, Costa Rica, Mexico, Russia, Japan, China, New Zealand, Germany, Italy and Iceland. The outstanding characteristic of these organisms have been investigated since the year of 1972, when Brock et al. coined the term "*Sulfolobus*" and described the first species of the group: *Sulfolobus acidocaldarius*[2] (DSM 639; former strain 98-3).

Since *Sulfolobus* spp. can be grown and manipulated under laboratory conditions, they are popular model organisms to study Archaea. Research has been focused on their biology and physiology. Currently, genomics (Bell et al., 2002; Dai et al., 2016), proteomics (Chong and Wright, 2005; Ellen et al., 2010; Pham et al., 2010; Kort et al., 2013), metabolomics (Ulas et al., 2012; Bräsen et al., 2014), composition and function of the archaeal membrane (Albers and Meyer, 2011) and the archaellum (Albers and Jarrell, 2015), as well as interaction with archaeal viruses (Prangishvili et al., 2006) are important fields of research. Nevertheless, there is also growing interest in the utilization of this genus in biotechnological applications and the development of engineered strains to exploit the organisms´ unique characteristics. *Sulfolobus* spp. are a source of unique enzymes (Littlechild, 2015), biomaterials (Benvegnu et al., 2009; Besse et al., 2015) and metabolic pathways (Bräsen et al., 2014). As most prominent examples, the branched Entner-Doudoroff pathway (Kouril et al., 2013) as well as Weimberg and Dahms pathways for the degradation of hexoses and pentoses (Nunn et al., 2010) should be named. These diverse catabolic pathways present a promising field for the exploitation of novel products (Ahmed et al., 2005; Siebers and Schönheit, 2005).

Among the eight *Sulfolobus* species established in literature, *S. islandicus, S. solfataricus* and *S. acidocaldarius* are by far the best described members of the genus. While *S. islandicus* is used as a model organism for comparative genomics and genetics (Reno et al., 2009) and for host-virus interactions (Held and Whitaker, 2009), no type strain has been designated and strains are not commercially available yet. *S. solfataricus* is the metabolically most diverse species and many catabolic enzymes have been investigated in detail (Bräsen et al., 2014). Unfortunately, this diversity comes along with a significant genetic instability caused by the presence of several hundred mobile elements identified in its genome (Brügger et al., 2002). By contrast, the genome of *S. acidocaldarius* is much more stable (Chen et al., 2005). This makes *S. acidocaldarius* interesting for industrial applications, where strain stability is of utmost importance.

A more in-depth review that highlights the potential of the genus *Sulfolobus* in future biotechnology can be found in Part I of this Thesis.

[2] The lexeme of the genus is a copulative compound consisting of the terms "Sulfo" (lat. *Sulfur*) and "lobus" (lat. *lobe*), while the species name is a compound of "acido" (lat. *acid*) and "caldarius" (lat. *warm* or *hot*), thereby describing the habitat and morphology of the organism.

State-of-the-art and Motivation for this Thesis

Before an organism can be utilized as production strain in the biotech industry several subjects have to be investigated and a certain basis of fundamental process technological and analytical tools has to be established. Physiology, metabolism and genetics of *S. acidocaldarius* are reasonably well characterized and reliable genetic tools are available and easily applicable. Moreover, *S. acidocaldarius* has a stable genome and thus low mutation rates of production strains can be expected. This genetic stability is especially important for continuous fermentations, a cultivation mode that is highly attractive for extremophiles due to the low risk of contamination. On the other hand important issues still remain to be tackled: Hardly any results are published on the use of sustainable, low-cost media derived from industrial waste streams for the production of sustainable bulk chemicals or the use of *Sulfolobus* species in biorefinery concepts. Also on the other side of the biotechnological spectrum, in the production of pharmaceuticals, the demand for an inexpensive and defined cultivation medium that meets the requirements of Quality by Design (QbD) guidelines is not met, since no such media formulations have been published yet. Strain characterization, development of regimes for high cell density cultivations, and development of sophisticated monitoring tools for advanced bioprocess development and characterization are further topics that still remain to be investigated in much more detail.

This Thesis is motivated by the promising properties of the potential future biotechnological host *S. acidocaldarius* and strives to clear the way for its utilization in an industrial context by shedding light on some of the above mentioned topics.

Goal of the Thesis

Goal of this Thesis is to describe the potential of *S. acidocaldarius* as host organism in industrial biotechnology, and to find and evaluate possible applications of the microorganism that are suitable for commercialization.

Scientific Questions

1. **Can lignocellulosic hydrolysates be used as carbon source for *S. acidocaldarius*?**

Approach: Conduction of feasibility studies for testing hydrolysates of two highly abundant sustainable sources of lignocellulose, namely wheat straw and beech wood, for their suitability as carbon sources for *S. acidocaldarius*. Potentially present inhibitors were to be removed via appropriate simple detoxification steps.

Hypotheses:

- The content of fermentable sugars in beech wood and wheat straw hydrolysates lead to a significant increase in growth of *S. acidocaldarius* compared to reference media that lack these carbon sources.
- Mild hydrolysis steps (enzymatic pretreatment and hot water hydrolysis at maximum 120 °C) lead to low amounts of inhibitors thereby facilitating growth of *S. acidocaldarius*.

2. **Can we find a composition for a defined cultivation medium that supports growth of *S. acidocaldarius* with biomass yields and specific growth rates comparable to a complex medium based on protein hydrolysates?**

Approach: Substitution of NZ-amine, a routinely used complex carbon source for the cultivation of *S. acidocaldarius*, with single, defined constituents aiming for highest specific growth rates and biomass yields and systematic reduction of salts and trace elements until the threshold of growth limitation, via one-factor-at-a-time changes. Investigation of the effect of potential growth promoting agents and search for a suitable buffer system.

Hypotheses:

- A complex carbon source can be substituted by one, or a mixture of, single constituent(s) without reduction of specific growth rate or biomass yield.
- The commonly used "Brock Medium" contains an excess of most salts and trace elements and a reduction of these components can be achieved without growth impairment.
- Substitution of vitamins has a growth promoting effect on *S. acidocaldarius*.

3. **Are the results of fluorescence based, at-line methods for the determination of the viability of *S. acidocaldarius* comparable with a plating assay that is considered the gold standard of viability assays?**

Approach: Screening of fluorescence dyes that are commonly used in Bacteria and Eukarya regarding their staining efficiency of *S. acidocaldarius* cells using a fluorescence microscope with subsequent transfer to a flow cytometer and fluorescence plate reader as more automated and statistically sound analysis techniques.

Hypotheses:

- Fluorescence based viability assays for *S. acidocaldarius* generate correlating results with a plating assay.
- Using fluorescence based viability assays it is possible to monitor an *S. acidocaldarius* culture over the course of a bioreactor cultivation so that changes in viability can be timely detected.

4. **Can *S. acidocaldarius* be used for the expression of a eukaryotic gene with the goal of intracellular conversion of xylose to xylitol?**

Approach: Literature search for a thermostable xylose reductase followed by biochemical characterization of the thermostable enzyme candidate. Subsequent expression of the xylose reductase gene in *S. acidocaldarius* strains with knock-outs in the pentose degradation pathway in order to produce xylitol at low pH and elevated temperature.

Hypotheses:

- The fungal kingdom can be tapped as source for a xylose reductase that is stable at temperatures of ~75 °C.
- Transcription, translation and folding of genes and proteins of eukaryotic origin are possible in *S. acidocaldarius*.
- Gene deletions in the pentose degradation pathway of *S. acidocaldarius* can increase the substrate flux towards xylitol formation.

5. **Do growth temperature and specific growth rate affect the lipid pattern of *S. acidocaldarius* regarding distribution of lipid classes and lipid species and if yes, how?**

Approach: Cultivation of *S. acidocaldarius* in a continuous stirred-tank reactor at different growth temperatures and specific growth rates and subsequent extraction and analysis of the generated lipid pattern with MALDI-MS analysis. Adoption of a stable physiological state and enabling of reproducibility of the lipid pattern by employing continuous cultivation conditions.

Hypotheses:

- In order to adapt to external influences *S. acidocaldarius* modulates its membrane composition by changing the ratio of lipid classes and lipid species within its membrane.
- Besides growth temperature, the nutrient supply is one such external influence.

Structure of the Thesis

The Thesis is structured into 3 parts containing 6 chapters in total. It comprises 3 scientific publications, 3 submitted manuscripts, 2 monographic chapters and 1 filed patent.

Part I Introduction to the organism *Sulfolobus acidocaldarius* and its biotechnological relevance

Chapter 1: Review – *Sulfolobus* in the biotech industry

A description of the properties that make *Sulfolobus* species useful hosts for industrial biotechnology and of the drawbacks and limitations of the organism, as well as potential applications that have been described in the literature.

Part II Fundamentals for the biotechnological utilization of *Sulfolobus acidocaldarius* as production host

Chapter 2: Growth on complex natural substrates

A feasibility study to evaluate 1) the potential of enzymatically treated beech wood hydrolysate as carbon source for *S. acidocaldarius* as well as 2) the utilization of *S. acidocaldarius* for the valorization of hot water treated wheat straw hydrolysate.

Chapter 3: Development of a defined cultivation medium

Description of a novel citrate buffered defined cultivation medium based on the carbon sources monosodium glutamate and glucose for the cultivation of *S. acidocaldarius*.

Chapter 4: At-line viability determination for efficient bioprocessing

Development of fluorescence based methods for the determination of viability of *S. acidocaldarius* during bioreactor cultivation.

Part III Potential applications of *Sulfolobus acidocaldarius* in industrial biotechnology

Chapter 5: Recombinant protein production and whole-cell catalysis

An investigation of the microbial conversion of xylose to xylitol by mutants of *S. acidocaldarius* with knock-outs in the pentose degradation pathway via use of a heterologous xylose reductase.

Chapter 6: Generation of tailor-made archaeal lipids

Investigation of the influence of growth temperature and specific growth rate on the lipid pattern of *S. acidocaldarius*, with the goal to steer the ratio of tetraether to diether lipids and number of cyclopentane rings via adjustment of the examined process parameters.

Achievements and Findings

In Part I of this Thesis I give a general overview of the current status of *S. acidocaldarius* in the biotech industry in form of a published review paper. In Part II I investigate 3 fundamental prerequisites for the adoption of *S. acidocaldarius* in biotechnological applications, described in the chapters on growth on complex natural substrates (submitted manuscript and monographic chapter), on the development of a defined cultivation medium (scientific publication) and the determination of cell viability for efficient bioprocessing (monographic chapter). Finally, in Part III I present the investigations of two concrete applications of *S. acidocaldarius* as heterologous host for whole-cell biocatalysis (scientific publication and monographic chapter) and source of commercially relevant tetraether lipids (submitted manuscript and filed patent).

Part I Introduction to the organism *Sulfolobus acidocaldarius* and its biotechnological relevance

Chapter 1: Review – *Sulfolobus* in the biotech industry

Overview: Biotechnological processes could benefit in several ways from the use of the extreme thermo-acidophile members of the genus *Sulfolobus*: greatly reduced contamination risk, high substrate solubility, adaption to harsh substrate pretreatment conditions, facilitated removal of volatile products and elimination of cooling costs. The genus is a source of a broad variety of temperature and acid stable enzymes and produces unique biomaterials and metabolites. A well-developed genetic toolset makes exploitation of these features possible and emergence of metabolically engineered production strains is reasonable in the near future.

However, there is still considerable need for careful bioprocess development. No continuous processes are reported in the literature and sophisticated tools for monitoring and control, like on-line measurement techniques for assessing cell viability, are lacking completely. Furthermore, media development and optimization have been neglected for too long. For the establishment of a competitive, long-lasting or continuous bioprocess, it is mandatory to expand process knowledge to be able to understand and control the bioprocess.

My contribution: I wrote the review on the potential of *S. acidocaldarius* in industrial biotechnology and refined it together with Oliver Spadiut. Sonja-Verena Albers, Bettina Siebers and Lu Shen contributed the chapters on genetic tools, central carbon metabolism and its graphical description, respectively.

Part II Fundamentals for the biotechnological utilization of *Sulfolobus acidocaldarius* as production host

Chapter 2: Growth on complex natural substrates

Overview: The utilization of industrial lignocellulosic waste steams has great potential as carbon source for heterotrophic biotechnological production hosts. Following the waste-to-value concept they represent a cheap alternative to food-based carbon source like glucose derived from wheat or corn, thereby greatly reducing their social and environmental footprint. A strong incentive for using lignocellulosic biomass as substrate for extremophilic bioprocesses with *S. acidocaldarius* is that the growth conditions of the organism (regarding pH and temperature) match the conditions for substrate pretreatment. This means, before its utilization, there is no need for substrate cooling and neutralization leading to savings in cooling costs and a significant reduction of chemicals required for pH regulation.

Goal: Evaluation of the potential of lignocellulosic hydrolysates derived from wheat straw and beech wood for the use as substrate for *S. acidocaldarius* and, if necessary, discovery of ways to remove unwanted inhibitors.

Examined Scientific Question: (1) Can lignocellulosic hydrolysates be used as carbon source for *S. acidocaldarius*?

Challenge: 1) Raw materials are very different depending on their origin and tend to exhibit strong variations depending on geographic production area, applied production techniques, production cycles, season and feedstock used in the lignocellulose processing industries; 2) during the pretreatment of lignocellulosic biomass, which is generally performed at high temperatures and extreme pH values, a significant amount of inhibitors (organic acids, furfural, hydroxymethyl furfural and phenolic compounds) is released or formed. These inhibitors have the potential to compromise growth of the production organism and therefore may have to be removed in a detoxification step; 3) hydrolysates often contain low concentration of fermentable sugars compared to media formulations that are used in traditional bioprocesses.

Results: The growth promoting effect of both enzymatically treated beech wood hydrolysate and hot water treated wheat straw could be shown. To achieve this effect a detoxification method based on activated carbon was developed. In the course of these studies a flow cytometry based method for biomass determination in turbid media was developed.

My contribution: The studies were designed together with Oliver Spadiut. Experiments and analytical measurements were planned, performed and evaluated. The methods for hydrolysate detoxification and biomass determination in turbid media were developed. The manuscript on the use of beech wood hydrolysate was written and the manuscript on the utilization of wheat straw was co-authored.

Chapter 3: Development of a defined cultivation medium

Overview: To date, many cultivation media used for the growth of *Sulfolobus* species are based on a formulation published in 1972 by Brock et al. ("Brock Medium"). This medium holds some substantial drawbacks: 1) complex media components, like NZ-amine or yeast extract, with high batch-to-batch variability, foil reproducibility, substrate analytics, strain characterization and selective media optimization; 2) the presence of inhibiting compounds in complex media is reported (Park and Lee, 2000); 3) the fluorescence background due to the presence of aromatic amino acids impedes direct fluorescence measurements in the medium (e.g. use of fluorescent biomarkers); 4) the formation of precipitates is a hindrance for reproducible media preparation; and 5) costs are significantly higher compared to formulations based on sugars or selected amino acids.

Goal: Substitution of NZ-amine, used in state-of-the-art media formulations for the cultivation of *S. acidocaldarius*, with a reasonable number of defined components while maintaining yield, maximum cell density and specific growth rate.

Examined Scientific Question: (2) Can we find a composition for a defined cultivation medium that supports growth of *S. acidocaldarius* with biomass yields and specific growth rates comparable to a complex medium based on protein hydrolysates?

Challenge: The exact composition of NZ-amine is not known and it is also uncertain which component or effect leads to the growth promotion upon addition of NZ-amine. Possible benefits can be a chemical buffer effect, compatible solute properties, the benefit of single nutrients or synergic effects, content of unknown trace elements, or a combination thereof.

Results: The presented results satisfy the urgent demand for a *standardized* and *defined* growth medium. During this study the following achievements were made:

- Substitution of complex substrate with monosodium glutamate
- Removal of precipitates
- Improved buffer capacity via addition of citric acid for higher final cell densities
- Reduction of salt and trace element concentrations for lower costs and reduced risk of growth inhibition

Using the described VD-Medium for shake flask cultivations of *S. acidocaldarius* yields 1.1 g/L dry cell weight (OD_{600}=1.7) after a typical incubation time of 95 h with an overall biomass yield of 0.33 g/g and a maximum specific growth rate of 0.08 h^{-1} compared to the Brock Medium with 1.2 g/L dry cell weight (OD_{600}=1.8), an overall biomass yield of 0.5 g/g and a maximum specific growth rate of 0.12 h^{-1}. The *average* specific growth rates of both media are nearly identical, when comparing the growth over the whole course of the cultivation.

My contribution: The study was designed together with Oliver Spadiut. Experiments and analytics were planned, performed and evaluated. The manuscript was written.

Chapter 4: At-line viability determination for efficient bioprocessing

Overview: Bioprocesses need to be monitored – for efficient process development, process control and in order to comply with QbD guidelines ("only viable biomass is producing biomass"). Today´s state-of-the-art method for viability determination of *Sulfolobus* species is based on plating assays. It is obvious that due to the long delay of at least 5 days until availability of results, the task of bioprocess monitoring cannot be achieved via a culture based assay. Therefore, there is a strong demand for the development of a fast and reliable at-line method for the assessment of cell viability.

Goal: Development of methods for the simple and timely at-line determination of viability parameters based on fluorescence staining.

Examined Scientific Question: (3) Are the results of fluorescence based, at-line methods for the determination of the viability of *S. acidocaldarius* comparable with a plating assay that is considered the gold standard of viability assays?

Challenge: The unique cell wall (S-layer) and membrane composition of *S. acidocaldarius* compared to Bacteria and mammalian cells, for which most fluorescence dyes have been developed, poses a serious challenge for finding suitable viability stains. *In-situ* methods but also at-line methods have to be evaluated carefully regarding the effect of elevated temperature (*in-situ*) and pH (*in-situ* and at-line) on reagents, probes and equipment; a straightforward method transfer from mesophilic hosts generally cannot be expected.

Results: A plate reader assay as well as a flow cytometry assay, both based on the enzymatic hydrolysis of fluorescein diacetate by endogenous esterases, were development. Comparison with a culture based plating assay, regarded the gold standard for viability determination, revealed a high correlation with the developed methods and the developed assays are capable of reflecting different physiological states of the host organism.

My contribution: I planned, performed and evaluated the experiments and analytical measurements for this study. The methods for fluorescence based viability determination were developed together with Andrea Tramontano. I wrote the monographic chapter on the determination of cell viability for efficient bioprocessing.

Part III Potential applications of *Sulfolobus acidocaldarius* in industrial biotechnology

Chapter 5: Recombinant protein production and whole-cell catalysis

Overview: Xylitol is an important C5-sugar alcohol which is in strong demand for food applications, pharmaceutics, cosmetics and dental care. Unfortunately, the state-of-the-art production of xylitol via Raney-nickel is environmentally harmful due to the use of energy intensive process conditions, the use of fossil fuel based reducing agents and huge amounts of organic solvents. To overcome these negative aspects a biotechnological production process for xylitol is desirable. The utilization of extremophiles for whole-cell biocatalysis combined in a one-pot reaction with hydrolyzed lignocellulosic substrates as source of xylose and energy is envisioned as sustainable solution for this problem.

Goal: Characterization of a thermostable xylose reductase and in vivo production of xylitol from xylose using *S. acidocaldarius* as whole cell biocatalyst.

Examined Scientific Question: (4) Can *S. acidocaldarius* be used for the expression of a eukaryotic gene with the goal of intracellular conversion of xylose to xylitol?

Challenge: An enzyme capable of catalyzing the reduction of xylose to xylitol at a temperature of ~75 °C with a reasonable activity has to be found. An *S. acidocaldarius* knock-out strain that does not metabolize xylose has to be found so that the sugar is still available for conversion to xylitol. The thermostable xylose reductase has to be integrated in the mutant strain, produced and correctly folded, so that the intracellular conversion can take place.

Results: We characterized a xylose reductase from *Chaetomium thermophilum* and introduced and translated the respective gene in a xylose negative mutant of *S. acidocaldarius*. Nevertheless the xylose reductase was not detectable via western blot analysis and as consequence also no conversion of xylose to xylitol was possible.

My contribution: The study was designed together with Oliver Spadiut. Cloning, expression experiments, enzyme characterization and analytics were planned, performed and evaluated. I wrote the manuscript on the biochemical characterization of the enzyme and the monographic chapter on the bioconversion experiments.

Chapter 6: Generation of tailor-made archaeal lipids

Overview: The unique structure of their membrane lipids is one of the most prominent phenotypic differences between Archaea vs. Bacteria and Eukarya. Due to their unusual chemical properties (bipolar structure, ether bonds, and linkage in the *sn*-2,3 position) archaeal lipids are significantly more resistant to high temperature, low pH, oxidation and enzymatic degradation compared to conventional lipids. Additionally, in members of the phylum Crenarchaeota condensation of two (monopolar) archaeal diether lipids to a single (bipolar) tetraether lipid as well as formation of cyclopentane rings in the isoprenoid core strongly reduce permeability of archaeal membranes.

A large number of studies exist that discuss archaeal lipids as building blocks for drug-, gene- and vaccine delivery vehicles. Also unconventional applications like the utilization as antimicrobial coating are proposed due to outstanding compactness and increased hydrophobicity (e.g. for coating of medicinal devices like catheters, and implants). Nevertheless, no such applications are currently realized on an industrial scale. The reason for the lacking commercialization is the expensive production of the archaeal lipids due to low growth rates, low cell densities and low biomass- and extraction yields combined with high downstream costs. A procedure for the production of tailor made archaeal lipids would greatly increase their economic potential due to mitigating downstream costs by already predetermining the lipid pattern during the upstream process.

Goal: Determination of critical process parameters during the upstream process that influence the lipid pattern regarding lipid classes and lipid species and tailoring the lipid pattern by purposeful manipulation of these parameters.

Examined Scientific Question: (5) Do growth temperature and specific growth rate affect the lipid pattern of *S. acidocaldarius* regarding distribution of lipid classes and lipid species and if yes, how?

Challenge: To ensure steady process conditions for the generation of reproducible lipid compositions the controlled environment of a bioreactor is crucial. Downstream processing and analytics are highly challenging due to generally low extraction yields of archaeal lipids.

Results: We showed that *S. acidocaldarius* changes its lipid composition as reaction to a shift in the growth rate caused by nutrient limitation. We thereby identified a novel factor for influencing the lipid composition of *S. acidocaldarius* and were able to determine the effect of this factor on the archaeal lipidome by using MALDI-MS: A shift in the specific growth rate from 0.011 h^{-1} to 0.035 h^{-1} during continuous cultivation of *S. acidocaldarius* led to a change in the ratio of diether to tetraether lipids from 1:3 to 1:5 and in the average cyclization number from 5.1 to 4.6. In other words, limitation of the growth rate led to an increase of the share of diethers, while the remaining tetraether lipids featured a higher average cyclization number.

My contribution: The study was designed together with Oliver Spadiut. Experiments were planned, performed and evaluated. Analytical measurements were performed under guidance and together with Ernst Pittenauer. I wrote the submitted manuscript.

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Manuscripts

Chapter 1

Quehenberger, Julian, Lu Shen, Sonja-Verena Albers, Bettina Siebers, and Oliver Spadiut. "*Sulfolobus* – A Potential Key Organism in Future Biotechnology." *Frontiers in Microbiology* 8 (2017).

Chapter 2

Quehenberger, Julian, Susanne Steudler, and Oliver Spadiut. "Evaluation of the potential of an enzymatically treated beech wood hydrolysate as carbon source for *Sulfolobus acidocaldarius*." Submitted to Bioresource Technology Reports (Oct 25, 2019), Manuscript ID: BITEB-D-19-00381

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Chapter 3

Quehenberger, Julian, Andreas Albersmeier, Holger Glatzel, Matthias Hackl, Jörn Kalinowski, and Oliver Spadiut. "A Defined Cultivation Medium for *Sulfolobus acidocaldarius*." *Journal of Biotechnology* 301 (April 15, 2019): 56–67.

Chapter 4

Chapter 5

Quehenberger, Julian, Tom Reichenbach, Niklas Baumann, Lukas Rettenbacher, Christina Divne, and Oliver Spadiut. "Kinetics and Predicted Structure of a Novel Xylose Reductase from *Chaetomium* Thermophilum." International Journal of Molecular Sciences 20, no. 1 (January 2019): 185.

Chapter 6

Quehenberger, Julian, Ernst Pittenauer, Günter Allmaier and Oliver Spadiut. "The influence of the specific growth rate on the lipid composition of *Sulfolobus acidocaldarius*." Submitted to *Extremophiles* (Oct 24, 2019), Manuscript ID: EXT-19-Oct-0198.

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Sulfolobus – A Potential Key Organism in Future Biotechnology

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REVIEW
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Sulfolobus – A Potential Key Organism in Future Biotechnology

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Extremophilic organisms represent a potentially valuable resource for the development of novel bioprocesses. They can act as a source for stable enzymes and unique biomaterials. Extremophiles are capable of carrying out microbial processes and biotransformations under extremely hostile conditions. Extreme thermoacidophilic members of the well-characterized genus *Sulfolobus* are outstanding in their ability to thrive at both high temperatures and low pH. This review gives an overview of the biological system *Sulfolobus* including its central carbon metabolism and the development of tools for its genetic manipulation. We highlight findings of commercial relevance and focus on potential industrial applications. Finally, the current state of bioreactor cultivations is summarized and we discuss the use of *Sulfolobus* species in biorefinery applications.

Keywords: *Sulfolobus*, biotechnology, thermophile, acidophile, bioprocessing, biorefinery

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INTRODUCTION

Thermophiles gain increasing attention in biotechnological applications due to their potential to expand the thermal range of industrial biotechnology and their unique metabolic capabilities (Littlechild, 2015; Zeldes et al., 2015; Beeler and Singh, 2016; Donati et al., 2016; Basen and Müller, 2017; Straub et al., 2017). In this review, we focus on the well-characterized members of the phylum Crenarchaeota, the extreme thermoacidophilic Archaea belonging to the genus *Sulfolobus*. Natural habitats of these organisms are solfataric fields all around the world, including the United States, Costa Rica, Mexico, Russia, Japan, China, New Zealand, Germany, Italy, and Iceland. The outstanding characteristic of these organisms, which have been investigated since the 1970s (**Supplementary Table S1**), is their ability to thrive at extremely low pH and high temperature, unprecedented in Eukaryotes and Bacteria.

Since *Sulfolobus* spp. can be grown and manipulated under laboratory conditions, they are popular model organisms to study Archaea. Research has been focused on their biology and physiology. Currently, genomics (Bell et al., 2002; Dai et al., 2016), proteomics (Chong and Wright, 2005; Ellen et al., 2010; Pham et al., 2010; Kort et al., 2013), metabolomics (Ulas et al., 2012; Bräsen et al., 2014), composition and function of the archaeal membrane (Albers and Meyer, 2011) and the archaellum (Albers and Jarrell, 2015), as well as interaction with archaeal viruses (Prangishvili et al., 2006) are important fields of research. Nevertheless, there is also growing interest in the utilization of this genus in biotechnological applications and the development of engineered strains to exploit the organisms' unique characteristics. *Sulfolobus* spp. are a source of unique enzymes (Littlechild, 2015), biomaterials (Benvegnu et al., 2009; Besse et al., 2015), and metabolic pathways (Bräsen et al., 2014). As most prominent examples, the branched Entner–Doudoroff (ED) pathway

(Kouril et al., 2013b) as well as Weimberg and Dahms pathways for the degradation of hexoses and pentoses (Nunn et al., 2010) should be named. These diverse catabolic pathways present a promising field for the exploitation of novel products (Ahmed et al., 2005; Siebers and Schönheit, 2005).

Among the eight *Sulfolobus* species established in the literature, *S. islandicus*, *S. solfataricus*, and *S. acidocaldarius* are by far the best described members of the genus. While *S. islandicus* is used as a model organism for comparative genomics and genetics (Reno et al., 2009) and for host–virus interactions (Held and Whitaker, 2009), no type strain has been designated and strains are not commercially available yet. *S. solfataricus* is the metabolically most diverse species and many catabolic enzymes have been investigated in detail (Bräsen et al., 2014). Unfortunately, this diversity comes along with a significant genetic instability caused by the presence of several hundred mobile elements identified in its genome (Brügger et al., 2002). By contrast, the genome of *S. acidocaldarius* is much more stable (Chen et al., 2005). This makes *S. acidocaldarius* interesting for industrial applications, where strain stability is of utmost importance. A phylogenetic tree of the genus *Sulfolobus* is shown in **Figure 1**.

In this review, we give an overview of the current state of knowledge on carbon metabolism, genetic tools, and fermentation techniques of *Sulfolobus* spp., describe relevant products, and discuss potential future applications of this genus.

CENTRAL CARBON METABOLISM

Sulfolobus spp. thrive at pH 2–3 and temperatures around 75–80°C. They are characterized by a chemoorganoheterotrophic lifestyle; however, chemolithoautotrophic growth using sulfur oxidation has been reported for some species (Huber et al., 1992; Schönheit and Schäfer, 1995). All *Sulfolobus* species exhibit an aerobic lifestyle and for *S. solfataricus* P2, a preferred growth at lower oxygen concentrations was reported (Grogan, 1989; Simon et al., 2009). The different *Sulfolobus* strains differ significantly in their metabolic potential. *S. solfataricus* possesses a broad substrate specificity and uses various sugars such as polysaccharides (e.g., cellulose, starch, dextrin), disaccharides (e.g., maltose and sucrose), hexoses (e.g., D-glucose, D-galactose, D-mannose, and L-fucose), pentoses (e.g., D-arabinose, L-arabinose, D-xylose), aldehydes, alcohols (e.g., ethanol, phenol), sugar acids as well as tryptone, peptides, and amino acids as carbon source (Grogan, 1989; Izzo et al., 2005; Brouns et al., 2006; Joshua et al., 2011; Comte et al., 2013; Wolf et al., 2016; Stark et al., 2017). For *S. solfataricus*, a genome scale model comprising 718 metabolic and 58 transport/exchange reactions and 705 metabolites was used to simulate growth on 35 different carbon sources (Ulas et al., 2012). While no such modeled data are published for *S. acidocaldarius*, traditional growth experiments suggest that this species is well adapted to proteolytic growth and can utilize only few other carbon sources such as dextrin, sucrose, D-glucose, D-xylose, and L-arabinose (Grogan, 1989; Joshua et al., 2011). The differences in the metabolic potential are also reflected by the respective genome size of 2.99 Mbp including 200 IS elements for *S. solfataricus* (She et al., 2001) and of 2.23 Mbp for *S. acidocaldarius* (Chen et al., 2005). In the following paragraphs, we sum up the knowledge on the central carbohydrate metabolism and give an illustration of these pathways in **Figure 2** (hexose and pentose degradation as well as glycogen, trehalose, and pentose formation).

Like most aerobic bacteria *Sulfolobus* spp. rely on the ED pathway for carbon degradation; however, in contrast to the classical pathway found, for example, in *Pseudomonas* species (Entner and Doudoroff, 1952), the archaeal pathway is branched and omits the initial phosphorylation of D-glucose. Instead, the sugar is directly oxidized to D-gluconate and dehydrated to 2-keto-3-deoxygluconate (KDG) as the characteristic intermediate of the pathway. In *S. solfataricus* KDG is either directly cleaved by the bifunctional aldolase to pyruvate and glyceraldehyde in the non-phosphorylative (np) branch of the ED pathway or first phosphorylated to 2-keto-3-deoxy-6-phosphogluconate (KDPG) and cleaved to pyruvate and glyceraldehyde 3-phosphate (GAP) in the semi-phosphorylated (sp) branch of the ED pathway. In the npED branch, glyceraldehyde is further oxidized and phosphorylated by glyceraldehyde:ferredoxin oxidoreductase and glycerate kinase to 2-phophoglycerate, which enters the lower shunt of the Embden–Meyerhof–Parnas (EMP) pathway (Ahmed et al., 2005). In the spED, GAP is oxidized to 3-phosphoglycerate by a non-phosphorylating GAP dehydrogenase (GAPN), activated by glucose 1-phophate, replacing the classical GAP dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK) couple (GAPDH/PGK). The pathway in *S. solfataricus* is promiscuous for D-glucose and D-galactose (Lamble et al., 2005). Metabolome analysis of the KDG kinase deletion strain revealed a major function of the spED pathway in providing GAP for gluconeogenesis (Kouril et al., 2013b).

Pyruvate is further oxidatively decarboxylated to acetyl-CoA via the pyruvate:ferredoxin oxidoreductase; the classical pyruvate dehydrogenase complex is absent in Archaea. Acetyl CoA enters the oxidative citric acid cycle and is finally completely oxidized to two molecules of CO_2. The substitution of the catabolic GAPDH and PGK couple by GAPN results in no net gain of ATP in the branched ED pathway. Only in the citric acid cycle, the succinyl-CoA synthetase is supposed to provide nucleoside triphosphate (NTP) by substrate level phosphorylation. Therefore, the major energy gain comes from aerobic respiration. The respiratory chain in several members of the Sulfolobales has been studied, and in *S. solfataricus* as well as in *S. acidocaldarius*, a branched electron transport chain with three terminal oxidases was reported (Schafer et al., 1999; Auernik and Kelly, 2008). For *S. solfataricus* the regulation at transcriptome level in response to different oxygen concentrations was demonstrated (Simon et al., 2009).

In *Sulfolobus* spp., the EMP pathway is only used for gluconeogenesis, although for glycolysis only a functional phosphofructokinase is missing (Kouril et al., 2013a). As key enzymes, especially the classical GAPDH and PGK are only active in the gluconeogenic direction. Further on, a bifunctional, gluconeogenic fructose bisphosphate aldolase/phosphatase (FBPA/ase) catalyzes the one-step formation of fructose

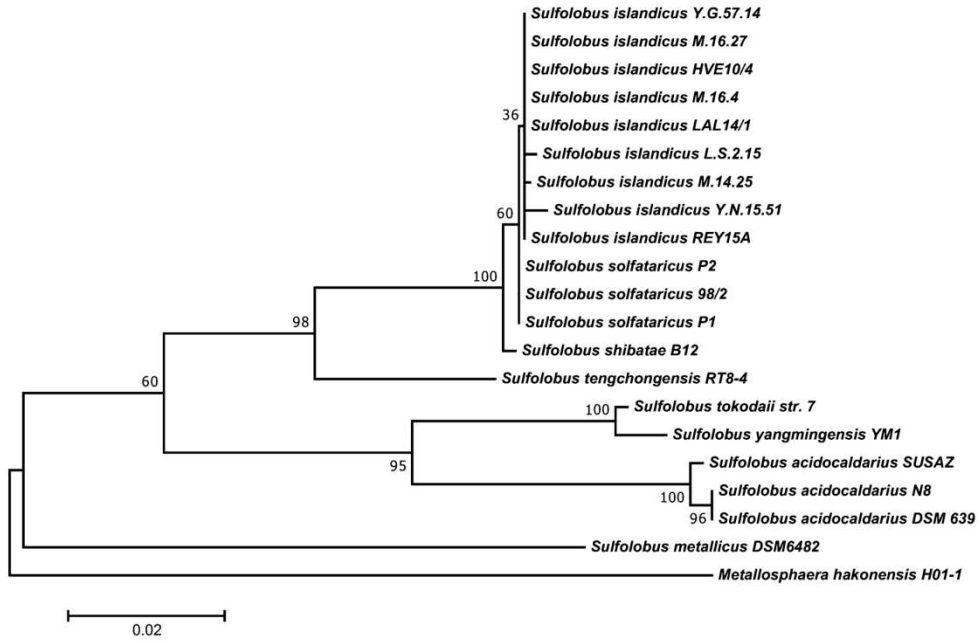

FIGURE 1 | Phylogenetic tree of the genus *Sulfolobus* based on all publicly available 16S rDNA sequences of acknowledged species. The tree was constructed with MEGA 7.0 using the maximum-likelihood method after automated alignment with clustalX2 and manual correction with GeneDoc. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Metallosphaera hakonensis* H01-1 was used as out-group.

6-phosphate from GAP and dihydroxyacetone phosphate (Say and Fuchs, 2010; Kouril et al., 2013a; Bräsen et al., 2014). Glycogen is formed as carbon storage compound (König et al., 1982) and as source for trehalose formation via the TreY/TreZ pathway [i.e., maltooligosyltrehalose synthase and maltooligosyltrehalose trehalohydrolase (Maruta et al., 1996)]. Trehalose is the only compatible solute reported so far in *Sulfolobus* spp.

Thus, like in all Archaea, the central carbohydrate metabolism in *Sulfolobus* spp. is characterized by unusual pathways and enzymes that – moreover – also confer unique regulatory properties. In contrast to the classical bacterial and eukaryotic EMP pathway, the regulation is established at the level of triose phosphates, which seems to be a general feature in (hyper)thermophilic Archaea with optimal growth close to 80°C. Triose phosphates are labile at high temperatures and it was shown that the thermal degradation of these pathway intermediates is a crucial bottleneck for efficient substrate conversion (Kouril et al., 2013a).

In addition, the upper part of the EMP pathway seems to play an important function for pentose generation. In *Sulfolobus* species, as in most Archaea, the classical pentose phosphate pathway is absent and pentoses are formed from fructose 6-phosphate via the reversed ribulose monophosphate pathway (RuMP) (Soderberg, 2005). The RuMP pathway was previously reported as formaldehyde fixation pathway in methylotrophic bacteria.

Pentose degradation has been studied in *S. solfataricus* and *S. acidocaldarius*. For *S. solfataricus* the D-arabinose degradation was resolved and an oxidative pathway with formation of α-ketoglutarate, which directly enters the citric acid cycle, was demonstrated (Brouns et al., 2006). Later studies revealed that the transporter and degradation pathway is partially promiscuous for L-fucose utilization (Wolf et al., 2016). The D-arabinose and D-xylose pathway merge at the identical intermediates 2-keto-3-deoxy-D-arabionoate (D-KDA) and 2-keto-3-deoxy-D-xylonate (D-KDX). For D-xylose degradation, a branched pathway with an aldolase-dependent branch forming pyruvate and finally glyoxylate (Dahms pathway), which enters the glyoxylate bypass, and an aldolase-independent branch forming the citric acid cycle intermediate α-ketoglutarate (Weimberg pathway) were proposed for *S. solfataricus* (Nunn et al., 2010). Important for cellulosic biomass conversion the absence of diauxic growth on D-glucose and D-xylose was reported for *S. acidocaldarius* (Joshua et al., 2011).

In general, the availability of genome scale models, functional genomics, and systems biology approaches for *Sulfolobales* under different stress and growth conditions in combination with biochemical and genetic studies enabled an in depth insight into metabolism and cellular processes [e.g., growth on L-fucose and casamino acids compared to D-glucose in *S. solfataricus* (Wolf et al., 2016; Stark et al., 2017)]. The established knowledge forms an important prerequisite for the establishment of *Sulfolobus* spp. as thermoacidophilic, archaeal platform organisms using

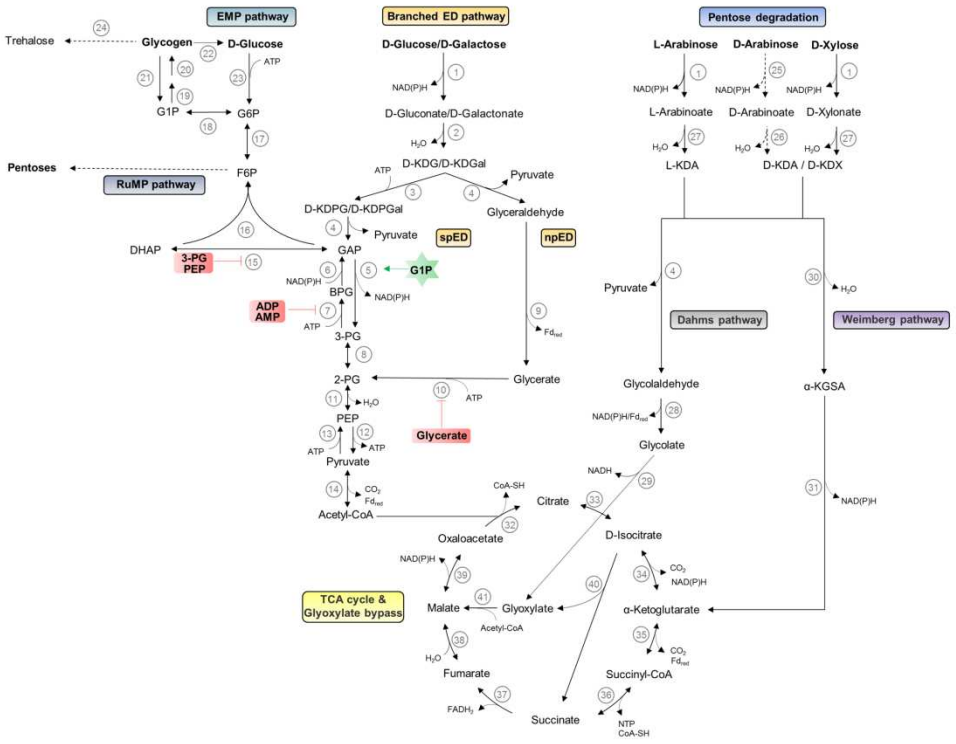

FIGURE 2 | Central carbohydrate metabolism in *Sulfolobus* spp. The pathways for hexose and pentose degradation as well as glycogen, trehalose, and pentose formation are shown. D-arabinose (dashed lines) can only be utilized as carbon source by *S. solfataricus* and not by *S. acidocaldarius*. The current understanding of regulation by effectors is indicated by green stars and red boxes for activator and inhibitors, respectively. Enzymes catalyzing different reactions are depicted as numbers: (1) glucose dehydrogenase (broad substrate specificity); (2) gluconate dehydratase; (3) 2-keto-3-deoxygluconate kinase; (4) 2-keto-3-deoxy-(6-phospho) gluconate aldolase (broad substrate specificity); (5) non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; (6) glyceraldehyde-3-phosphate dehydrogenase; (7) phosphoglycerate kinase; (8) phosphoglycerate mutase; (9) glyceraldehyde:ferredoxin oxidoreductase; (10) glycerate kinase; (11) enolase; (12) pyruvate kinase; (13) phosphoenolpyruvate synthetase; (14) pyruvate:ferredoxin oxidoreductase; (15) triosephosphate isomerase; (16) fructose-1,-6-bisphosphate aldolase/phosphatase; (17) phosphoglucose/phosphomannose isomerase; (18) phosphoglucomutase/phosphomannomutase; (19) NTP-glucose-1-phosphate uridylyltransferase; (20) glycogen synthase; (21) glycogen phosphorylase; (22) glucan-1,4-α-glucosidase; (23) hexokinase; (24) maltooligosyltrehalose synthase/maltooligosyltrehalose trehalohydrolase; (25) D-arabinose dehydrogenase; (26) D-arabinoate dehydratase; (27) L-arabinoate/D-xylonate dehydratase; (28) glycolaldehyde dehydrogenase/glycolaldehyde:ferredoxin oxidoreductase; (29) glycolate dehydrogenase; (30) 2-keto-3-deoxy-arabinoate/xylonate dehydratase; (31) α-ketoglutarate semi-aldehyde dehydrogenase; (32) citrate synthase; (33) aconitase; (34) isocitrate dehydrogenase; (35) α-ketoglutarate:ferredoxin oxidoreductase; (36) succinyl-CoA synthetase; (37) succinate dehydrogenase; (38) fumarase; (39) malate dehydrogenase; (40) isocitrate lyase; (41) malate synthetase. EMP, Embden–Meyerhof–Parnas; ED, Entner–Doudoroff; spED, semi-phosphorylative ED; npED, non-phosphorylative ED; RuMP, reversed ribulose monophosphate; TCA, tricarboxylic acid; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; BPG, 1,3-bisphosphoglycerate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; D-KDG, 2-keto-3-deoxy-D-gluconate; D-KDGal, 2-keto-3-deoxy-D-galactonate; D-KDPG, 2-keto-3-deoxy-6-phosphate-D-gluconate; D-KDPGal, 2-keto-3-deoxy-6-phosphate-D-galactonate; L-KDA, 2-keto-3-deoxy-L-arabinoate; D-KDA, 2-keto-3-deoxy-D-arabinoate; D-KDX, 2-keto-3-deoxy-D-xylonate; α-KGSA, α-ketoglutarate semi-aldehyde.

metabolic engineering, and synthetic biology approaches for future biotechnological applications.

GENETIC TOOLS

The lack of genetic tools has been a major drawback for the establishment of archaeal model organisms for basic research and biotech industries. The major problem was that most of the traditionally used antibiotics and resistance cassette genes cannot be used in archaeal phyla and therefore auxotrophies have to be used as selectable markers. However, nowadays very well-developed genetic toolboxes exist for the euryarchaea *Thermococcus kodakarensis*, *Pyrococcus furiosus*, *Haloferax volcanii*, and a number of methanogenic Archaea (Leigh et al., 2011). For *Pyrococcus*, it has been demonstrated that large gene clusters can be introduced for the production of several compounds (Lipscomb et al., 2014). Also for the genus *Sulfolobus*, a number of genetic systems have been established (Leigh et al., 2011). Early in the 1990s, the first transformation protocols by electroporation were established for *S. solfataricus* strains and self-transmissible vectors based on a conjugative

plasmid, pNOB8, and the virus SSV1 were developed (Schleper et al., 1992; Elferink et al., 1996). The virus vector-based pMJ0503 was successfully used for the overexpression of tagged proteins in *S. solfataricus* (Albers et al., 2006). For the expression of proteins in *S. islandicus*, the plasmid pSeSD1 proved to be very useful (Peng et al., 2012). The first targeted deletion mutants were obtained in a *S. solfataricus* 98/2 PBL2025, which had a large deletion of 50 kB in the genome including many genes coding for proteins involved in sugar metabolism. As this strain was unable to grow on lactose as single carbon source, the β-galactosidase LacS could be used as marker cassette (Worthington et al., 2003). However, in this case no counterselection could be used to remove the marker cassette and therefore double deletion mutants could not be obtained. In the meantime, three model systems have developed, namely two in *S. islandicus* strains and one in *S. acidocaldarius*, which use mainly uracil auxotrophy for the selection and counterselection of mutants (She et al., 2009; Wagner et al., 2012; Zhang and Whitaker, 2012). Whereas the two *S. islandicus* strains contain a large number of transposable elements, which can lead to large genome rearrangements, the *S. acidocaldarius* genome is remarkably stable (Chen et al., 2005), which was shown by sequencing several strains isolated from North America, Russia, and Japan (Mao and Grogan, 2012). For *S. acidocaldarius* currently two uracil auxotrophic mutants are being used, MW001 (Wagner et al., 2012) and MR31 (Reilly and Grogan, 2001). For MW001 a whole set of genetic tools has been established. This includes several plasmids for the construction of markerless deletion mutants or for the insertion of tags into the genome (Wagner et al., 2012). Using these, the glucose ABC transporter of *S. solfataricus* was ectopically integrated into the MW001 genome and successfully expressed (Wagner et al., 2012). Based on the cryptic plasmid pRN1 from *S. islandicus* (Zillig et al., 1993), *Escherichia coli–Sulfolobus* shuttle vectors and expression vectors were established, which enabled the homologous or heterologous expression of tagged proteins of interest (Berkner et al., 2007, 2010). The *S. acidocaldarius* MW001 genetic system has been successfully used in a number of laboratories and helped to establish *S. acidocaldarius* as a model crenarchaeon. In a recent achievement, it was possible to harness the endogenous CRISPR/Cas system of *S. islandicus* for targeted genome editing (Li et al., 2016). This is a great next step in the direction of facilitated and accelerated manipulation of the genus *Sulfolobus*. **Table 1** gives an overview of robust and highly cited expression systems and tools for gene disruption/deletion and genomic integration for the genus *Sulfolobus*.

The availability of potent genetic tools (Wagner et al., 2012; Peng et al., 2017) makes the transfer of heterologous genes to *Sulfolobus* species possible, allowing to benefit from both the metabolic diversity of *S. solfataricus* and the stability of *S. acidocaldarius*. In fact, the simpler, less promiscuous catabolism of *S. acidocaldarius* is an advantage over *S. solfataricus* in biotechnological applications, making it much easier to partly knockout metabolic pathways with the aim to redirect substrate fluxes toward a desired product.

UNTAPPING THE RESOURCE *Sulfolobus*

To date, extremophiles are exploited as source of thermostable enzymes, so-called extremozymes, for food and feed industry, textile and cleaning industry, pulp and paper industry, but also in scientific research and diagnostics. Starch-hydrolyzing (Elleuche and Antranikian, 2013), (hemi)cellulolytic (Beg et al., 2001; Kuhad et al., 2011), pectinolytic (Sharma et al., 2013), chitinolytic (Chavan and Deshpande, 2013), proteolytic (Li et al., 2013), and lipolytic (Hasan et al., 2006) enzymes are in high demand in industry (Elleuche et al., 2015). Enzymes of *Sulfolobus* spp. are especially interesting for such applications not only because of their great catalytic diversity, but also mainly due to their superior pH and temperature stability, which comes hand-in-hand with increased resilience toward organic solvents and

TABLE 1 | A selection of expression systems and tools for gene disruption/deletion and genomic integration for the genus *Sulfolobus*.

Organism	Expression vectors	Gene disruption/deletion and genomic integration
Sulfolobus acidocaldarius	Expression plasmid pCmalLacS with a maltose inducible promoter, *lacS* marker gene, *pyrEF* selection, and amp^r cassette (Berkner et al., 2010)	Construction of markerless insertion and deletion mutants via double crossover based on *pyrEF*/5-FOA counterselection (Wagner et al., 2012)
Sulfolobus solfataricus	pSVA expression plasmid series with an arabinose inducible *araS* promoter, *pyrEF* selection, and amp^r cassette (Albers et al., 2006)	Gene disruption by homologous recombination via permanent insertion of the *lacS* marker gene (Albers and Driessen, 2007)
Sulfolobus islandicus	Expression plasmid pSeSD with a modified arabinose inducible *araS* promoter, two 6xHis tags and two protease sites for tag removal, *pyrEF* selection and an amp^r cassette (Peng et al., 2012)	Improved method for markerless gene deletion by combining the established *pyrEF*/5-FOA and *lacS* markers with the stringent *argD* selection (Zhang et al., 2013) Markerless gene deletion using *apt*/6-MP counterselection (Zhang et al., 2016) CRISPR-based gene knockout and integration via homologous recombination (Li et al., 2016)

These examples represent only a fraction of the developed genetic tools, but based on their frequent usage can be considered highly reliable and successful systems. A more detailed insight into the development of genetic tools for the genus is given, for example, in a very recent review by Peng et al. (2017). lacS, gene coding for a β-galactosidase from S. solfataricus for lactose selection and blue/white screening; pyrEF, genes for the complementation of uracil auxotrophy; pyrEF/5-FOA counterselection, based on the resistance to pyrimidine analog 5-fluoroorotic acid (5-FOA) due to inactivation of the orotate phosphoribosyltransferase (pyrE) and orotidine 5′-phosphate decarboxylase (pyrF); argD, gene for the complementation of agmatine auxotrophy; apt/6-MP counterselection, based on the resistance to purine analog 6-methylpurine (6-MP) due to inactivation of a putative adenine phosphoribosyltransferase (apt); amp^r, ampicillin resistance cassette for selection in Escherichia coli.

resistance toward proteolysis (Daniel et al., 1982; Unsworth et al., 2007; Stepankova et al., 2013). However, also tetraether lipids, membrane vesicles with antimicrobial properties, the storage component trehalose, and novel β-galactooligosaccharides are gaining importance nowadays. The most important products are shortly described below and summarized in **Table 2**.

Proteases

Stable proteases are of great interest for the industry and a vast number of different proteases from both *S. solfataricus* (Hanner et al., 1990; Burlini et al., 1992; Colombo et al., 1995; Guagliardi et al., 2002; Gogliettino et al., 2014) and *S. acidocaldarius* (Fusek et al., 1990; Lin and Tang, 1990) has been described in detail. Condò et al. (1998) described an active, chaperonin-associated aminopeptidase from *S. solfataricus* MT4. Sommaruga et al. (2014) were able to significantly improve stability and reaction yield of a well-characterized carboxypeptidase also from *S. solfataricus* MT4 by immobilizing the enzyme on magnetic nanoparticles.

Esterases/Lipases

A serine arylesterase from *S. solfataricus* P1 was expressed. Besides its broad arylesterase activity, it was found to exhibit paraoxonase activity toward organophosphates (Park et al., 2008). With a temperature optimum of 94°C, a half-life of approximately 50 h at 90°C and high stability against detergents, urea and organic solvents, the enzyme has a high potential for industrial applications. An esterase from *S. tokodaii* strain 7 was expressed in *E. coli* and in addition to its optimal activity at 70°C remained active in a mixture of water and organic solvents such as acetonitrile and dimethyl sulfoxide (Suzuki et al., 2004).

Chaperonins

A small heat shock protein (S.so-HSP20) from *S. solfataricus* P2 was successfully used to increase the tolerance in response to temperature shocks (50, 4°C) of *E. coli* cells (Li et al., 2012). The chaperonin Ssocpn, which requires ATP, K^+, and Mg^{2+} but no additional proteins for its function, produced in *S. solfataricus* GΘ has been shown to yield folded and active protein from denatured materials. For this application, the chaperonin (920 kDa) was retained on an ultrafiltration cell, while the renatured substrates passed through the membrane (Cerchia et al., 2000).

Liposomes/Membrane

The membrane of extreme thermophilic Archaea is unique in its composition due to its tetraether lipid content. Archaeal lipids are a promising source for liposomes with outstanding temperature and pH stability and tightness against solute leakage. These so-called archaeosomes are potential vehicles for drug, vaccine, and gene delivery (Patel and Sprott, 1999; Krishnan et al., 2000; Benvegnu et al., 2009; Mahmoud et al., 2015). Also the use as components for bioelectronics has been proposed (De Rosa et al., 1994; Hanford and Peeples, 2002). Unfortunately, no such applications using archaeal lipids have been published yet.

Sulfolobicins

Sulfolobus spp. produce an interesting class of antibiotic proteins and peptides which are known under the term archaeocins, or more specifically sulfolobicins (Prangishvili et al., 2000; O'Connor and Shand, 2002; Besse et al., 2015). Sulfolobicins are potent and highly specific growth inhibitors targeting species closely related to the producing organism. Sulfolobicins have been identified as proteins of a size of 20 kDa in *S. islandicus* (Prangishvili et al., 2000) or heterodimers of 22 kDa per subunit in *S. acidocaldarius* (Ellen et al., 2011). They are associated with the cell membrane as well as with membrane vesicles of 50–200 nm in diameter. Known producers of sulfolobicins are *S. islandicus* strain HEN2/2 (Prangishvili et al., 2000), *S. acidocaldarius* DSM639, *S. tokodaii* strain 7, and *S. solfataricus* P2 and P1 (all strains: Ellen et al., 2011). Sulfolobicins are among the most resilient antimicrobial biomolecules withstanding temperatures of 78°C, SDS treatment, a broad pH range from 3 to 10.7, trypsin treatment, and longtime storage (Besse et al., 2015).

TABLE 2 | Products and applications of *Sulfolobus* spp. reported in the literature.

Enzymes or products	Application	Citations
Extremozymes		
Proteases	Food, textile, and cleaning industry	Fusek et al., 1990; Hanner et al., 1990; Burlini et al., 1992; Colombo et al., 1995; Condò et al., 1998; Guagliardi et al., 2002; Gogliettino et al., 2014
Esterases/lipases	Textile and cleaning industry; synthesis of chiral fine chemicals	Suzuki et al., 2004; Park et al., 2008
Chaperonins	Biopharmaceutical protein production	Cerchia et al., 2000; Li et al., 2012
Polysaccharide degrading enzymes	Biorefinery applications for the conversion of lignocellulose into value-added products	Grogan, 1989; Moracci et al., 1995, 2000; Haseltine et al., 1996; Cannio et al., 2004; Kim et al., 2004; Kufner, 2011
Novel biomolecules and interesting metabolites		
Archaeal membrane components	Liposomes for drug delivery	De Rosa et al., 1994; Patel and Sprott, 1999; Krishnan et al., 2000; Benvegnu et al., 2009; Mahmoud et al., 2015
Sulfolobicins	Antibiotic agents	Prangishvili et al., 2000; O'Connor and Shand, 2002; Besse et al., 2015
Trehalose	Preservation of enzymes and drugs	Nicolaus et al., 1988; Kobayashi et al., 1996; Lernia et al., 2002
β-galactooligosaccharides	Food industry/dietary additives	Reuter et al., 1999; Petzelbauer et al., 2000

Trehalose

Trehalose is crucial for anhydrobiosis in many organisms and is widely used for the preservation of enzymes and antibodies (Ohtake and Wang, 2011). On top of that it serves as a valuable chemical in the food and cosmetics industry (Richards et al., 2002). It is a known metabolite of *Sulfolobus* spp. and the biosynthetic pathways are identified (Nicolaus et al., 1988; Kobayashi et al., 1996). Since its biosynthesis is regarded to be a stress response, the selective production of trehalose is a promising target for process engineering. The enzymatic capability of *S. solfataricus* to efficiently produce trehalose was already proven by Lernia et al. (2002): In a cell-free environment, trehalose was produced from dextrins with enzymes from *S. solfataricus* MT4 in an immobilized bed reactor with a conversion rate of 90%.

Unique Enzymes for the Synthesis of High-Value Chemicals

A number of applications for enzymes from *Sulfolobus* spp. in the synthesis of high-value chemicals have been suggested and many innovative processes have been reported: Petzelbauer et al. (2000) developed a high-temperature process for enzymatic hydrolysis of lactose for the generation of novel di- and trisaccharides (Reuter et al., 1999) using β-glycosidases from *S. solfataricus* MT4 and *Pyrococcus furiosus*. Sayer et al. (2012) characterized a thermostable transaminase from *S. solfataricus* P2. This enzyme is part of the non-phosphorylated pathway for serine synthesis which is not described in bacteria, but found in animals and plants (Walsh and Sallach, 1966; Liepman and Olsen, 2001). In *S. tokodaii*, an L-haloacid dehalogenase was found and characterized by Rye et al. (2009). This enzyme could potentially be used for the chiral production of halo-carboxylic acids which are important precursors in the fine chemical and pharmaceutical industries, as well as for bioremediation. An NAD^+/NADH-dependent medium-chain alcohol dehydrogenase with remarkably broad substrate specificity toward primary, secondary, branched as well as cyclic alcohols and their corresponding aldehydes and ketones has been described by Raia et al. (2001). Lactonases have been described both from *S. solfataricus* MT4 (Merone et al., 2005) and from *S. islandicus* (Hiblot et al., 2012). These enzymes are attractive for biotechnological and pharmaceutical applications. An aldolase from *S. solfataricus* P1 catalyzing the reversible C-C bond formation between non-phosphorylated substrates pyruvate and glyceraldehyde to KDG was described by Buchanan et al. (1999). A sterioselective amidase from *S. solfataricus* MT4 has been described by Scotto d'Abusco et al. (2001).

BIOPROCESSING WITH *Sulfolobus*

It is evident that *Sulfolobus* spp. accommodate a huge variety of high value-added products useful in different fields of research and industry. However, this resource has basically remained untapped until now, due to a lack of proper bioprocessing tools. Of course, many of these products can also be produced recombinantly in mesophilic hosts. Benefits of the heterologous production in mesophilic hosts are much faster growth rates, highly efficient expression, extremely well-developed process technology, and facilitated downstream processing of thermostable proteins, since a considerable amount of host cell proteins can be readily removed via heat precipitation. Nevertheless, the production of proteins difficult to express and products remaining inactive due to differences in the expression and folding machinery, call for protein production in the archaeal host (Eichler and Adams, 2005; Kim and Lee, 2006). Furthermore, certain products are native cell constituents of *Sulfolobus* spp. (e.g., archaeal membrane containing tetraether lipids), which underlines the need to generate biomass and thus of bioprocess technology.

We are convinced that thermophilic bioprocesses have the potential to compete with conventional bioprocesses, since the drawbacks of typically lower growth rates and protein expression rates can be outweighed by a number of advantages resulting from the elevated process temperature:

(1) Probably the most significant advantage is the reduced risk of contamination. Loss of complete batches or reduced productivity due to chronical basal contamination levels poses serious threats for an economically feasible bioprocess based on mesophiles (Skinner and Leathers, 2004). In case of bioprocesses with *Sulfolobus* spp. not only the high cultivation temperature, but also the low pH reduce the contamination risk.

(2) While often limited at moderate temperatures, the solubility of substrates is significantly increased at elevated process temperatures (Gray et al., 2007). This is especially crucial in applications where oligomers and polymers are used as substrates, like in waste-to-value processes based on the conversion of lignocellulosic biomass.

(3) Considering energy requirements, a further advantage over mesophilic fermentations is the reduced need for expensive, active cooling of the fermenter in large scales for the removal of excess metabolic heat. Here, high-temperature fermentations benefit from the greater difference between ambient air temperature and fermentation broth (Abdel-Banat et al., 2010).

(4) Expression systems based on so-called cold shock promoters are well known and commercialized for mesophilic hosts (e.g., the pCold expression system from Takara Bio Europe, Saint-Germain-en-Laye, France). Nevertheless, the utilization in large-scale processes is not feasible due to high costs for cooling. In high-temperature processes, cooling is much more cost-efficient due to fast heat transfer. This way, temperature-regulated expression with shifts from growth phase to production phase becomes an option.

(5) The production of volatile compounds like short-chained alcohols benefits from high process temperatures. These compounds can be continuously recovered via the off-gas stream, while no additional separation is required. Furthermore, product inhibition, a common issue when producing toxic substances like alcohols, is prevented (Zeldes et al., 2015).

Although there is a steadily growing interest in the development of extremophilic bioprocesses, no industrial process utilizing *Sulfolobus* spp. has been developed yet. Doubling times of at least 5–8 h (Brock et al., 1972; Grogan, 1989) and low biomass titers in batch cultures [max. 2 g/L dry cell weight (Schiraldi et al., 1999)] are the main obstacles for establishing efficient bioprocesses. The low biomass titer not only is a severe hindrance for biotechnological applications, but also poses a limitation for basic research because biomass and enzyme production of *Sulfolobus* spp. in shake flasks is painfully inefficient. As a result, archaeal enzymes are still mainly produced recombinantly in mesophilic hosts like *E. coli*, despite the aforementioned limitations.

In order to realize a competitive bioprocess, high cell densities in a reasonable time and economically feasible space-time yields must be achieved. This can be done by genetic engineering, optimized nutrient supply, and adjustment of process parameters. On the other hand, for bioconversion reactions, the issue of a low growth rate is not necessarily a neck-breaking drawback, if it is possible to integrate a cell-retention system combined with continuous cultivating. In that case, rather the maximum cell density, which is proportional to the volumetric catalytic activity, is a critical process parameter. However, studies on bioreactor cultivations with *Sulfolobus* spp. are still scarce.

As shown in **Table 3**, a high cell density cultivation is only reported for *S. shibatae* B12. However, it is evident that a sophisticated bioreactor setup including a cell-retention system is needed to realize a competitive bioprocess with *Sulfolobus* spp. Such a bioreactor setup is exemplarily depicted in **Figure 3**.

Remarkably, in none of the fermentations reported to date, defined media were used. Nevertheless, this is of high importance for the generation of platform knowledge and science-based process development. Use of defined media does allow not only the characterization and comparison of the variety of strains, but also the generation of comprehensive process understanding enabling process control and prediction. Furthermore, the use of defined media facilitates the transfer of process knowledge and speeds up process development and optimization. Another aspect worth considering is that bioprocesses that follow good manufacturing practice guidelines call for defined media to

TABLE 3 | Bioreactor cultivations with *Sulfolobus* spp. described to date.

Strain	Final biomass titer (g_{DCW}/L)	Fermentation time (h)	Average volumetric productivity (g_{DCW}/L/h)	$Yield_{X/S}$ ($g_{DCW}/g_{substrate}$) and carbon sources	Cultivation mode and working volume (L)	Source
Sulfolobus shibatae B12 (DSM 5389)	114	358	0.32	0.156 g/g at an Yeast extract/D-glucose ratio of 1:15	Dialysis reactor, 1 L	Krahe et al., 1996
Sulfolobus solfataricus P2 (DSM 1617)	22.6	170	0.13	0.17 g/g at an Yeast extract/D-glucose ratio of 1:4	Constant volume fed batch, 13.8 L	Park and Lee, 1997
Sulfolobus solfataricus P2 (DSM 1617)	21.7	213	0.10	Yeast extract/D-glucose ratio of 1:4	Fed batch, 2.3 L	Park and Lee, 1999
Sulfolobus solfataricus GΘ	35	310	0.11	Yeast extract/D-glucose ratio of 1:15	Fed batch with microfiltration,10 L	Schiraldi et al., 1999
Sulfolobus shibatae B12 (DSM 5389)	10	200	0.05	Yeast extract/D-glucose ratio of 1:15	Fed batch, 1.3 L	Krahe et al., 1996

DCW, dry cell weight.

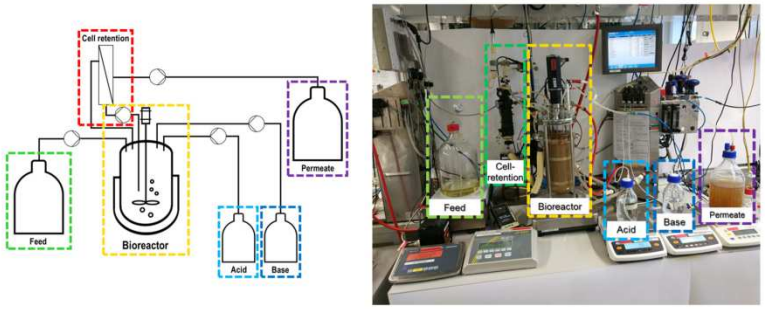

FIGURE 3 | Scheme and setup of bioreactor system capable of reaching high cell densities via simultaneously applying a feed and cell-retention strategy. Nutrients can be continuously fed and at the same time spent medium containing metabolites and possibly inhibiting substances is removed via a membrane, while cells are retained.

avoid batch-to-batch variability. These aspects underline the importance of the substitution of complex carbon sources, like yeast extract or protein hydrolysates, for the application of *Sulfolobus* spp. in industrial biotechnology for the production of high value-added products. Summarizing, to move *Sulfolobus* spp. into industrial biotechnology, (1) sophisticated bioreactor solutions and (2) defined media must be available.

Sulfolobus AS POTENTIAL PLAYER IN THE BIOREFINERY OF THE FUTURE?

Besides being a native source of high value-added products like extremozymes, extreme thermoacidophiles are predestined for the task of sustainably converting lignocellulosic biomass into value-added products due to their resilience toward harsh process conditions and their hemicellulolytic and cellulolytic properties (Turner et al., 2007). *S. solfataricus* in particular can grow on a very broad range of carbon sources (Grogan, 1989) and harbors a variety of polymer-degrading enzymes such as cellulases (Kufner, 2011), glucoamylases (Kim et al., 2004), alpha-amylases (Haseltine et al., 1996), beta-glucosidases (Moracci et al., 1995), xylanases (Cannio et al., 2004), and xylosidases (Moracci et al., 2000). Optimal growth in a hot, acidic environment means perfect synergy with the state-of-the-art method of substrate pretreatment utilizing high temperature and low pH. Although a variety of concepts for substrate pretreatment exists, the most favored process is the one of dilute sulfuric acid hydrolysis where concentrations of 0.5–1.5% sulfuric acid and temperatures between 120 and 180°C are commonly used (Carvalheiro et al., 2008; Maurya et al., 2015). Thus, pretreated substrate can be utilized in biorefinery applications based on *Sulfolobus* spp. with little to no need of neutralization and cooling of the medium. During the pretreatment process, a mixture of sugar monomers (mainly D-xylose, D-glucose, D-mannose, and L-arabinose) is released. In contrast to mesophilic hosts like *Saccharomyces cerevisiae* or *E. coli*, *S. acidocaldarius* lacks carbon catabolite repression (Ulas et al., 2012), thus allowing the efficient simultaneous utilization of a variety of sugars.

The combination of broad substrate specificity, lack of carbon catabolite repression, expression of polymer degrading enzymes, and extreme growth conditions make *Sulfolobus* spp. promising candidates for biorefinery applications. Following this approach, waste streams of the chemical and pulp and paper industry can be converted into value-added products. These processes would greatly benefit from the increased substrate solubility due to high temperatures and low pH. The availability of genetic tools and a broad variety of different strains are the basis for an application of *Sulfolobus* spp. in the biorefinery – however, the challenge of realizing a competitive bioprocess remains.

CONCLUSION

There are several reasons to be optimistic with respect to the use of *Sulfolobus* spp. in biotechnology. Greatly reduced contamination risk, high substrate solubility, adaption to harsh substrate pretreatment conditions, facilitated removal of volatile products, and elimination of cooling costs are benefits of high-temperature processes with *Sulfolobus* spp. The genus is a source of a broad variety of temperature and acid stable enzymes as well as a producer of unique biomaterials and metabolites. A well-developed genetic toolset makes exploitation of these features possible and emergence of metabolically engineered production strains is reasonable in the near future.

However, there is still a great need for careful bioprocess development. No continuous processes are reported in the literature and sophisticated tools for monitoring and control, like on-line measurement techniques for assessing cell viability, are lacking completely. Furthermore, media development and optimization have largely been neglected. For the establishment of a competitive, long-lasting, or continuous bioprocess, it is mandatory to generate basic process knowledge to be able to understand and control the bioprocess. Thus, we will tackle this challenge to be able to add *Sulfolobus* spp. as key player in industrial biotechnology in the future.

AUTHOR CONTRIBUTIONS

OS conceived the idea for writing this review. JQ drafted the manuscript, while S-VA and BS contributed the chapters on genetic tools and central carbon metabolism, respectively. LS contributed the figure describing the central carbon metabolism. OS critically reviewed and corrected the manuscript and gave substantial input.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02474/full#supplementary-material

TABLE S1 | Milestones in *Sulfolobus* research.

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Enzymatically treated beech wood hydrolysate as carbon source for *Sulfolobus acidocaldarius*

Contribution to Chapter 2

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Evaluation of the potential of an enzymatically treated beech wood hydrolysate as carbon source for *Sulfolobus acidocaldarius* for the production of high value chemicals

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Abstract

In this case study we investigated the potential of enzymatically treated beech wood hydrolysate as substrate for the well-studied thermo-acidophilic archaeal model organism *Sulfolobus acidocaldarius*. Although the mild enzymatic treatment led to the absence of the common inhibitors furfural and hydroxymethylfurfural, a detoxification step with activated carbon was still necessary to allow efficient growth on the hydrolysate. In addition to the optimization of this detoxification process we developed a flow cytometry based method for biomass determination in turbid cultivation media, like the investigated hydrolysate. This feasibility study showed that it is possible to utilize the tested hydrolysate as carbon source when supplemented with monosodium glutamate as growth promoting substance.

Introduction

The Archaeon *Sulfolobus acidocaldarius* is a thermo-acidophilic microorganism growing optimally at 75 °C and pH 3. The organism is a source of unique enzymes (Littlechild, 2015) and metabolic pathways (Bräsen et al., 2014) not existent in any mesophilic microbial hosts. Its unique characteristics and the fact that it is amenable to genetic manipulation make the organism a promising candidate for numerous biotechnological applications, like bioconversion processes (e.g. treatment of process wastewater), bioleaching for the economic mining of low grade ores, for prospecting of highly stable enzymes possibly with unusual catalytic activity, or for the production of unique biological materials (Quehenberger et al., 2017). From a process technological point of view, the organism is predestined for applications where sterility is difficult to achieve and/or to maintain due to its harsh growth conditions. Especially continuous processes run over extended periods of time, which are undoubtedly becoming more and more popular in the biotechnological industry, are important examples.

Tetraether lipids (TELs), the major membrane constituents of *S. acidocaldarius*, are gaining rapidly increasing attention as a unique biological material for pharmaceutical applications due to their remarkable stability at elevated temperatures and low pH values and resistance towards enzymatic degradation (He et al., 2018; Jacobsen et al., 2017; Kaur et al., 2016; Mahmoud et al., 2018; Uhl et al., 2017). TELs are mainly used in medical applications for the formation of drug delivery vehicles. Despite the numerous promising publications on the pharmaceutical utilization of TELs, no commercial applications exist to date. Reasons are too high production costs and low availability of the lipids. As expenditures for cultivation media are the most important cost driver in microbial bioprocesses we wanted to tackle the problem of exceeding costs by investigating the use of a cheap and sustainable alternative carbon sources to supplement or replace expensive routinely used substrates, like glucose or NZ-amine, for *S. acidocaldarius*. This role can be fulfilled especially well by substrates based on biomass waste streams originating from forestry, food or agricultural industries. In this work we chose to investigate the use of hydrolyzed beech wood chips as lignocellulosic model substrate, since beech is the most abundant hard wood species in Austria (Austrian Research Centre for forests (BFW), 2011) and after spruce the second most frequently used industrial wood species contributing to an estimated annual total of 7.8 million m^3 of industrial wood residues in Austria (Austrian Ministry for Sustainability and Tourism (BMNT), 2015). Apparently, lignocellulose hydrolysates represent a cheap, available and sustainable raw material for microbial bioprocesses. Nevertheless, due to the harsh pretreatment conditions of high temperature and extreme pH values, inhibitors formed during classical hydrolysis processes of acid hydrolysis or steam explosion oftentimes restrict the applicability as carbon source. Therefore, we chose to investigate hydrolysate produced by enzymatic treatment, where comparatively mild conditions of pH 4 and 75 °C were employed. Goal of this feasibility study was the evaluation of the potential of enzymatically treated beech wood hydrolysate as cheap and sustainable carbon source for the growth of *S. acidocaldarius*, an extremophilic microorganism with great potential as production host for pharmaceutically relevant tetraether lipids.

Material and Methods

Lignocellulose pretreatment

Enzymatically treated beech wood hydrolysate was prepared from 10 g/L beech wood saw dust incubated at 75 °C for 48 h with a 1:1 mix of cellulases and xylanases (Ecopulp TX-800 A (AB Enzymes, Germany) and CelluPract AL 100 (Biopract GmbH, Germany)). 10 mL enzyme mix were added at 0, 12 and 24 h (30 mL total).

Strains and growth conditions

Sulfolobus acidocaldarius DSM 639 was grown aerobically at 75 °C in a reciprocal shaking oil bath at 100 rpm in 100 mL long neck Erlenmeyer flasks to avoid evaporation. The flasks were filled with 20 to 50 mL hydrolysate or culture medium and the initial pH was adjusted to 4.0

with 4.8 w% H_2SO_4. Salts and trace elements were provided by addition of appropriate stock solutions as described elsewhere (Quehenberger et al., 2019). When indicated, the hydrolysate was supplemented with 1 g/L NZ-amine and 2 g/L glucose to support faster growth. As reference for the evaluation of biomass growth the defined VD-Medium described by Quehenberger et al. (2019) was used.

Analytical methods

Growth of *S. acidocaldarius* was monitored with a photometer (Genesys 20 photometer (Thermo Scientific, USA) via determination of the optical density at 600 nm. Supernatant for determination of sugar concentrations was prepared by centrifugation (12 000 rcf, 10 min, 4 °C). Sugar analysis of the hydrolysate was performed with HPLC equipped with a refraction index detector (Agilent G1362A, USA) using an Aminex HPX-87H column (300x7.8 mm) at 60 °C. 4 mM H_2SO_4 was used as eluent with an isocratic flow of 0.6 mL/min for 30 min. Flow cytometry analysis was performed with a Cube 8 flow cytometer (Sysmex, Germany). No stains were added to the cells and samples were diluted with 0.85 w/v% NaCl solution prepared with MQ water to a range appropriate for the instrument. Samples were measured timely (max 30 min) after harvest.

Results and Discussion

Preliminary growth experiments

To determine whether growth on the enzymatically treated beech wood hydrolysate (composition in Table 1) was possible, shake flask cultivations were performed on hydrolysate supplemented with 1 g/L NZ-amine and 2 g/L glucose to support faster growth.

Table 1: Composition of the enzymatically treated beech wood hydrolysate. No sugars other than xylose and glucose were found; also neither acetate nor the known inhibitors furfural and hydroxymethylfurfural were detected in the substrate.

Component	[g/L]
Glucose	0.91
Xylose	0.14
Arabinose	n.d.
Galactose	n.d.
Mannose	n.d.
Reducing sugars (total)	2.10
Acetate	n.d.
Furfural	n.d.
Hydroxymethylfurfural	n.d.
Protein	6.22

n.d. not detected

During the initial growth experiments the hydrolysate was pre-incubated for 24 h at 75 °C and subsequently centrifuged to precipitate the majority of the hydrolytic enzymes. Following this pre-incubation step, a dilution series of the hydrolysate (1:1, 1:2, 1:5 and 1:10;

all supplemented to a final concentration of 1 g/L NZ-amine and 2 g/L glucose) was performed. The results of these growth experiments are shown in Figure 1. It turned out that the beech wood hydrolysate indeed contained inhibitors that prohibited growth on the undiluted substrate even though only treated gently with enzymes. 1:2 dilution of the medium already allowed reasonable biomass growth, while at a dilution of 1:5 best growth was observed. Interestingly, at a dilution of 1:10 growth decreased again. A reason for better growth on the 1:5 dilution could be the presence of growth promoting factors in the hydrolysate, like amino acids or oligopeptides derived from the hydrolytic enzymes. Unfortunately, the inhibitors present in the beech wood hydrolysate could not be identified. Possible substances would include phenolic compounds or fatty acids. Furfural and hydroxymethyl furfural could be ruled out based on HPLC analysis. It should be noted that the determined growth curves were subject to high variation due to persistent formation of suspended particles despite the precipitation step. Therefore, we had to develop a more sophisticated method of flow cytometry based biomass determination.

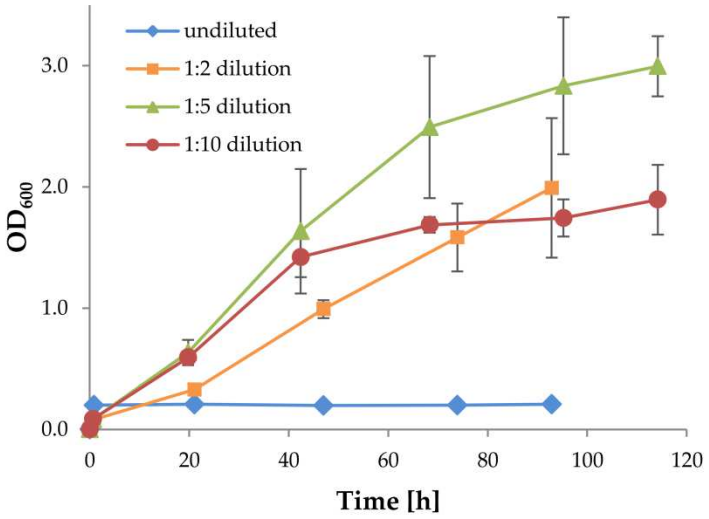


Figure 1: Growth experiments on complex medium based on enzymatically hydrolyzed beech wood. Experiments were performed in different dilutions to investigate the potential presence of growth inhibitors. Best growth was observed with the 1:5 diluted hydrolysate, representing the optimal balance between elimination of inhibitors and supply with growth promoting substances.

Biomass determination in turbid media

Although the investigated beech wood hydrolysate was centrifuged and filtered over a 0.2 µm membrane, precipitates formed during incubation at growth conditions (75 °C, pH 4) and the broth became cloudy even if kept sterile. Under these conditions routinely used methods for biomass determination, like measurement of OD_{600} or gravimetric determination, cannot be applied since suspended solids are also counted as biomass with these methods. In order to be able to follow the biomass growth in the turbid media and thus allow process monitoring in beech wood hydrolysates a flow cytometry based method capable of discriminating between cells of *S. acidocaldarius* and media particles due to differences in shape and size of the particles was developed. The method is applicable without the use of fluorescent dyes – the signals of side scatter (SSC) and forward scatter (FSC) were sufficient to distinguish cells of *S. acidocaldarius* from media particles (Figure 2). This way, media components that add to OD_{600} and gravimetrically determined dry cell

weight are efficiently excluded from the biomass measurement. A direct comparison of 5 exemplary growth curves acquired with OD_{600} measurements in comparison to the flow cytometry method is show in Figure 3. For verification of the flow cytometry based method the colony counts from a plating assay (as orthogonal method for biomass determination) are displayed. The results reveal that with an average error between the two methods of only 4 %, the flow cytometry based method is in accordance with the plating assay, while there is only little correlation with the OD_{600} results, yielding an average error of 16 % between the photometric method and the plating assay.

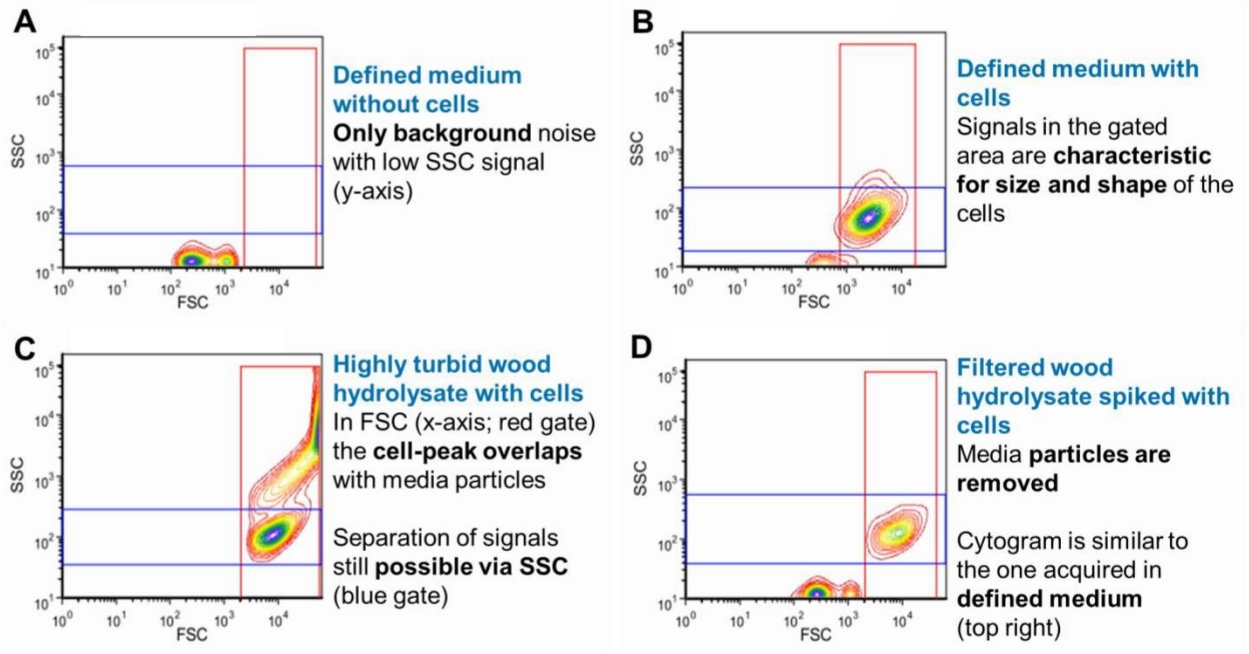


Figure 2: A combination of forward- (FSC) and side scatter (SSC) signals can be used to distinguish cells from (differently shaped) media particles. While SSC represents the shape of a particle, FSC is linked to the particle size. A: In the gated area (overlap of blue and red rectangle) no signal can be detected. B: the same medium was inoculated with cells and a sample was measured during exponential growth phase. Signals are visible in the gated area, characteristic for the shape and size of the cells. C: a culture grown in highly turbid wood hydrolysate was measured. Although in FSC (x-axis; red gate) the cell-peak overlaps with the signals from the media particles, a separation of these signals can be achieved via different intensities of their SSC (blue gate). D: wood hydrolysate was filtered and spiked with cells. The resulting cytogram is similar to the one acquired in the standard medium (B).

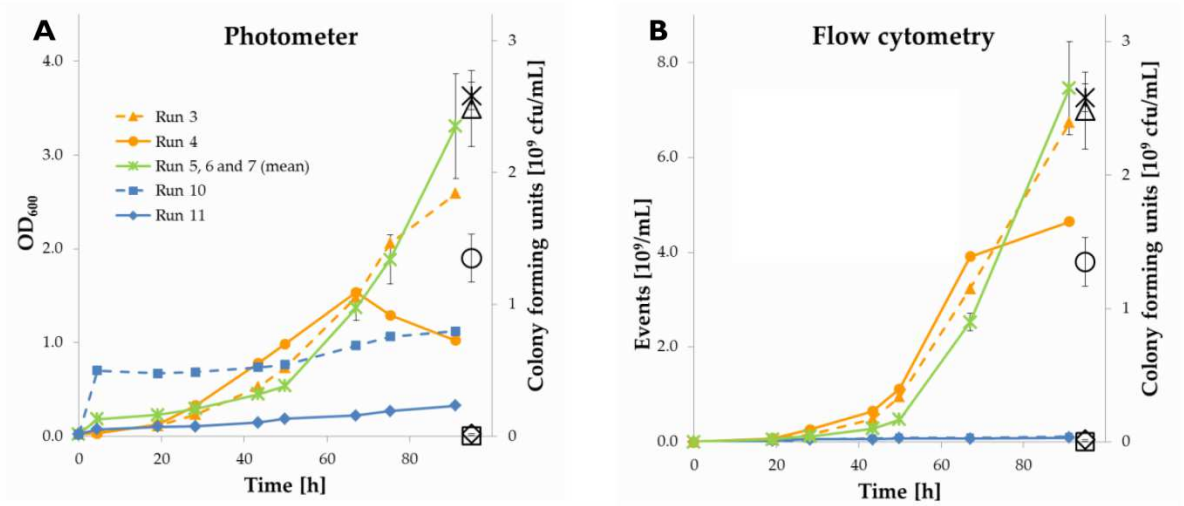


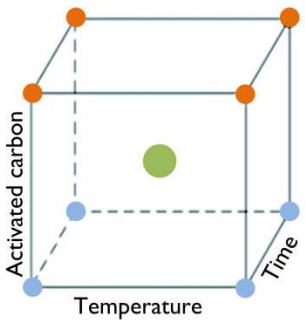
Figure 3: Comparison of cell density measurement using a photometer vs. flow cytometry, exemplarily shown for 5 differently treated hydrolysates (see Table 2 for a description of the treatment conditions of the different runs). Using flow cytometry for the cell density measurements resulted in significantly different growth curves compared to OD_{600}. Results of the former method correlate well with the outcomes of a plating assay (determined in triplicates for the final time points and displayed as empty symbols [10^9 cfu/mL]) with an average error between the two methods of only 4 %, while the error between the photometric method and the plating assay is 16 %. A: Growth curve on differently treated substrates according to OD_{600}. B: Growth curve on differently treated substrates acquired with flow cytometry.

Optimization of a detoxification step using activated carbon

Since growth was not possible on the investigated undiluted hydrolysate, and further dilution could not be considered an option due to the already low content of fermentable sugars, it was necessary to develop a simple detoxification step for the removal of inhibitors present in the beech wood hydrolysates. Treatment with activated carbon (AC) turned out to be highly efficient in removing inhibitors from the employed substrate. Following a DoE approach the AC treatment was optimized regarding temperature, time and AC concentration (Table 2) using subsequent growth of *S. acidocaldarius* as response factor. The samples were supplemented with trace elements, 1 g/L NZ-amine and 2 g/L glucose, owing to the low sugar concentration in the used beech wood hydrolysate.

Table 2: Conditions applied in the DoE for the investigation of the substrate treatment with activated carbon. Growth of *S. acidocaldarius* determined via flow cytometry after 90 h cultivation time was taken as response factor. $R^2 = 0.762$. $Q^2 = 0.681$.

Exp. No.	Time [min]	Temperature [°C]	Activated carbon [w%]	Response [10^9 events]
1	30	25	7.5	**3.21**
2	120	25	7.5	**4.94**
3	30	75	7.5	**6.73**
4	120	75	7.5	**4.65**
5	75	50	5.0	**6.50**
6	75	50	5.0	**7.41**
7	75	50	5.0	**8.47**
8	30	25	2.5	**0.11**
9	120	25	2.5	**0.16**
10	30	75	2.5	**0.15**
11	120	75	2.5	**0.09**



The resulting growth curves on the differently treated media are shown in Figure 4. Growth was monitored with the previously described flow cytometry based method for biomass determination in turbid media. Compared to incubation time and temperature the concentration of AC had the strongest effect on growth. 2.5 w% AC was not enough for detoxification of the wood hydrolysate (no growth in any experiments with 2.5 w% AC). Incubation time and temperature correlated positively with higher cell densities. For the investigated hydrolysate we empirically determined conditions of 75 min incubation time at 50 °C using 5 w% AC to be most beneficial for growth rate and biomass yield of *S. acidocaldarius* when grown on the AC treated hydrolysates. Nevertheless, based on the conducted growth experiments we conclude that it will be necessary to empirically test new hydrolysates and that platform knowledge is hard to come by due to the strong influence of feedstock and various pretreatment steps. Therefore, we propose the use of fractional DoEs as a fast and simple way to determine the optimal detoxification conditions for other feedstocks.

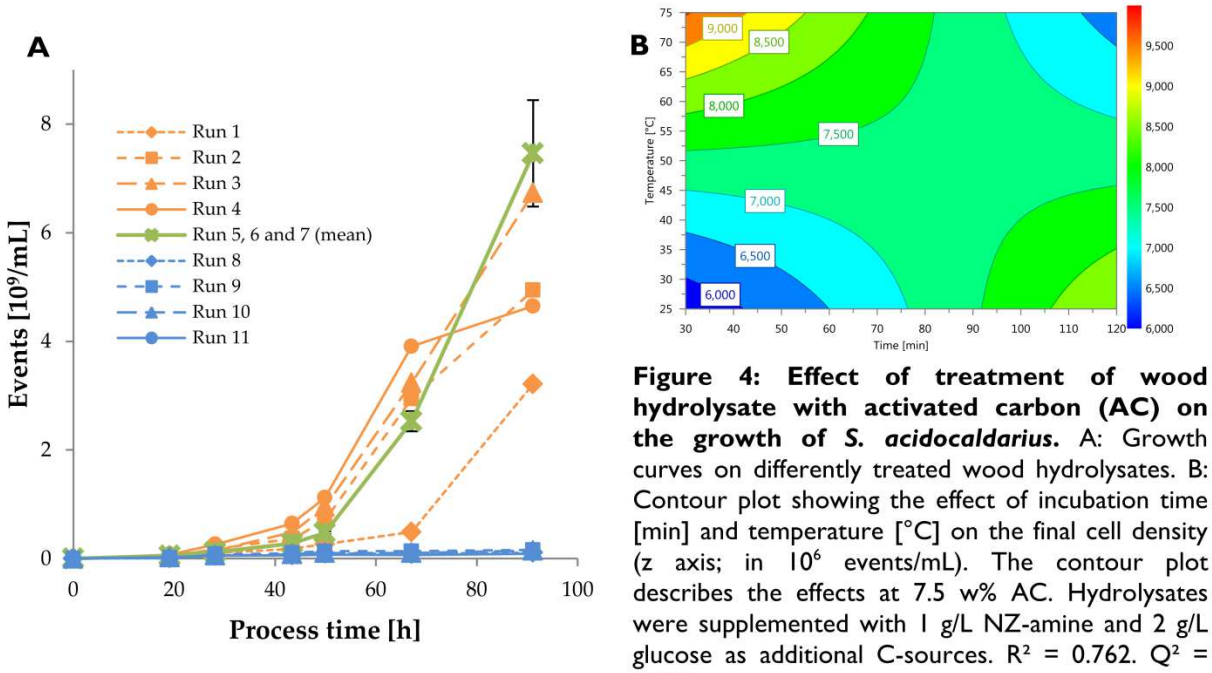


Figure 4: Effect of treatment of wood hydrolysate with activated carbon (AC) on the growth of *S. acidocaldarius*. A: Growth curves on differently treated wood hydrolysates. B: Contour plot showing the effect of incubation time [min] and temperature [°C] on the final cell density (z axis; in 10^6 events/mL). The contour plot describes the effects at 7.5 w% AC. Hydrolysates were supplemented with 1 g/L NZ-amine and 2 g/L glucose as additional C-sources. R^2 = 0.762. Q^2 = 0.681.

Growth performance of *S. acidocaldarius* on detoxified beech wood hydrolysate

Based on the preliminary growth experiments and the results of the DoE for the hydrolysate detoxification we used undiluted beech wood hydrolysate treated with 5 w% AC for 75 min at 50 °C for concluding growth experiments. Goal was to compare the growth performance with a defined reference medium that contained 1.75 g/L sodium glutamate (MSG) and 3 g/L glucose as carbon source (Quehenberger et al., 2019) and to determine whether supplementation with an additional carbon source (0.5 g/L MSG) was necessary for efficient growth. The growth promoting effect of the hydrolysate itself was assessed by comparing

the growth with a suitable "blank" medium where the beech wood hydrolysate was substituted with water and the same amount of additional carbon source (0.5 g/L MSG). The results of these experiments are shown in Figure 5. Compared to the defined reference medium growth rate and maximum cell density were significantly lower. Without supplementation of any other C-source growth was very slow (~10% cell density compared to the reference after 100 h). Addition of 0.5 g/L MSG led to a significant boost in growth and enabled the organism to utilize the sugars in the hydrolysate much more efficiently. Thereby leading to higher growth compared to a medium with the same amount of MSG but with only water instead of the beech wood hydrolysate. Also in continuous cultivations the addition of MSG would be highly beneficial. In order to obtain the MSG from a cheap and environmentally friendly source, yeast biomass, that naturally contains high amounts of MSG (Pacheco et al., 1997), could be utilized. Yeast biomass can be either acquired from pharmaceutical or beverage production processes, where it is generally considered a waste product and is cheaply sold as feed or to biogas plants (Ferreira et al., 2010). To ensure availability of MSG yeast biomass could be supplied as autohydrolysate or, following the concept of process intensification, could be directly added during the beech wood hydrolysis step.

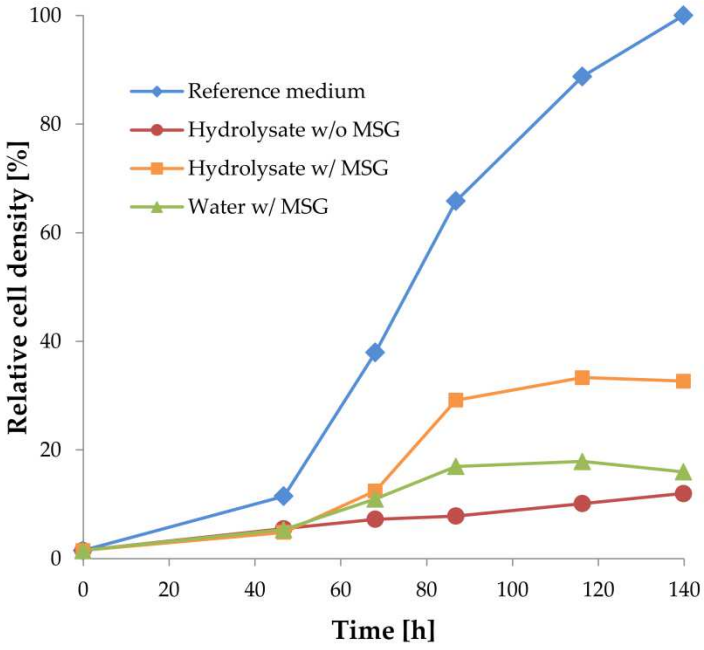


Figure 5: Growth of *S. acidocaldarius* on undiluted beech wood hydrolysate treated with activated carbon (incubation for 75 min at 50 °C with 5 w% activated carbon). Compared to the defined reference medium growth rate and maximum cell density were significantly lower. Without supplementation of any other C-source ("Hydrolysate w/o MSG") growth was very slow (~10% cell density compared to the reference after 100 h). Addition of 0.5 g/L sodium glutamate (MSG) led to a significant boost in growth ("Hydrolysate w/ MSG") and enabled the organism to utilize the sugars in the hydrolysate much more efficiently. Thereby leading to higher growth compared to a medium with the same amount of MSG but with only water instead of the beech wood hydrolysate ("Blank w/ MSG").

Conclusion

This feasibility study showed once again that complex natural substrates are unpredictable in their nature and their utilization as carbon source is challenging even for an extremophile, like *S. acidocaldarius*. The required additional process steps for substrate preparation add additional costs to any envisioned industrial application. Therefore it has to be stated that the utilization of lignocellulose hydrolysates as substrate for extremophiles is not a straightforward process and always comes with a high demand for development and

optimization efforts before its implementation as confirmed in this study by the necessary development of a detoxification step and method for biomass determination.

A possibility that remains to be investigated is the utilization of these substrates (with high inhibitor concentrations and low amounts of fermentable sugars) in continuous perfusion processes, where unwanted inhibitors are continuously removed while high cell concentrations can be maintained. As additional benefit of a perfusion process, fermentable sugars could be supplied in sufficient quantity by employing feed rates greater than the critical dilution rate.

Author contribution

OS conceived the idea for this study. SS prepared beech wood hydrolysate. JQ designed and performed the experiments for cultivation of *S. acidocaldarius* on beech wood hydrolysate and developed the methods for biomass determination in turbid media and detoxification of the hydrolysate. JQ drafted the manuscript. OS critically reviewed and corrected the manuscript and gave substantial input.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Exploitation of wheat straw biorefinery side streams as sustainable substrates for microorganisms - a feasibility study

Contribution to Chapter 2

(Submitted manuscript)

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Exploitation of wheat straw biorefinery side streams as sustainable substrates for microorganisms – a feasibility study

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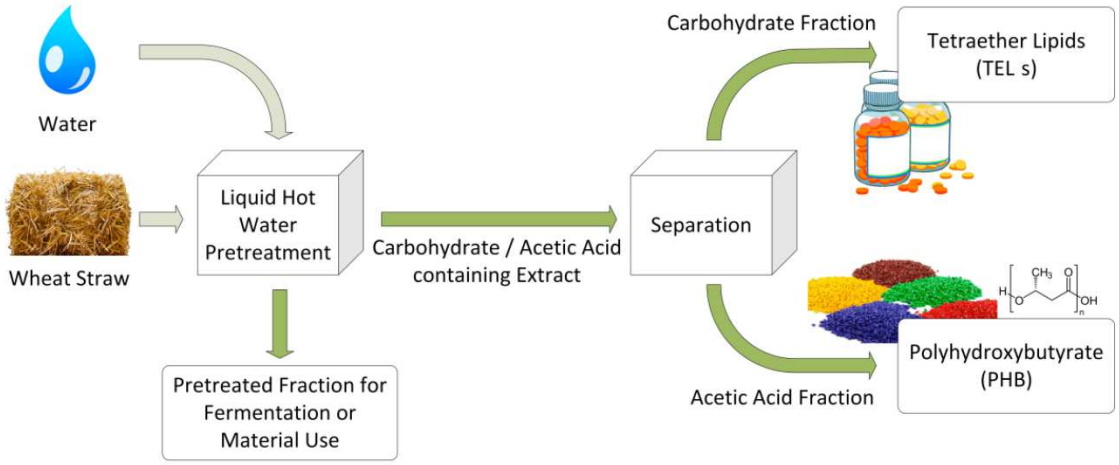

Abstract:

Lignocellulosic agricultural side products, like wheat straw, are widely seen as an important contribution to a future sustainable economy. However, optimization of biorefinery processes and exploitation of all side streams are crucial for an economically viable biorefinery. Pretreatment of lignocellulosic raw material, which is necessary for further processing steps, can generate low-value side streams. In this feasibility study, side streams from a liquid hot water pretreatment of wheat straw were utilized for the production of polyhydroxybutyrate (PHB) and highly valuable tetraether lipids (TELs). Additional value created by these products can benefit the biorefinery´s economic operation. Especially TELs as a biological material for pharmaceutical applications are anticipated to create high value. The utilized side stream consists mainly of carbohydrates from hemicelluloses and fermentation inhibitors such as acetic acid. In order to achieve a successful production of both products, an acetic acid separation step was necessary. Subsequently, the acetic acid fraction was utilized for the PHB production using cyanobacteria. The carbohydrate rich fraction was applied in the cultivation of *S. acidocaldarius* and resulted in the successful production of TELs.

Keywords: lignocellulose; biorefinery; liquid hot water; side streams; carbohydrates; acetic acid; polyhydroxybutyrate; lipids; tetraether

1. Introduction

The world is currently confronted with the progressive depletion of its resources, mainly based on non-renewable feedstocks [1]. Lignocellulosic biomass residues are estimated to exceed 2×10^{11} t/year worldwide and offer a vast source for the production of renewable products and chemicals in so-called biorefineries [2,3]. In order to achieve an economic operation and efficient processing of renewable feedstocks into bio-based products all biomass fractions are supposed to be turned into a marketable products [4].

A biorefinery approach involves a multi-step process in which the first step, subsequent to the feedstock selection, typically involves treating the biomass to pre-separate the main components cellulose, hemicellulose and lignin and to make it more amenable for further processing [5]. This step is conventionally referred to as pretreatment and can account for up to 20-40% of the overall production costs which puts special focus on its optimization [6].

Among the various pretreatment methods, hydrothermolysis using water at elevated temperature and under pressure has shown to be effective in removing and solubilizing hemicellulose, thus improving the subsequent processing. The so-called liquid hot water pretreatment (LHW) of lignocellulosic biomass dissolves hemicelluloses and some of the lignin while minimizing the formation of inhibitors for subsequent use as substrate for the cultivation of microorganisms [7]. The process results in a solid stream containing mainly cellulose and lignin and an aqueous side stream containing mainly dissolved hemicelluloses and acetic acid. In terms of the improvement of the process economics, mentioned components in the aqueous side stream can potentially be used as carbon source for microbial fermentation processes. In this feasibility study we exemplarily tested the utilization of this aqueous side stream as substrates for the cultivation of cyanobacteria producing polyhydroxybutyrate (PHB; e.g. [8,9]) as well as for the cultivation of *Sulfolobus acidocaldarius*, a thermoacidophilic archaeon and producer of highly valuable tetraether lipids (TELs; e.g. [10]). We chose these two very different hosts due to the synergies that emerge from the combination of the hydrolysate and the organisms: *S. acidocaldarius* can directly grow on the hot and acidic hydrolysate without the need for extensive cooling and neutralization, while the photoautotrophic cyanobacteria can thrive on acetate as additional energy source. At the same time *S. acidocaldarius* benefits from the removal of acetate since it is a potent growth inhibitor for the archaeon.

PHB is the best-characterized member of the polyhydroxyalkanoate (PHA) family and is widespread in various bacterial species as storage material [11,12]. PHB is biodegradable (and compostable according to EN 13432), insoluble in water, non-toxic and biocompatible. Therefore, PHB could be an attractive alternative to petroleum-based plastics [13]. It resembles the commodity polymer polypropylene in its properties [14]. PHB is commercially produced by heterotrophic bacteria such as *Alcaligenes eutrophus* [15], *Alcaligenes latus* [16] and recombinant *Escherichia coli* [17]. Despite relatively high yields of PHB, production from bacterial fermentation requires sugar supplementation and continuous oxygen supply which results in high substrate and operation costs [11,18]. In addition, public discussion about bioplastics production from sugar feedstocks is similar to the discussion about first generation biofuels. Competition of material with food and feed production for the same resources potentially leads to shortages and price increases and is also contributing to climate change through direct and indirect land use change [19].

TELs, the major membrane constituents of *S. acidocaldarius*, are gaining rapidly increasing attention as a unique biological material for pharmaceutical applications [20–24]. These TELs are highly stable at elevated temperatures and low pH values and resent towards enzymatic degradation. TELs are mainly used in medical applications for the formation of drug delivery vehicles. Despite the numerous promising publications on the pharmaceutical utilization of TELs no commercial applications exist to date. Reason for that are too high production costs and low availability of the lipids. As expenditures for cultivation media are the most important cost driver in microbial pharmaceutical bioprocesses we wanted to tackle this problem by investigating the use of a cheap and sustainable alternative carbon source to supplement or replace routinely-used carbon sources, like glucose or NZ amine, as substrate for *S. acidocaldarius*. This role can be fulfilled

especially well by substrates based on biomass waste streams from forestry, food or agricultural industries, like the here investigated agricultural by-product wheat straw.

This feasibility study shows the pretreatment of the residual lignocellulose wheat straw in a LHW process in laboratory and pilot scale. The resulting liquid side streams containing hemicellulose and acetic acid were investigated in terms of their suitability for the recombinant production of PHB and TELs in laboratory experiments.

2. Materials and Methods

2.1. Materials

The wheat straw used was harvested in 2015 in lower Austria and stored under dry conditions until use. The composition is given in **Fehler! Verweisquelle konnte nicht gefunden werden.**.

Table 1. Wheat straw composition on dry basis in wt%.

Arabinan	Galactan	Cellulose	Xylane	Mannan	Lignin	Ash
2.68	0.872	32.5	19.8	0.409	16.1	0.539

2.2. Wheat Straw Pretreatment

Liquid Hot Water (LHW) treatment applies only water at elevated temperatures in liquid state to pretreat the wheat straw. Experiments were conducted in lab-scale and pilot-scale equipment.

The lab-scale LHW experiments were conducted in 45 ml pressure vessels (PARR, Model 4716) in electrical heating jackets. Certain amounts of water and wheat straw were added to the pressure vessels to achieve a 9.2 wt% wheat straw content. The reactors were rapidly heated to 120°C and 170°C, respectively, and held at this temperatures for the desired time of 0, 30, 60, 90 and 120 min. Afterwards, the pressure vessels were immersed in a cooled water bath in order to achieve fast cooling of the reactor content and the solid and liquid phase was separated for analysis.

The pilot-scale LHW experiments were conducted in a 10 l DIG-MAZ extraction system (Samtech Extraction Systems, Austria). In brief, the extraction plant consists of a jacketed fixed bed reactor and extraction solvent is circulated through the bed of wheat straw. Identical to the lab-scale experiments, the wheat straw content was around 9.2 wt% and extraction temperature was 120°C. Extract samples were taken after 0, 30, 60, 90 and 120 min. The pilot-scale extracts were then concentrated by a factor of 5 in a thin film evaporator unit of the DIG-MAZ extraction system (Samtech Extraction Systems, Austria). The surface temperature was set to 95°C and the pressure to 70 mbar.

2.3. Extract Separation

Acetic acid was separated in a rotary evaporator R-220 EX (Buchi, Switzerland) by lowering the pH value to around 3 with sulfuric acid. The separation was conducted in 5 steps, the nonvolatile fraction was filled up to the initial volume with water and the pH value was set back to 3 after each individual step. The heating bath temperature was set to 57°C and the pressure to 60 mbar.

The acetic acid-containing distillate was concentrated by neutralizing the pH value to 7 with sodium hydroxide and the separation of water in the before mentioned setup.

2.4. Cultivation of S. acidocaldarius

Sulfolobus acidocaldarius DSM 639 was grown aerobically at 75 °C in a reciprocal shaking oil bath at 100 rpm in 100 mL long neck Erlenmeyer flasks to avoid evaporation. The flasks were filled with 20 to 50 mL LHW extract or culture medium and the initial pH was adjusted to 3.0 with 4.8 wt% H_2SO_4. Unless stated otherwise, salts and trace elements were provided by addition of appropriate stock solutions as described elsewhere. The medium described by Brock et al. [25] was used as reference for the evaluation of biomass growth.

2.5. Cultivation of cyanobacteria

Synechocystis sp. PCC 6714 was grown in basal BG-11 medium [26] with 10 mM HEPES buffer, pH 8.5. The medium was either supplemented with the acetic acid fraction from the distillation step, or, in case of the reference medium, with 5 mM $NaHCO_3$ as carbon source.

2.6. Analytics

The carbohydrate content was determined by the sample preparation following the National Renewable Energy Laboratory (NREL) laboratory analytical procedure (LAP), "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples" [27], but no neutralization of the samples after hydrolysis was conducted and a Thermo Scientific ICS-5000 HPAEC-PAD system (Thermo Scientific, Waltham, MA, USA) with deionized water as the eluent was used for the determination of arabinose, glucose, mannose, xylose and galactose. The concentration of the degradation products acetic acid, hydroxymethylfurfural (HMF) and furfural were determined with a Shimadzu HPLC system and a Shodex SH1011 column (Showa Denko, Tokyo, Japan) at 40°C, with 0.005 M H_2SO_4 as eluent.

The carbohydrate yields are defined as the percentage of carbohydrates (sum of the monomeric and oligomeric carbohydrates arabinose, glucose, mannose, xylose and galactose) dissolved in the LHW extract based on the carbohydrates in the raw materials. The yields of acetic acid and degradation products are based on the dry mass of the initial wheat straw.

Microbial growth was monitored with a photometer (Genesys 20 photometer (Thermo Scientific, USA) via determination of the optical density at 600 nm and 750 nm for *S. acidocaldarius* and *Synechocystis sp.* PCC 6714, respectively.

3. Results & Discussion

3.1. Wheat Straw Pretreatment

Wheat straw needs to be pretreated in order to pre-separate the main components of the biomass and to make it more amenable for further processing [5]. In this study LHW was used as pretreatment method. Thereby, only water at elevated temperatures is used. Factors influencing the pretreatment severity include the reaction temperature and time. On one hand, higher pretreatment temperatures and time increase the efficiency, but on the other hand, also increase the concentration of degradation products which are potential inhibitors for subsequent fermentation processes [28]. Figure 1 shows the carbohydrate yields of the pretreatments in laboratory and pilot scale. Increasing temperatures shows a sharp increase of the carbohydrate yields. The yield is increasing from 3.5 % to 24 % after 90 min pretreatment time for the 120°C and 170°C lab-scale experiment, respectively.

Comparing the carbohydrate monomer percentage on the total carbohydrates dissolved during the process, the monomer percentage in the 120°C pilot scale pretreatments was 22.6 ± 2.2% and decreased to 14.1 ± 5.2% in the 170°C laboratory-scale experiments. Changes of the monomer percentages were not evident over the pretreatment time. Higher monomer concentrations are favorable for subsequent microbial fermentation steps since otherwise required additional enzymatic hydrolysis steps can be avoided.

Figure 2 shows the average distributions of the carbohydrates arabinose, galactose, xylose, glucose and mannose in the pretreatment extracts of the pilot-scale pretreatment at 120°C and the lab-scale pretreatment at 170°C. The predominant carbohydrate species at the pretreatment temperature of 120°C was glucose with 57.3%. However, at 170°C pretreatment temperature, xylose showed a share of 50.8%. Glucose is a hexose whereas xylose is a pentose and subsequent fermentation steps based on classical biotechnological hosts are likely to be suitable for only one of the carbohydrate types due to the phenomenon of carbon catabolite repression. Therefore, pretreatment temperature can have a significant influence on subsequent process steps and needs to be considered. However, a pretreatment duration dependency was not evident.

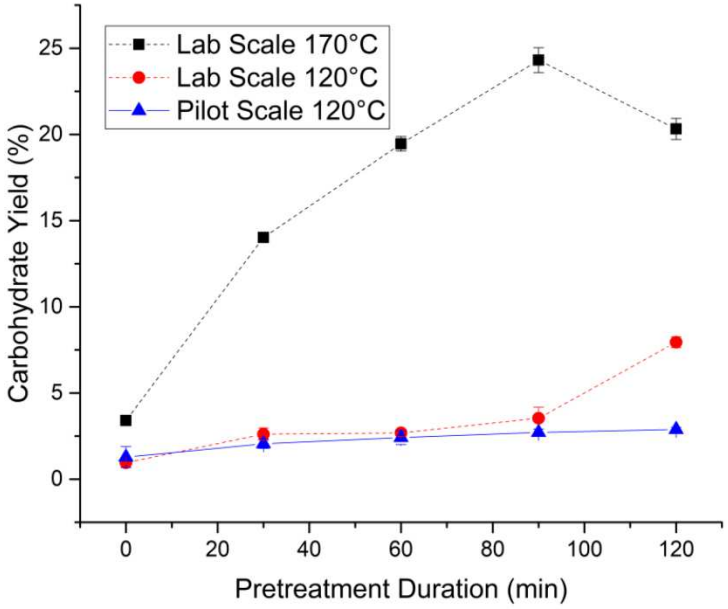

Figure 1. Total carbohydrate yields during the LHW pretreatments.

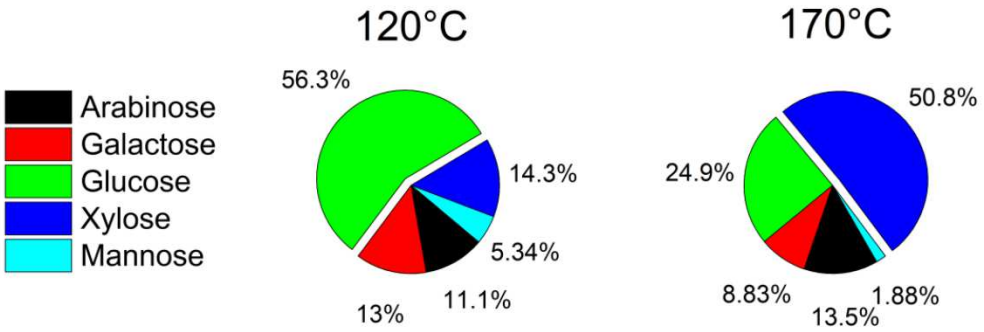

Figure 2. Average total carbohydrate distributions over the pretreatment time in the extracts of the pilot-scale pretreatment at 120°C and the lab-scale pretreatment at 170°C.

However, increasing the temperature also increased the production of degradation products acetic acid, hydroxymethylfurfural (HMF) and furfural which can inhibit subsequent microbial fermentation processes [29–31]. The pretreatment temperature and time dependency of the quantitatively highest degradation product acetic acid is shown in Figure 3 B. Acetic acid yields were increasing over pretreatment duration under all tested pretreatment conditions. However, the temperature had the main influence on the acetic acid formation. Comparing the acetic acid yields after 120 min pretreatment, the temperature increase from 120°C to 170°C led to an approximate 3.4-fold increase of the acetic acid formation. Similar trends can be seen in the formation of furfural and HMF. The time dependency of their formation at 170°C pretreatment temperature is shown in Figure 3 A. Longer pretreatment increases the formation of both, HMF and furfural, whereas the latter is the predominant of the mentioned species. However, these components were not detectable at pretreatment temperatures of 120°C.

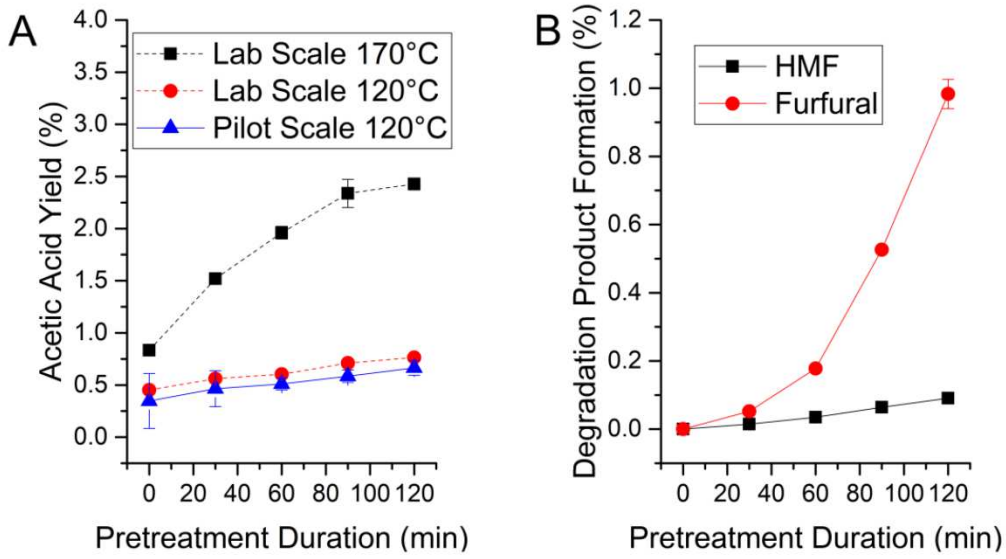

Figure 3. A) Acetic acid generated during the LHW pretreatment; B) Degradation Product formation during the lab scale pretreatments at 170°C. Shown degradation products were below detection limit (1.3 mg/l for Furfural; 0.6 mg/l for HMF) at pretreatments at 120°C.

In order to achieve higher carbohydrate concentrations in the extract, it was concentrated in a thin film evaporator by a factor of 5.04. The composition of the extract after the concentration step is shown in Table 1.

Table 1. Composition of the concentrated wheat straw extract.

	Arabinose	Galactose	Glucose	Xylose	Mannose	Acetic Acid
Monomeric Carbohydrates (g/l)	0.549	0.176	5.32	0.459	0.533	-
Total Carbohydrates (g/l)	0.833	0.953	9.03	2.17	0.732	-
Degradation Products[1] (g/l)	-	-	-	-	-	1.84

[1] HMF and Furfural were below detection limit

3.2. Fractionation of the Extract

The high acetic acid content of the extract caused growth inhibition of *S. acidocaldarius* when the extract was directly used as carbon source. Therefore, acetic acid was separated using distillation under acidic conditions. The separated aqueous acetic acid solution was subsequently concentrated in rotary evaporator at neutral pH values. The process resulted in two fractions, an acetic acid rich fraction and a carbohydrate fraction. The composition of the latter is shown in Table 2 and the acetic acid fraction reached an acetic acid concentration of 3.11 g/l.

Table 2. Composition of the Carbohydrate Fraction

	Arabinose	Galactose	Glucose	Xylose	Mannose	Acetic Acid
Monomeric Carbohydrates (g/l)	1.22	0.090	3.67	0.0860	0.254	-
Total Carbohydrates (g/l)	2.39	2.08	11.4	2.56	0.664	-
Degradation Products[1] (g/l)	-	-	-	-	-	0.128

[1] HMF and Furfural were below detection limit

3.3. *Cultivation of* S. acidocaldarius

In order to lower the inhibitor concentrations after the acetic acid removal, the carbohydrate faction of the LHW extract was diluted 1:2. However, growth of *S. acidocaldarius* was still not possible on the medium. Due to the already low content of fermentable sugars further dilution was not considered a feasible option and therefore, the substrate was detoxified by incubation with 5 wt% activated carbon (AC) at 50 °C for 75 min to reduce the concentration of potential inhibitors. The sugar content of the 1:2 diluted carbohydrate fraction before and after the treatment with AC is shown in Table 2. When supplemented with only 0.5 g/l NZ-amine as additional carbon source the AC treated hydrolysate supported a growth rate of *S. acidocaldarius* similar to a reference medium containing 2 g/l glucose and 1 g/l NZ-amine until growth ceased at an approximate OD_{600} of 1.2 due to nutrient depletion (Figure 4).

Table 3. Monomeric Carbohydrate content before and after detoxification with activated carbon (AC)

mg/l	Arabinose	Galactose	Glucose	Xylose	Mannose	Acetic Acid
Before AC Treatment	609	45	1835	43	127	64
After AC Treatment	167	25	1534	-	41	56

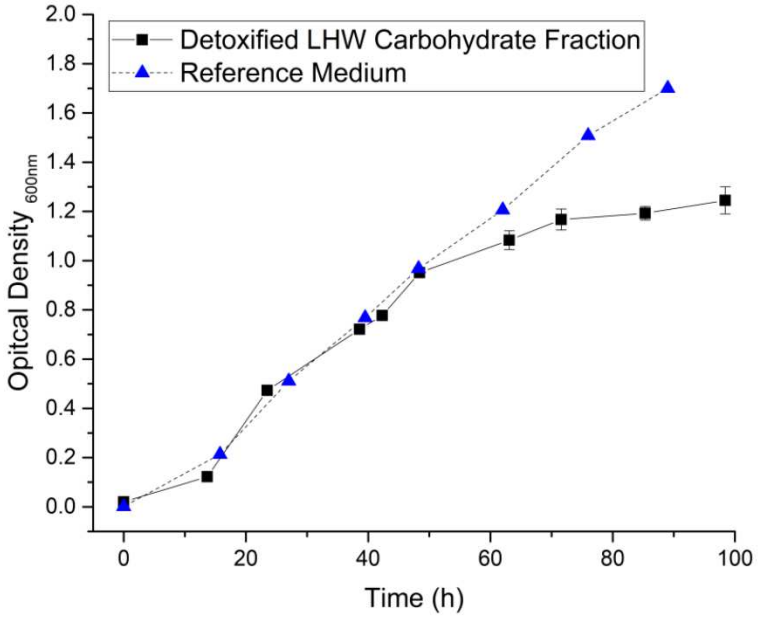

Figure 4. Time dependent growth of *S. acidocaldarius* in reference medium and LHW carbohydrate fraction detoxified with activated carbon (AC).

Figure 4 shows that wheat straw hydrolysate could be successfully used to replace the two carbon sources glucose and NZ-amine contained in a reference medium to a large part. However, the utilization of the wheat straw hydrolysate, separation of acetate and detoxification for the removal of inhibitors was necessary.

3.4. *Cultivation of cyanobacteria*

PHB-producing cyanobacteria were cultivated on a medium containing 3.1 g/l acetate from the distillation step. Microbial growth was boosted in comparison with the reference medium

containing only an inorganic carbon source (5 mM $NaHCO_3$) as described by Kamravamanesh et al. [32].

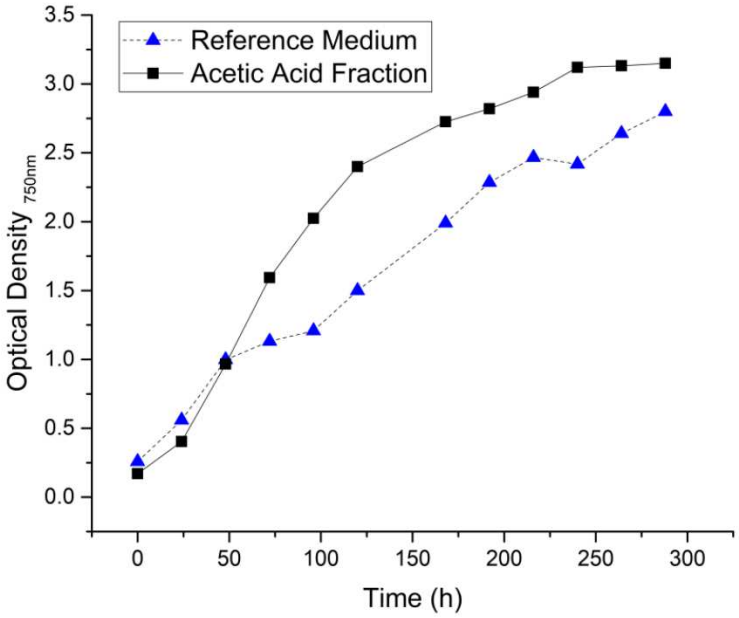

Figure 5. PHB production using cyanobacteria in a reference medium and the acetic acid fraction of the LHW extract.

As shown in Figure 5, addition of acetate led to an increased growth rate compared to a reference medium without an organic carbon source.

4. Conclusions

A complete process chain starting from wheat straw pretreatment using a liquid hot water process, the fractionation of the pretreatment waste-stream and the successful utilization of these fractions for the cultivation of PHB- and TEL-producing microorganisms was shown. Severe pretreatment conditions lead to higher concentrations of degradations products such as acetic acid, furfural and HMF. Whereas it was shown that acetic acid can be separated from the pretreatment waste stream and used as carbon source for cyanobacteria, the higher concentrations of the degradation products made a further detoxification step necessary for the cultivation of *S. acidocaldarius*. Both cultivations by applying lignocellulosic biorefinery side streams were found to be feasible. This opens the opportunity for the improvement of the economics of a biorefinery by the production of high value products from low value side streams and leads direction to an intended 100% usage of the applied raw material.

Author Contributions: S.B. designed and performed the pretreatment experiments. J.Q. designed and performed the cultivation of *S. acidocaldarius*. D.K. designed and performed the cultivation of cyanobacteria. S.B., J.Q. and O.S. wrote and edited the manuscript with significant input and editing from D.K. and A.F.

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A defined cultivation medium for *Sulfolobus acidocaldarius*

Contribution to Chapter 3

(Scientific publication)

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A defined cultivation medium for *Sulfolobus acidocaldarius*

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ABSTRACT

The thermoacidophilic Crenarchaeon *Sulfolobus acidocaldarius* is an important model organism for Archaea and genetic systems are well established. To date, the organism is routinely cultivated on complex media based on protein hydrolysates and no common defined medium is established. In this work we address this lack of a standardized defined medium and replaced the complex protein hydrolysate with sodium glutamate as primary substrate. Starting from an existing medium formulation we stepwise managed to improve the medium regarding formation of precipitates, buffer capacity, concentration of basal salts and trace elements, and optimized growth rates. The differences on the cellular level between the original medium and our new formulation, called VD Medium, were investigated by comparative gene expression analysis and significant differences were discussed. The final formulation of the VD Medium contains 1.75 g/L Na-glutamate, 3 g/L D-glucose and 0.5 g/L citric acid as carbon sources. Using the described medium for the cultivation of *S. acidocaldarius* DSM 639 in shake flasks yields 1.1 g/L dry cell weight (OD_{600} = 1.7) after a typical incubation time of 95 h with an overall biomass yield of 0.33 $g_{DCW}/g_{substrate}$.

1. Introduction

First discovered in 1972, the genus *Sulfolobus* is one of the best described members of the phylum Crenarchaeota. A total of eight species has been isolated from solfataric fields all over the world, which are characterized by an obligate aerobic lifestyle and optimal growth at 75–85 °C and pH 3–3.5. To date, many cultivation media for the cultivation of the extreme-thermoacidophilic genus *Sulfolobus* are based on a formulation published in 1972 by Brock et al. (subsequently referred to as "Brock Medium"). This medium is in turn based on "Allen´s Medium", originally used for the cultivation of *Cyanidium caldarium*, an algae living in acidic hot springs (Allen, 1959). Since then no efforts on media development have been published in the literature. Therefore we presume that there is significant potential for improving the composition of the Brock Medium both regarding its bulk and trace elements.

Although the Brock Medium has proven its applicability countless times and can be considered the gold standard in biologic research with *Sulfolobus* spp., it holds some substantial drawbacks for reproducible cultivation and physiological strain characterization in a controlled environment: 1) complex media components, like NZ-amine or yeast extract, with high batch-to-batch variability foil reproducibility, substrate analytics, strain characterization and selective media optimization; 2) the presence of inhibiting compounds in complex media based on protein hydrolysates or yeast extract is reported (Park and Lee, 2000); 3) the fluorescence background due to the presence of aromatic amino acids impedes direct fluorescence measurements in the medium (e.g. use of fluorescent biomarkers); and 4) the medium´s tendency to form precipitates over time is a hindrance for reproducible media preparation. Another substantial drawback of a complex medium based on protein hydrolysates are significantly higher costs for the carbon source compared to formulations based on cheap sugars or selected amino acids. At the time of writing this manuscript 1 kg of NZ-amine AS costs ~4-times as much as 1 kg of monosodium L-glutamate and ~11-times as much as 1 kg of D-glucose (€187.00 (N4517-1KG, Merck) vs. €49.00 (49621-1KG, Merck) vs. EUR €17.50 (6887.1, Roth)).

To address these issues, we developed a defined cultivation medium for shake flask cultivations (subsequently referred to as "Vienna Defined Medium" or "VD Medium"). The goal of the present work was to turn the widely used Brock Medium based on complex carbon sources into a defined medium in compliance with Quality by Design (QbD) guidelines (ICH Q9/Q10). Specifically, the following five aspects were investigated:

1) Substitution of complex media components (for higher

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reproducibility and to facilitate substrate analytics, physiological strain characterization and selective media optimization)
2) Elimination of precipitation (to ensure medium homogeneity)
3) Increasing the buffer capacity (for higher final cell densities)
4) Evaluation of growth promoting agents (for an increased specific growth rate)
5) Reduction of basal salt and trace element concentrations (to save costs and avoid growth inhibition)

In this publication we describe the process of media development and compare growth kinetics of *Sulfolobus acidocaldarius* DSM 639 on the final formulation of the VD Medium with the state-of-the-art Brock Medium. Also the growth performance of 4 additional *Sulfolobus* strains (*Sulfolobus solfataricus* P2, *Sulfolobus islandicus* Rey15A, *Sulfolobus tokodaii* str. 7 and *Sulfolobus shibatae* B12) is shown.

2. Material and methods

2.1. Strain and growth conditions

S. acidocaldarius DSM 639, *S. solfataricus* P2, *S. islandicus* Rey15A, *S. tokodaii* str. 7 and *Sulfolobus shibatae* B12 were grown aerobically at 75 °C in a reciprocal shaking oil bath at 100 rpm in 100 mL long neck Erlenmeyer flasks to minimize evaporation (determined evaporation rate: 1% after 70 h; data not shown). The flasks were filled with 54 mL culture medium and the initial pH was adjusted to 3.0 with 4.8 w% H_2SO_4. The basal medium (= medium without carbon sources; further on referred to as "Brock Basal") used in the initial experiments was prepared according to the original medium formulation described by Brock et al. (1972; Table 1). During the course of the study the medium composition was adapted stepwise according to emerging findings.

2.2. Preparation of inoculum

All growth experiments were started with freshly grown cells derived from an active preculture. Cells from the preculture were washed once (4000 g, 15 min) and resuspended in basal medium mirroring the salt concentrations of the following experiment. All cultures were inoculated with a calculated starting OD_{600} of 0.02 to 0.03. In the initial experiments with *S. acidocaldarius* NZ-amine and D-glucose were used as carbon sources in the preculture. After finding a substitute for NZ-amine (Monosodium L-glutamate (MSG) + D-glucose) these substrates were used instead. Precultures for all other *Sulfolobus* strains were grown on VD Medium supplemented with Wolin´s vitamin solution (Wolin et al., 1963).

Table 1
Composition of the Brock Medium (Brock et al., 1972).

Basal salts and trace elements				
$(NH_4)_2SO_4$	1.30	g/L	9.84	mM
KH_2PO_4	0.28	g/L	2.06	mM
$MgSO_4 \cdot 7\ H_2O$	0.25	g/L	1.01	mM
$CaCl_2 \cdot 2\ H_2O$	0.07	g/L	0.48	mM
$FeCl_3 \cdot 6\ H_2O$	20.00	mg/L	73.99	µM
$Na_2B_4O_7 \cdot 10\ H_2O$	4.50	mg/L	11.80	µM
$MnCl_2 \cdot 4\ H_2O$	1.80	mg/L	9.10	µM
$ZnSO_4 \cdot 7\ H_2O$	0.22	mg/L	0.765	µM
$CuCl_2 \cdot 2\ H_2O$	0.05	mg/L	0.293	µM
$Na_2MoO_4 \cdot 2\ H_2O$	0.03	mg/L	0.124	µM
$VOSO_4 \cdot 2\ H_2O$	0.03	mg/L	0.221	µM
$CoSO_4 \cdot 7\ H_2O$	0.01	mg/L	0.036	µM
C-sources				
NZ-amine or Yeast extract	1.0	g/L	n.d.	
Dextrine or Sucrose	2.0	g/L	n.d./5.84	mM
Adjust to pH 3 with H_2SO_4				
n.d. not determinable				

2.3. Calculation of dry cell weight, specific rates and biomass yield

Dry cell weight, DCW [g/L], was calculated from OD_{600} values using a conversion factor of DCW = $OD_{600} \times 0.651$. The factor was derived from a DCW-OD_{600} correlation curve shown in Supplementary Fig. S1.

Specific substrate uptake rate, q_S [g/g/h], between two sampling points was calculated as the difference in substrate concentration (MSG, D-glucose, or citric acid) divided by the mean DCW value of the two sampling points and the respective time difference.

Specific growth rate, µ [g/g/h], between two sampling points was calculated as the difference in DCW divided by the mean DCW value of the two sampling points and the respective time difference.

Biomass yield, $Y_{X/S}$ [g/g], was calculated as the quotient of the DCW value of the final sample and the sum of the differences in substrate concentrations (MSG, sum of all 20 amino acids, D-glucose, or citric acid) between the first and the final sample in the supernatant.

2.4. Analytical methods

Growth was monitored photometrically (WPA CO8000, Biochrom, Germany or Genesys 20, Thermo Fisher Scientific, USA) via determination of the optical density at 600 nm. Within single experiments always the same photometer was used. Supernatant for determination of substrate concentrations was prepared by centrifugation (12,000*g*, 10 min, 4 °C). Amino acids were measured with HPLC equipped with a fluorescence detector operating at 340/450 nm (excitation/emission) and 266/305 nm (ex./em.), using an Agilent Eclipse AAA 3.5 µm column (150 x 3 mm, Agilent, USA) as described by Hofer et al. (2018). Glucose and TCA-cycle intermediates were measured with HPLC equipped with a refraction index detector (Agilent G1362A, USA) and a diode array detector operating at 210 nm, using an Aminex HPX-87H column (300 x 7.8 mm) at 60 °C. 4 mM H_2SO_4 was used as eluent with an isocratic flow of 0.6 mL/min for 30 min. MSG was measured with a Cedex Bio HT Analyzer (Roche, Germany) using an enzymatic assay (Glutamate V2 Bio HT, Roche, Germany). pH was measured with a pH meter (WTW, Germany) or for small sample volumes with indicator strips.

For the determination of the elemental biomass composition of *S. acidocaldarius* 400 mL culture volume (grown on Brock Basal supplemented with 4 *g*/L D-glucose and 1.75 *g*/L MSG) were harvested at an OD_{600} of 0.35 via centrifugation (7000 *g*, 10 min) and washed 3 times with 0.85% NaCl solution (pH 4.5). Lyophilization yielded 125 mg of a dry cell pellet. The dried biomass was analyzed in triplicates at an external institute (Mikroanalytisches Laboratorium Universität Wien, Vienna, Austria) to determine the share of C, H, N, S, O and P.

2.5. Transcriptome analysis

For transcriptome analysis, *S. acidocaldarius* was grown on complex Brock Medium containing NZ-amine, and on the defined VD Medium. From each condition 10 mL broth were harvested in the late exponential phase at an OD_{600} of 0.8. After shock cooling to room temperature using liquid nitrogen samples were centrifuged (19,000 *g*, 1 min, 4 °C). The resulting pellet was frozen immediately in liquid nitrogen and stored at −80 °C. For each sample (Brock Medium and VD Medium) three independent biological replicates were prepared. Samples were then shipped on dry ice to the site of transcriptome analysis.

RNA was isolated for all samples and replicates with Trizol (ThermoFisher, Waltham, USA) as described previously (Hottes et al., 2004). The obtained RNA samples were treated with RNase-free DNase (Qiagen) according to the protocol and afterwards purified using an ethanol precipitation. Total RNA is routinely checked using an Agilent RNA 6000 Nano Kit on a Bioanalyzer (Agilent, Böblingen, Germany). Afterwards, ribosomal RNA was depleted using a RiboZero magnetic kit

for bacteria (Illumina, San Diego, USA) with a modified protocol. Only 90 µl instead of 225 µl magnetic beads were used and for the rRNA removal reaction and only 1 µg RNA was mixed with 4 µl removal solution in a total volume of 20 µl instead of 40 µl.

Sequencing libraries for all samples and replicates were prepared with the TruSeq® Stranded mRNA HT kit (Illumina) starting with the RNA fragmentation step after elution of precipitated RNA in 19 µl of the Fragment-Prime-Mix.

Sequencing libraries were quantified with a High-Sensitivity Chip on a Bioanalyzer (Agilent, Böblingen, Germany) and a measurement with a Quant-iT PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, USA) on a Microplate Reader Tecan Infinite 200 (Tecan, Männedorf, Switzerland). Sequencing was performed on a HiSeq1500 instrument (Illumina) in rapid mode with a read length of 2 × 25nt. Sequencing reads were mapped with Bowtie2 (Langmead and Salzberg, 2012) against the reference genome (*S. acidocaldarius* DSM639, genome size: 2,225,959 nt, RefSeq ID: NC_007181.1). The data was visualized in the software ReadXplorer and mapped reads were counted for each gene (Hilker et al., 2014). For determination of regulated genes, a statistical analysis was performed using DESeq2 (Love et al., 2014). For significance, all genes with an adjusted p-value below 0.01 and an at least two-fold change in transcription were considered as differentially transcribed.

3. Results and discussion

3.1. Substitution of complex media components

As shown in Fig. 1, common complex substrates vary significantly in their amino acid patterns. Nevertheless, while *Sulfolobus* is capable of growing on a number of defined sugars like maltose, sucrose, D-glucose, D-galactose, D-mannose, L-fucose D-arabinose, L-arabinose and D-xylose as single carbon source (Grogan, 1989; Joshua et al., 2011; Wolf et al., 2016; Stark et al., 2017), it is common practice to add one or another protein-based, complex substrate to the medium for the benefit of faster growth. These protein-based media components are mainly a source of amino acids and small oligo peptides and are taken up preferentially during the initial 40 h of growth (Supplementary Fig. S2).

Our goal was to mimic this effect (a boost in the initial growth phase) by exchanging the complex carbon source with a single defined amino acid or a combination of amino acids. The amino acids tested for increased growth were selected based on the amino acid uptake pattern of *S. acidocaldarius* grown on Brock Basal supplemented with NZ-amine and glucose. From all amino acids contained in this medium, 7 amino acids were found to be taken up to more than 50% of their initial concentration within the early growth phase of 40 h (L-glutamic acid, L-aspartic acid, L-arginine, L-lysine, L-phenylalanine, L-isoleucine, L-leucine; Supplementary Fig. S3). We tested the growth of *S. acidocaldarius* using these 7 amino acids as substrate either separately or in combination. These initial growth experiments were always carried out with an amino acid concentration of 0.5 g/L for the single amino acid experiments, while for the mix of all 7 amino acids the amino acids were supplied in the same mass-ratio as found in NZ-amine (free amino acids, determined with HPLC), adding up to a total amount of 0.5 g/L. Additionally 2 g/L D-glucose were added as secondary carbon source (the tested amino acids being the primary carbon source). D-glucose was used instead of the commonly used sucrose, since in our experience sucrose was subject to hydrolysis due to the growth conditions during cultivation (75 °C, pH 3).

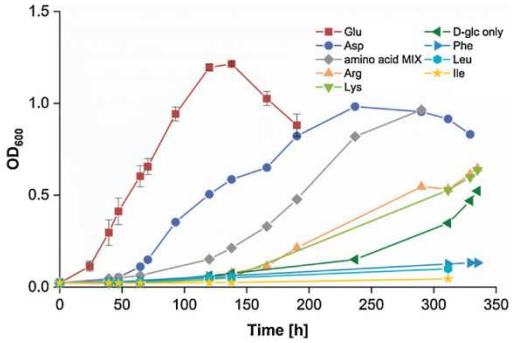

Fig. 2. Effect of amino acids on the growth of *Sulfolobus acidocaldarius*. 0.5 g/L of the respective amino acids were added to the medium in addition to 2.0 g/L D-glucose. In case of the amino acid mix the concentration of the sum of amino acids added up to 0.5 g/L. L-glutamic acid (Glu) and L-aspartic acid (Asp) led to an increased growth rate in comparison to a medium with D-glucose as only carbon source (D-glc only). Although the mix of all 7 amino acids (amino acid MIX) also led to an increased growth rate compared to D-glc only, the effect was much weaker compared to Glu or Asp. L-arginine (Arg) and L-lysine (Lys) had a minor positive effect on growth, while addition of L-phenylalanine (Phe), L-leucine (Leu) and L-isoleucine (Ile) strongly inhibited growth.

Addition of L -glutamic acid and L -aspartic acid led to an increased growth rate in comparison to a medium with D-glucose as sole carbon source. Although the mix of all 7 amino acids also led to an increased growth rate compared to D-glucose only, the effect was much weaker compared to the single amino acids. L-arginine and L-lysine had a small positive effect on growth, while addition of L-phenylalanine, L-leucine and L-isoleucine inhibited growth (Fig. 2). L-glutamic acid was found to have by far the highest growth promoting effect of the tested amino acids. This result is in accordance with the findings of Park and Lee (2000), who investigated the growth promoting effect of amino acids on *S. solfataricus*. We wondered whether this effect is due to L-glutamic acid being an almost immediate substrate for the tricarboxylic acid

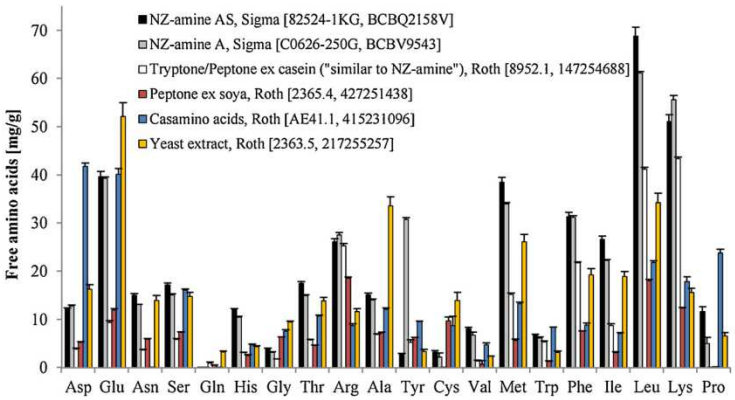

Fig. 1. Amino acid composition of various complex substrates. The amino acid pattern varies considerably between substrates from different sources (casein vs. soy vs. yeast) but also enzymatically treated casein samples (NZ-amine AS, NZ-amine A, Tryptone/Peptone ex casein and Casamino acids) exhibit great variation in their composition. Concentrations were determined in triplicates from single lots. Order number and lot number of the substrates are shown in square brackets.

cycle (TCA-cycle), since it enters the TCA-cycle after just a single reaction to 2-oxoglutarate catalyzed by the enzyme glutamate dehydrogenase. Interestingly, neither 2-oxoglutarate nor other TCA substrates like malic acid and citric acid had a growth promoting effect comparable to L-glutamic acid (Supplementary Fig. S4). Possibly, the different charge of the molecules at the low media pH and consequently different uptake kinetics play a critical role for the different growth promoting effects of these substrates.

In all following experiments MSG was used instead of glutamic acid due to its 2-fold lower price (49 vs. 100 €/kg, Merck) and ~100-fold higher solubility (7.5 vs. 740 g/L at 20 °C). This adaption is also justified, since no differences in growth could be observed, when deployed in equimolar concentrations (data not shown).

We concluded the search for a substitute for complex media components by determining the ratio of MSG to D-glucose and their respective concentrations to obtain highest growth rates. A design space ranging from 1 to 5 g/L D-glucose and 0.5 to 3.5 g/L MSG was investigated. Although higher final OD_{600} values were reached with highest MSG concentrations, we opted to choose an intermediate MSG concentration of 1.75 g/L since at high MSG levels growth was inhibited during the initial growth phase (Fig. 3A). In this initial experiment we could not detect significant differences in growth within the tested D-glucose concentration range. In a later experiment we found a concentration of 3.0 g/L D-glucose to have a slightly better effect on growth compared to concentrations of 2.0 and 4.0 g/L (Fig. 3B). Summarizing, the following concentrations were chosen for optimal growth rates: 1.75 g/L MSG and 3.0 g/L D-glucose.

3.2. Elimination of precipitation

The original Brock Medium forms precipitates during storage. To avoid precipitation and ensure medium homogeneity we tested the effect of the chelating agents ethylenediaminetetraacetate (EDTA) and citric acid. As the precipitates are formed upon addition of iron, most probably due to formation of insoluble iron phosphate, the chelating agents were supplied in equimolar concentrations to iron. In both cases precipitates could be avoided and no negative effect on growth was observed (Fig. 4). Since citric acid was later shown to have a positive effect on the buffer capacity of the medium, we chose to use citrate as chelator (by exchanging $FeCl_3$ with Fe(III)-citrate) to keep the number of media compounds as low as possible. No negative effects were observed upon the loss of chloride, since it is still supplied as $CaCl_2$.

3.3. Increasing the buffer capacity

The original Brock Medium suffers from a very low buffer capacity

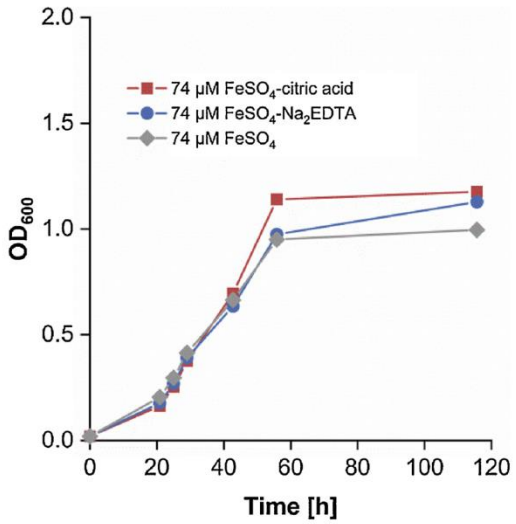

Fig. 4. The influence of iron chelators EDTA and citric acid on the growth of *S. acidocaldarius* was tested. No significant differences among the chelators and between chelators and a control containing only $FeSO_4$ were detected during the growth phase. Growth curves were determined in single experiments.

that is mostly derived from NZ amine (β_{Brock} = 3.20 mM/pH; Supplementary Fig. S5). Consequently, depending on amount and type of available substrate, stationary growth can be caused not by substrate depletion but rather due to the pH rising above the critical level of 6.0 as a result of metabolite formation. This is especially an issue when nitrogen containing substrates, like amino acids, are used as carbon source, since the surplus nitrogen is excreted as basic metabolites. To circumvent this problem and allow for extended batch cultivation times and higher final biomass concentrations, we searched for potential buffer substances with limited direct interference with the growth of *Sulfolobus*. Unfortunately, the choice of buffer substances in the range of pH 3–3.5 is very limited and no suitable inorganic buffer substances could be found. We tested the effect of potassium hydrogen tartrate at its saturation point (5.7 g/L) which is a standard buffer at pH 3.5, glycine and citric acid (buffer range from pH 3.0–6.2) in different concentrations (addition of 0.5, 1.0, 1.5, 2.0 g/L citric acid; Fig. 5). As potassium hydrogen tartrate had a significant negative effect on growth (Supplementary Fig. S6), we investigated glycine and citric acid in more detail. Glycine is described as a buffer substance for a *Sulfolobus* medium by Zillig et al. (1993) at a concentration of 0.7 g/L. Since citric

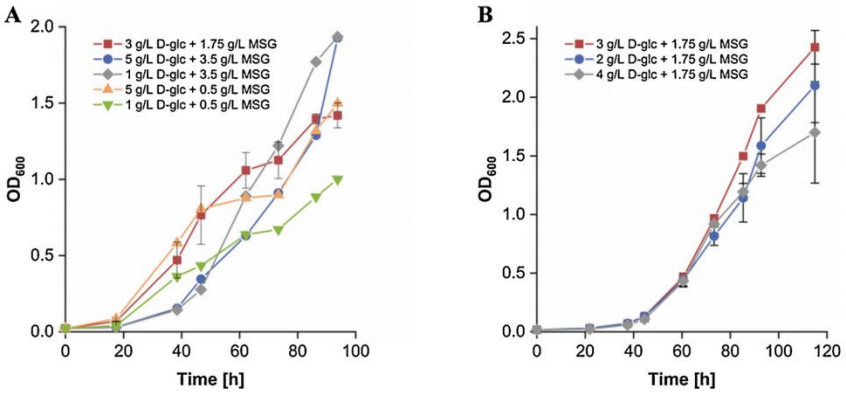

Fig. 3. A: MSG concentrations of 3.5 g/L (blue and grey curves) led to growth inhibition in the initial growth phase despite higher final OD_{600} values. An intermediate MSG concentration of 1.75 g/L (red curve) led to consistently highest growth rates while at 0.5 g/L MSG a similar growth rate could only be sustained when the D-glucose concentration was at 5.0 g/L (orange curve). A reduction of both MSG and D-glucose (green curve) showed a significantly lower growth rate. Within the tested D-glucose concentration range not significant differences in growth could be observed. B: Since the experiment in Fig. 3A did not reveal a clear growth dependency on the D-glucose concentration, we tested the effect of the D-glucose concentration again in a separate experiment with fixed MSG concentration: 3.0 g/L D-glucose were found to have a slightly better effect on the growth rate compared to concentrations of 2.0 and 4.0 g/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

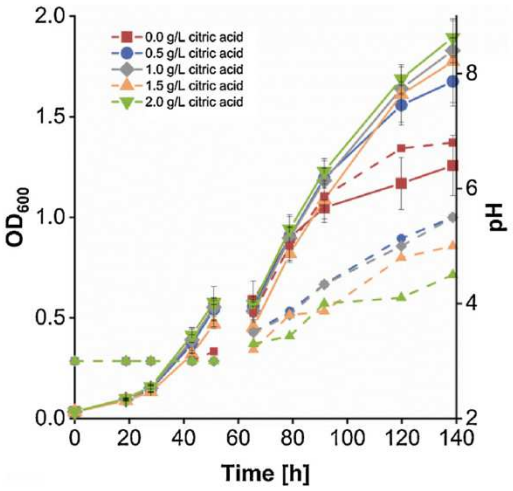

Fig. 5. Increasing the buffer capacity via addition of citric acid led to a significant increase of the growth rate compared to the unbuffered medium (red curve). pH values corresponding to the growth curves are shown with dashed lines. Note: Between 51.0 and 65.5 h growth was halted due to oxygen limitation caused by failure of the shaker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

acid has a similar effect on growth but, as shown in Supplementary Fig. S5, a higher buffer effect compared to glycine, we decided to add 0.5 g/L citric acid as buffer substance. On top of that, small amounts of citrate are already present in the new media formulation due to the exchange of $FeCl_3$ with Fe(III)-citrate as described before. The buffer capacity is increased to 7.60 mM/pH (Supplementary Fig. S5) and growth is no longer limited by increasing pH.

3.4. Addition of growth promoting agents

The substitution of NZ-amine with MSG led to slower biomass growth. To mitigate this effect we investigated potentially growth promoting agents. The influence of a mixture of vitamins (known as "Wolin´s vitamin solution", (Wolin et al., 1963); Table 2) was tested as well as addition of inositol and exchange of Fe^{3+} with Fe^{2+}.

Although Wolin´s vitamin solution is added in some laboratories when cultivating *S. solfataricus or S. islandicus* (e.g. Snijders et al., 2006; Deng et al., 2009), no effect on the growth of *S. acidocaldarius* could be observed in our experiments (Fig. 6A).

Inositol is a carbocyclic sugar that plays an important role for signal transduction in Eukaryotes. It is used as supplement in some yeast media, in Bacteria and Archaea it is considered a growth factor and was detected to be part of the S-layer as well as a precursor for compatible solutes, e.g. di-*myo*-inositol-phosphate (Komatsu and Chong, 1998; Müller et al., 2005; Roberts, 2006). Nevertheless, growth experiments with addition of 2 mg/L inositol showed no effect on the growth of *S. acidocaldarius* (Fig. 6B). The employed inositol concentration is based on a medium formulation for *Saccharomyces* (Zonneveld, 1986).

Several species of *Sulfolobus* are described as Fe^{2+} oxidizers (e.g. *Sulfolobus metallicus* (Huber and Stetter, 1991), *S. tokodaii* (Bathe and Norris, 2007), *Sulfolobus yangmingensis* (Jan et al., 1999), *S. solfataricus* (De Rosa and Gambacorta, 1975)). To test a possible growth promoting effect of Fe^{2+}, we substituted Fe^{3+} with equimolar amounts of Fe(II) SO_4. No effect on growth could be observed (Fig. 6C) and since Fe^{2+} is unstable in oxidative environments, we chose Fe^{3+} as the preferred iron source. Summarizing, we could not find a positive effect on the growth of *S. acidocaldarius* by any of the tested agents; nevertheless, as shown below, addition of the vitamin mix with inositol promoted the growth of *S. tokodaii*.

Table 2
: Composition of Wolin´s vitamin solution (x100 stock solution; Wolin et al., 1963). *Myo*-inositol is not part of Wolin´s vitamin solution, but was added to this table for the sake of completeness.

	Concentration [mg/L]
Biotin	2
Folic acid	2
Pyridoxine · HCl	10
Riboflavin	10
Thiamine	5
Nicotinic acid	5
Pantothenic acid	5
Vitamin B12	0.1
4-aminobenzoic acid	5
Lipoic acid	5
(*myo*-inositol)	(200)

3.5. Reduction of basal salt and trace element concentrations

To save costs and to simplify media preparation we reduced the overall number of media components, as well as the concentration of trace elements (TE) and basal salts based on the elemental biomass composition of *S. acidocaldarius*. As a side effect the reduced salt concentrations led to increased long term stability of stock solutions due to elimination of precipitation (data not shown).

When comparing the concentrations of the bulk elements nitrogen, phosphorus and sulfur in the original formulation from Brock with the elemental biomass composition of *S. acidocaldarius* much higher concentrations can be found in the Brock Medium (Table 3). The total amount of nitrogen found in the biomass at the end of a shake flask cultivation (stationary phase) accounts for only 45% of the nitrogen supplied by the Brock Medium. Phosphorus and sulfur are (relatively) even more abundant in the medium as their accumulation in biomass accounts only for 18% and 0.4% of the supplied quantity. Based on these finding we decided to stepwise decrease the concentrations of basal salts and TEs.

In initial experiments we were able to stepwise reduce TEs to 30% and basal salts to 50% (with exception of $(NH_4)_2SO_4$ which was kept at the original level) without negative effects on neither growth rate nor biomass yield of *S. acidocaldarius* (Fig. 7). Eventually also the $(NH_4)_2SO_4$ concentration was reduced to 80% of the value in the Brock Medium and, with exception of $ZnSO_4$ which was kept at 30%, TEs were reduced to 20% (data not shown).

Additionally, it was possible to omit $VOSO_4$ completely in the medium. This is especially beneficial since this chemical is regarded unstable in presence of oxygen and has to be handled in an oxygen free atmosphere.

Our proposed regime for stock preparation is shown in Supplementary Table S1. $CaCl_2{\cdot}2\ H_2O$ and Fe-citrate solutions are prepared separately to avoid precipitation.

The final VD Medium is given in Table 4.

3.6. Comparative gene expression analysis

We performed comparative gene expression analysis based on an RNASeq experiment to reveal differences on a cellular level upon growth on a complex medium containing NZ-amine (Brock Medium) vs. the VD Medium.

103 genes showed an at least 4-fold change in the transcriptional level, of which 62 were lower and 41 higher expressed during growth on the defined medium (Fig. 8).

When clustering the differently transcribed genes according to their metabolic pathways, we could identify 6 main groups with significant changes in the expression levels. These groups are (1) transporters for

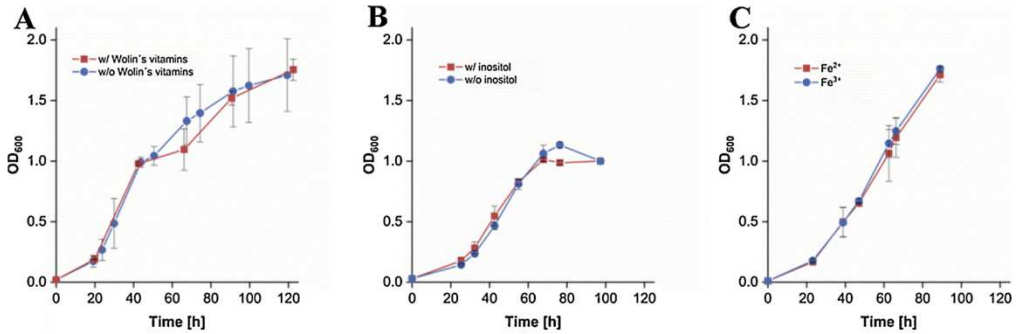

Fig. 6. Several substances were tested for their growth promoting effect. Unfortunately, neither of the tested substances led to increased growth compared to their control. A: Wolin´s vitamin solution (composition shown in Table 2) was tested. B: addition of *myo*-inositol. Although a slight depression in the OD_{600} curve can be seen in the stationary phase (at ~75 h), we deemed the influence of inositol as non-significant, since the growth curves were well matching at all other sampling points. C: exchange of Fe^{3+} with Fe^{2+}.

Table 3
Elemental biomass composition of *Sulfolobus acidocaldarius* and calculated recovery of nitrogen, phosphorus and sulfur in the biomass in respect to the Brock Medium.

	Biomass composition [w%]	Calculated elemental composition at stationary phase (OD_{600} = 1.8) [g/L]	Concentration in Brock Medium [g/L]	Calculated recovery in Biomass [%]
DCW	100.00	1.17		
C	45.53	0.53		
H	7.01	0.082		
O	29.70	0.35		
N	10.69	0.13	0.28	45%
P	1.01	0.012	0.064	18%
S	0.50	0.0059	1.55	0.4%
n.d.	5.55			

n.d. not determined

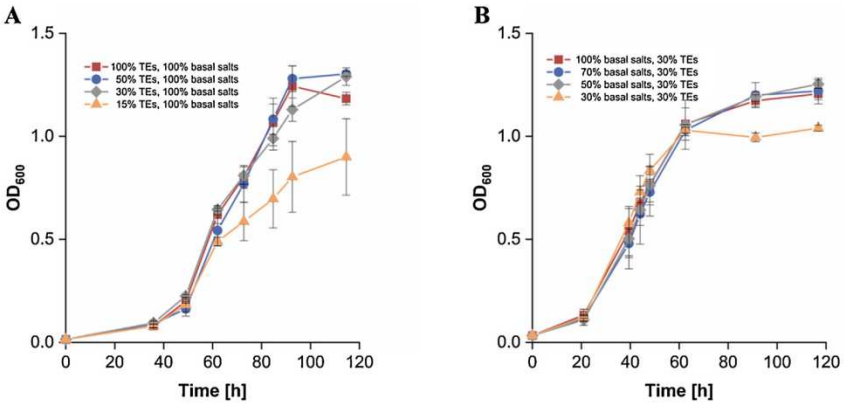

Fig. 7. A: Stepwise reduction of trace elements (TEs). Growth was severely slowed down at 15% of the value in the original formulation by Brock. B: Stepwise reduction of basal salt (with exception of $(NH_4)_2SO_4$ which was kept at the original level). The biomass yield was reduced at 30% of the value in the original formulation by Brock.

substrate uptake, proteins associated with (2) amino acid- and (3) acyl-CoA metabolism, as well as enzymes of the (4) respiratory chain (mostly cytochromes), (5) sulfur metabolism and (6) biotin synthesis. (Table 5). Genes with an at least 4-fold change in the transcriptional level not clustered into these groups are shown in Supplementary Table S2.

All 13 differentially transcribed (1) transporter genes were expressed to a lower extend on the defined medium with an up to 48-fold change in the transcriptional level. This reflects the fact that under this condition the number of different substrates was strongly reduced compared to the complex substrate condition. Interestingly, 8 of the differentially transcribed transporters are annotated as transporters for carbohydrates (Saci_0885, Saci_1136, Saci_1163-1165, Saci_1782, Saci_1835 and Saci_2095) while only 2 are associated with amino acid transporter function (Saci_1127 and Saci_1745). Possibly, the annotation is incomplete or the transporters in question could be more promiscuous than assumed.

Besides differential transcription of trnsporter genes, modifications in the expression of genes associated with (2) amino acid metabolism were anticipated. A shift from degradation of a diverse mix of amino acids to the necessity of biosynthesis of all amino acids apart from L-glutamic acid on the defined medium had to take place. As an example, genes involved in the biosynthesis of L -lysine like Saci_0753, Saci_0754 and Saci_1304 have a 5 to 17-fold increased transcription on the VD medium.

Since very little is known about the fatty acid metabolism in Archaea in general and in the genus *Sulfolobus* in particular, our attempts to interpret the expression pattern of genes associated with (3) acyl-CoA metabolism are highly speculative. Genes related to β-

Table 4
Composition of the VD Medium.

Basal salts and trace elements				
$(NH_4)_2SO_4$	1.04	g/L	7.87	mM
KH_2PO_4	0.14	g/L	1.03	mM
$MgSO_4 \cdot 7\ H_2O$	0.125	g/L	0.51	mM
$CaCl_2 \cdot 2\ H_2O$	0.035	g/L	0.24	mM
$Fe(III)Citrate \cdot H_2O$	9.75	mg/L	37.08	µM
$Na_2B_4O_7 \cdot 10\ H_2O$	0.90	mg/L	2.36	µM
$MnCl_2 \cdot 4\ H_2O$	0.36	mg/L	1.82	µM
$ZnSO_4 \cdot 7\ H_2O$	0.066	mg/L	0.23	µM
$CuCl_2 \cdot 2\ H_2O$	0.010	mg/L	0.059	µM
$Na_2MoO_4 \cdot 2\ H_2O$	0.006	mg/L	0.025	µM
$CoCl_2 \cdot 6\ H_2O$	0.002	mg/L	0.007	µM
C-sources				
Na-L-glutamate	1.75	g/L	10.35	mM
D-glucose	3.00	g/L	16.65	mM
Citric acid	0.50	g/L	2.60	mM
Adjust to pH 3 with H_2SO_4			~14.2	mM

oxidation (Acyl-CoA ligase, Acetyl-CoA acetyltransferase, Acyl-CoA dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase) are 5 to 13-fold expressed more weakly on the defined medium. Since neither of the two media contains fatty acids and the existence of a reverse β-oxidation pathway for fatty acid biosynthesis is postulated in Archaea, we speculate that the pathway is used in an anaplerotic manner for fatty acid synthesis. Possibly, during growth on the complex medium the organism experiences a surplus of nutrients and fatty acid biosynthesis is activated as form of overflow metabolism to generate high energy storage compounds. It should be noted, that although the majority of the Acyl-CoA related genes were expressed to a lower extend on the defined medium, we also found two genes that were expressed to a 5-fold higher extend which are annotated as Acetyl-CoA acetyltransferase and Acyl-CoA dehydrogenase.

The 4 to 44-fold higher expression of ten genes (Saci_0097-99, Saci_1858-61, Saci_2089/90, Saci_2200) coding for (4) membrane associated cytochromes of the respiratory chain can be a hint for an increased level of metabolic stress on the defined medium. The energy demanding process of biosynthesis of all amino acids except L -glutamate induces the need for higher respiratory activity to generate the required ATP for these anaplerotic reactions. This hypothesis is supported by an observation described in Fig. 9B and C: When grown on the defined medium, the culture has a lower biomass yield resulting from a higher specific substrate uptake rate while the specific growth rate is at a similar level. This indicates that a greater share of substrate is used for energy generation (and CO_2 production) and a smaller portion is actually incorporated in the biomass.

Six genes encoding enzymes involved in (5) sulfate reduction (Fig. 9) are 5 to 84-fold higher expressed when *S. acidocaldarius* is grown on the VD Medium. A likely explanation is that the organism is metabolizing sulfate to counteract the higher sulfate concentrations in the VD Medium. The increased sulfate concentration is a result of a higher requirement of sulfuric acid for pH adjustment compared to the Brock Medium (14.2 mM vs. 12.3 mM). The surplus sulfur species could then be exported via a higher expressed sulfite exporter. In contradiction, analysis of culture supernatant did not reveal increased sulfite concentrations when cultivated on the VD Medium. An explanation for the lack of detectable sulfite concentrations could be the generally low metabolic rate of *S. acidocaldarius* combined with the possibility of abiologic re-oxidation of exported sulfite under the oxic cultivation conditions. Another reason for the increased activity of the sulfate reduction pathway could be that the organism is generating reduced sulfur species for the incorporation in sulfur containing building blocks (e.g. cysteine, methionine, Acetyl-CoA, Fe-S clusters, vitamins, etc.). When grown on the complex Brock Medium, reduced sulfur is already provided via the amino acids L-cysteine and L -methionine. This could be the reason for downregulation of the assimilatory sulfate reduction pathway on the complex medium, since the pathway is energetically costly, produces the toxic compounds sulfide and sulfite, and is not required for the production of organo-sulfur compounds.

The 4 to 5-fold lower expression of Saci_0345 and Saci_0351, both

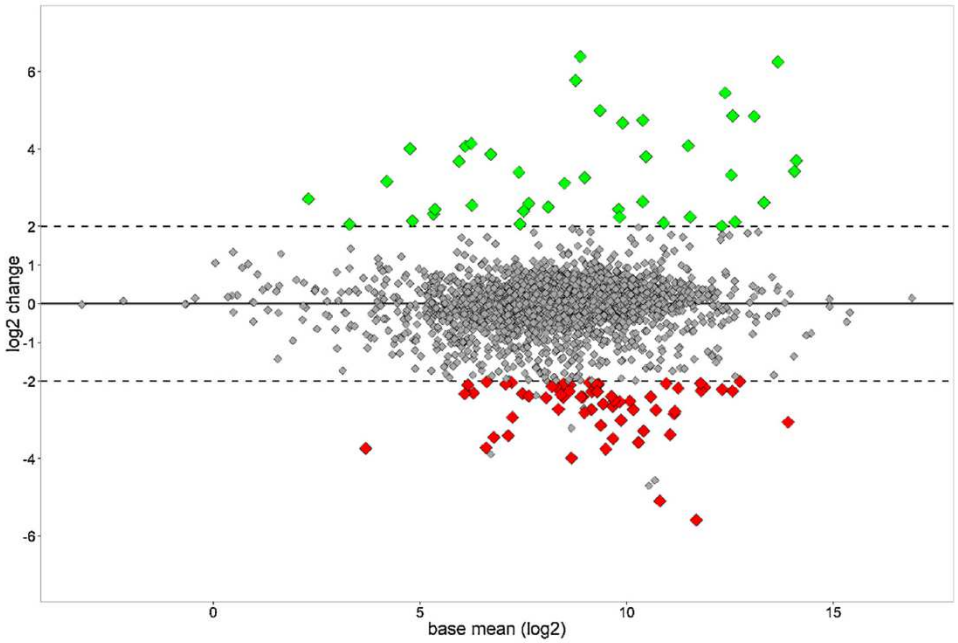

Fig. 8. Results of differential gene expression analysis. Genes with a significant change in the number of transcripts are shown in green (4-fold higher number of transcripts on the VD Medium) or in red (4-fold lower number of transcripts during growth on the VD Medium); genes with no significant change according to the analysis with DESeq2 are shown in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 5
Selected genes with a minimum of 4-fold different expression. Classification into one of the six groups discussed in the text is shown in the final column. A positive log2-fold change means higher expression on the VD Medium compared to the Brock Medium; a negative log2-fold change means lower expression.

Locus	arCOG	Annotation	log2-fold change	pvalue	Group
Saci_0886	G	MFS family permease	−5.584	2.49E-132	Transporter
Saci_1127	E	Amino acid transporter	−2.254	2.09E-22	Transporter
Saci_1136	G	MFS family permease	−3.405	9.07E-17	Transporter
Saci_1163	G	ABC-type maltose transport system. permease component	−2.260	5.55E-10	Transporter
Saci_1164	G	ABC-type sugar transport system. permease component	−2.274	1.48E-09	Transporter
Saci_1165	G	Maltose-binding periplasmic protein	−2.214	4.25E-15	Transporter
Saci_1701	F	Xanthine/uracil/vitamin C permease. AzgA family	−2.058	1.90E-10	Transporter
Saci_1745	E	Na+/proline symporter	−2.321	2.49E-05	Transporter
Saci_1782	G	MFS family permease	−2.379	2.04E-08	Transporter
Saci_1809	H	Energy-coupling factor transporter transmembrane protein EcfT	−2.091	9.24E-12	Transporter
Saci_1810	P	Energy-coupling factor transporter ATP-binding protein EcfA2	−2.732	8.59E-14	Transporter
Saci_1835	G	MFS family permease	−2.515	8.54E-22	Transporter
Saci_2095	G	MFS family permease	−3.989	2.23E-18	Transporter
Saci_0252	E	3-isopropylmalate dehydratase small subunit	2.090	7.86E-15	Amino acid metabolism
Saci_0350	E	Glycine cleavage system H protein (lipoate-binding)	−2.495	9.55E-13	Amino acid metabolism
Saci_0753	E	Lysine biosynthesis protein LysW	2.397	9.43E-10	Amino acid metabolism
Saci_0754	H	Glutathione synthase/glutaminyl transferase/ alpha-L-glutamate ligase	2.499	4.32E-18	Amino acid metabolism
Saci_1304	E	Isopropylmalate/homocitrate/citramalate synthase	4.087	1.02E-92	Amino acid metabolism
Saci_1714	E	Thermopsin-like protease	−2.036	3.08E-06	Amino acid metabolism
Saci_2041	E	N-methylhydantoinase A/acetone carboxylase. beta subunit	−5.094	3.48E-29	Amino acid metabolism
Saci_2056	E	Kynurenine formamidase	−2.935	3.70E-16	Amino acid metabolism
Saci_1054	I	Acyl-CoA synthetase (AMP-forming)/AMP-acid ligase II	−3.140	1.27E-12	Acetyl-CoA
Saci_1114	I	Acetyl-CoA acetyltransferase	2.241	4.05E-07	Acetyl-CoA
Saci_1774	I	Acyl-coenzyme A synthetase/ AMP-(fatty) acid ligase	−2.309	2.95E-06	Acetyl-CoA
Saci_2208	I	3-hydroxyacyl-CoA dehydrogenase. some fused to Enoyl-CoA hydratase	−2.816	1.36E-11	Acetyl-CoA
Saci_2209	I	Acetyl-CoA acetyltransferase	−2.408	7.68E-12	Acetyl-CoA
Saci_2217	I	Acyl-CoA dehydrogenase	2.443	5.28E-08	Acetyl-CoA
Saci_2232	I	Acetyl-CoA acetyltransferase	−2.750	2.00E-09	Acetyl-CoA
Saci_2233	I	Acetyl-CoA acetyltransferase	−2.837	1.35E-10	Acetyl-CoA
Saci_2234	I	Acyl-CoA dehydrogenase	−3.724	3.86E-15	Acetyl-CoA
Saci_2235	I	Acyl-coenzyme A synthetase/ AMP-(fatty) acid ligase	−3.008	2.13E-09	Acetyl-CoA
Saci_0097	C	Heme/copper-type cytochrome/quinol oxidase. subunit 1	3.700	4.64E-44	Cytochromes
Saci_0098	C	Terminal oxidase. subunit doxC	3.323	4.32E-36	Cytochromes
Saci_0099	C	Terminal oxidase. subunit doxE	3.115	6.17E-18	Cytochromes
Saci_1858	Q	Ethylbenzene dehydrogenase subunit A. cytochrome b558/566	4.843	5.23E-104	Cytochromes
Saci_1859	C	Cytochrome b558/566. subunit B	4.739	2.75E-51	Cytochromes
Saci_1860	C	Rieske Fe-S protein	5.451	2.83E-76	Cytochromes
Saci_1861	C	Cytochrome b subunitof the bc complex	4.857	3.18E-69	Cytochromes
Saci_2200	H	Uroporphyrinogen-III methylase	3.396	1.717E-13	Cytochromes
Saci_2089	C	Heme/copper-type cytochrome/quinol oxidase. subunit 2	2.117	6.933E-08	Cytochromes
Saci_2090	C	Rieske Fe-S protein	2.006	1.450E-05	Cytochromes
Saci_2069	O	Metal-sulfur cluster biosynthetic enzyme	2.442	1.427E-05	Sulfur metabolism
Saci_2151	P	Sulfite exporter. TauE/SafE family	4.069	2.264E-14	Sulfur metabolism
Saci_2198	P	Rhodanese-related sulfurtransferase	2.614	3.017E-22	Sulfur metabolism
Saci_2201	P	Sulfite reductase. beta subunit (hemoprotein)	4.996	5.514E-72	Sulfur metabolism
Saci_2202	E	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase)/FAD synthetase or related enzyme	6.391	8.149E-56	Sulfur metabolism
Saci_2203	P	ATP sulfurylase (sulfate adenylyltransferase)	5.774	2.361E-53	Sulfur metabolism
Saci_0351	H	Lipoate-protein ligase A	−2.392	5.866E-15	Biotin
Saci_0345	H	Lipoate-protein ligase A	−2.097	1.818E-08	Biotin

encoding a lipoate-protein ligase involved in (6) biotin synthesis on the complex medium could be explained by the presence of traces of this vitamin in the complex carbon source NZ-amine. Nevertheless, as already mentioned before, addition of biotin did not lead to a measurable increase of the growth rate.

3.7. Final considerations and performance of various Sulfolobus strains

The final formulation of the VD Medium fulfills the requirement of a defined medium capable of supporting fast growth. We aimed to keep the number of media components at a minimum in order to facilitate media preparation and reduce costs. In total 3 different carbon sources (MSG, D-glucose and citric acid) are added to the medium. As shown in Fig. 10 citric acid is directly fed into the citric acid cycle (TCA-cycle), while MSG is converted to the TCA-cycle substrate 2-oxoglutarate via a single enzymatic step. To avoid accumulation of TCA-cycle substrates, MSG and citric acid can be eventually converted to pyruvate via anaplerotic pathways. This means that the Entner-Doudoroff pathway (for degradation of D-glucose) and the TCA-cycle are the only catabolic pathways that are switched on for energy generation.

Fig. 11A and B show growth and substrate concentrations during cultivation of *S. acidocaldarius* on the VD Medium and on the complex Brock Medium. Using either medium the final cell density is reached at an OD_{600} of 1.7–1.8 with non-significant differences between both media formulations. When applying an exponential fit during the initial growth phase (exponential phase, $OD_{600} < 0.8$) the maximum specific growth rate (μ_{max}) is 0.08 h^{-1} on the VD Medium and 0.12 h^{-1} on the Brock Medium. Notably, the slopes of the growth curves are identical during the following phase, while the reason for the reduced maximal growth rate on the VD Medium is only a prolonged lag phase.

When looking at the substrate uptake on the VD Medium, no mechanism like carbon catabolite repression can be observed. All 3 carbon sources are taken up simultaneously. In contrast to D-glucose, the uptake of citric acid is nearly halted after reaching the stationary phase (Fig. 11A). Both specific substrate uptake rates as well as the growth rate are *increasing* during the initial growth phase (lag phase). After the

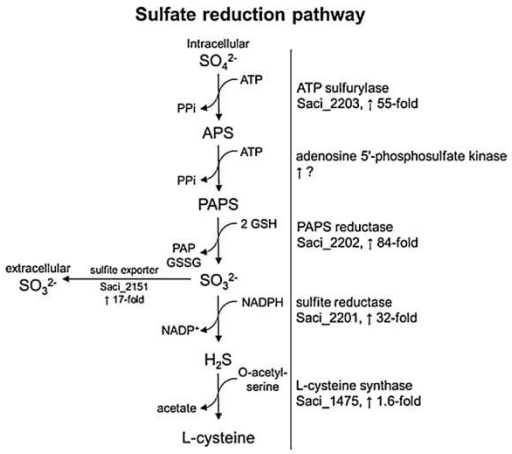

Fig. 9. Sulfate reduction pathway and -fold change in expression between cultivation of *S. acidocaldarius* on VD Medium vs. the complex Brock Medium. APS: adenosine 5′-phosphosulfate; PAPS: 3′-phosphoadenosine-5′-phosphosulfate; GSH: glutathione; PAP: adenosine 3′,5′-bisphosphate; GSSG: glutathione disulfide.

initial lag phase, the specific uptake rates for D-glucose and citric acid settle at 0.023 g/g/h and 0.010 g/g/h and in the case of citric acid the rate is decreasing again towards the end of the growth phase. The initial increase of the specific MSG uptake rate ($q_{MSG,\ max} = 0.15$ g/g/h) as well as the growth rate ($\mu_{max} = 0.08\ h^{-1}$) is followed by a decline which strongly correlates with the decline of the MSG concentration. This indicates a concentration-dependent MSG uptake and biomass growth. Unfortunately, as shown earlier in Fig. 3, also too high MSG concentrations quickly start to inhibit growth. We explain these two contrary effects by two distinct processes: on the one hand the uptake of MSG is accelerated due to enhanced diffusion over the membrane caused by the increased concentration gradient, while at a certain critical point (in this study we showed an inhibiting effect for concentrations of 3.5 g/L MSG) intracellular MSG accumulates and disturbs the host physiology (e.g. disturbance of the intracellular buffer system, product inhibition of enzymes or unfavorable side reactions yielding toxic products). This phenomenon of growth inhibition of *Sulfolobus* spp. caused by relatively low concentrations of organic acids is well documented in the literature (e.g. (Grogan, 1989; Park and Lee, 2000). During batch cultivations this creates only a small concentration window for optimal growth, which could be deliberately exploited in controlled fed batch or continuous cultures. The biomass yield (biomass per sum of carbon sources) over the course of the whole fermentation is 0.33 g/g.

On the Brock Medium the specific growth rate is high from beginning and no lag phase can be observed. Nevertheless, the decrease of the specific growth rate during the cultivation is very similar to what can be observed on the VD Medium. Although generally lower, the trend of the specific uptake rate of free amino acids is similar to the specific MSG uptake rate on the VD Medium. This lower uptake of free amino acids is compensated by a higher specific uptake rate of D -glucose. The biomass yield over the course of the whole fermentation is 0.5 g/g. It should be noted that the discrepancy of low substrate uptake rate and high growth rate at the initial growth phase can be an indication for uptake of another substrate, possibly oligopeptides which are also present in NZ amine.

The time courses of the specific uptake rates for the VD Medium and Brock medium are shown in Fig. 11C and D, respectively.

The performance of other common *Sulfolobus* strains (*S. solfataricus* P2, *S. islandicus* Rey15A, *S. tokodaii* str. 7 and *S. shibatae* B12), was tested. *S. tokodaii* str. 7 initially showed very low biomass yield on the VD Medium. Upon addition of Wolin´s Vitamin solution with inositol, growth was increased substantially (Fig. 12A–D).

The starting point for this study was media optimization for industrial bioprocesses. Therefore, we want to conclude this work by highlight the benefits of the VD Medium for the usage in biotechnological applications:

In biotechnology the major part of variable costs during the upstream process are allocated to media costs. This makes the VD Medium highly attractive for its adoption in high temperature *Sulfolobus*-based bioprocesses since the main component is ~4-times cheaper compared to

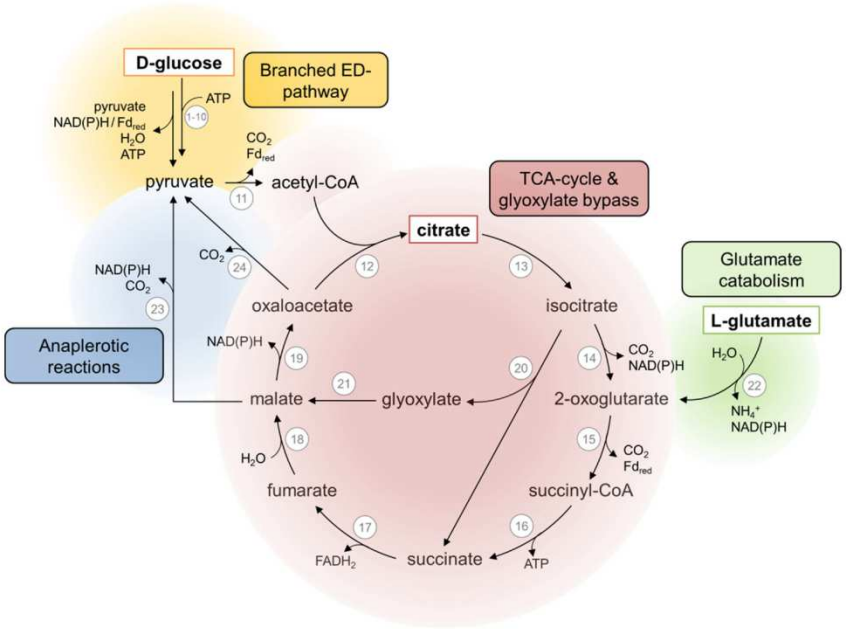

Fig. 10. Metabolic pathways for the utilization of the carbon substrates MSG (depicted as L-glutamate), D-glucose and citric acid contained in the VD Medium. 1–10, Enzymes of the non-phosphorylative and semi-phosphorylative Entner-Doudoroff pathway: glucose dehydrogenase, gluconate dehydratase, 2-keto-3-deoxy-gluconate kinase, 2-keto-3-deoxy-(6-phospho)gluconate aldolase, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, glyceraldehyde:ferredoxin oxidoreductase, glycerate kinase, enolase, pyruvate kinase; 11, pyruvate:ferredoxin oxidoreductase; 12, citrate synthase; 13, aconitase; 14, isocitrate dehydrogenase; 15, α-ketoglutarate:ferredoxin oxidoreductase; 16, succinyl-CoA synthetase; 17, succinate dehydrogenase; 18, fumarase; 19, malate dehydrogenase; 20, isocitrate lyase; 21, malate synthetase; 22, glutamate dehydrogenase; 23, malate dehydrogenase; 24, oxaloacetate decarboxylase. For the sake of readability some cosubstrates are not shown in this metabolic map.

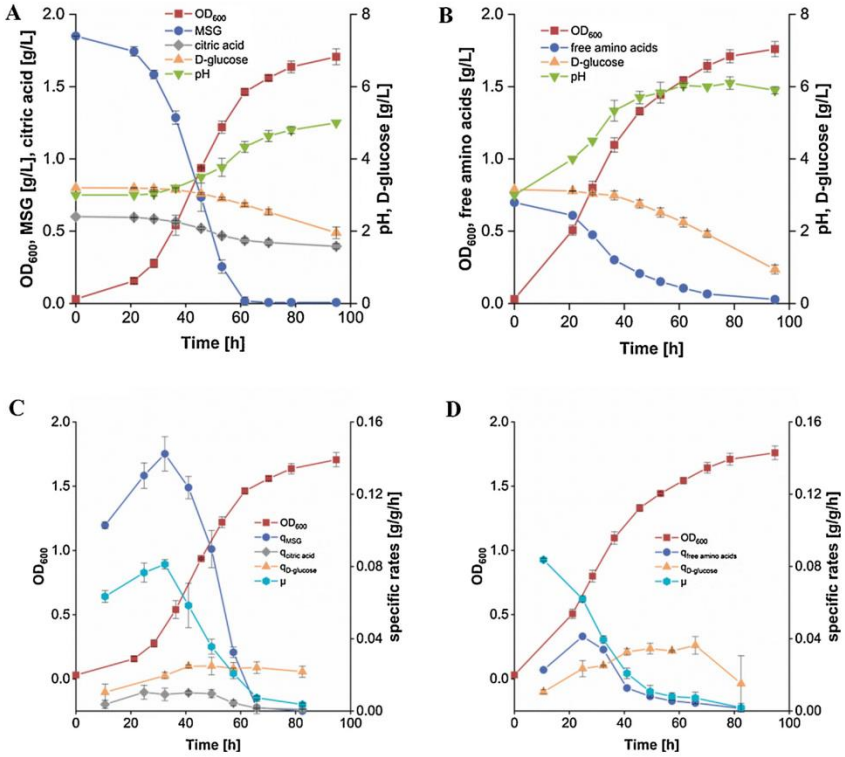

Fig. 11. A: Growth of *S. acidocaldarius* and substrate concentrations on VD Medium. B: Growth of *S. acidocaldarius* and substrate concentrations on Brock Medium. C: Specific substrate uptake rates as well as the growth rate on the VD Medium. D: Specific substrate uptake rates as well as the growth rate on the Brock Medium.

the main component of the Brock Medium (monosodium L-glutamate: €49.00 (49621-1KG, Merck) vs. NZ-amine AS: (€187.00 (N4517-1KG, Merck)). Additionally, downstream processing, the overall cost driver in microbial and cell culture processes alike, is heavily facilitated due to easier removal of media components in the VD Medium (protein hydrolysates containing peptides vs. single amino acids in the defined medium). Furthermore, mechanistic understanding, one of the pillars of the state-of-the-art QbD approach in bioprocess engineering, is very hard to come by in complex media due to the overwhelming number of possible interactions between media components as well as presence of unknown substances in trace amounts and unknown in-process stability of components.

Although these issues do not hinder process development a priori, in practice, the sum of these drawbacks deters potentially interested companies and ultimately leads to the focus of process development divisions on production systems with more predictive behavior (e.g. mesophilic hosts like *Escherichia coli* or yeasts), while their involvement with innovative hosts like *Sulfolobus* remains limited. We see the development of the defined medium as one important factor to overcome these limitations.

To further contribute to a better standing of *Sulfolobus* in the field of industrial application, during the development of the VD Medium we focused on *S. acidocaldarius* as host, since its industrial potential is generally considered the highest among the commonly known *Sulfolobus* strains due to its unmatched genome stability, simper carbon catabolism and ease of genetic manipulation. For that matter we limited the experiments with other *Sulfolobus* strains to the investigation of the effect of vitamins on their growth rate for reasons of comparison, since such effects were described for the investigated strains but not for *S. acidocaldarius*. Consequently, we do not claim that the VD Medium is optimized for any *Sulfolobus* strains other than *S. acidocaldarius*.

Unfortunately, although the development of a defined medium is a major step towards biotechnological applications, until now, no production processes using *Sulfolobus* can be expected in the nearest future, due to the general novelty of the field of high temperature fermentation, and hence limited process knowledge and limited know-how of high cell density cultivation techniques with *Sulfolobus*.

Therefore, on the short run, we see the usefulness of the medium in the fields of transcriptomics and metabolomics as here defined media backgrounds are of great importance. Also omitting complex media in favor of the VD Medium is highly beneficial when using fluorescent protein markers like GFP fusion tags or fluorescence based analytical methods like cell cycle analysis or flow cytometry based viability determination with fluorescent dyes. This applies for an increase of sensitivity in off-line methods, although here cell washing is still an option, but is most crucial for on-line and in-line applications which are employed during the cultivation process.

4. Conclusion

The described cultivation medium represents a substantial improvement of the successful Brock Medium for the cultivation of *S. acidocaldarius* and the undertaken efforts of media development are a novelty in *Sulfolobus* literature.

The presented results satisfy the urgent demand for a *standardized* and *defined* growth medium which is crucial for selective media design and strain characterization. During this study the following achievements were made:

- Substitution of complex substrate with monosodium glutamate
- Removal of precipitates
- Improved buffer capacity via addition of citric acid for higher final

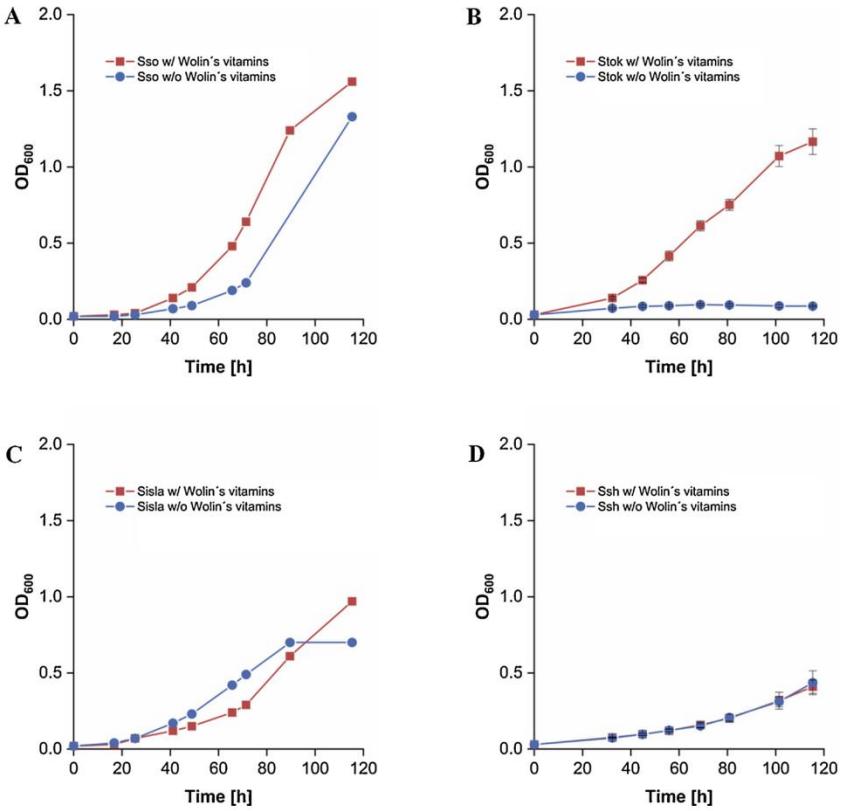

Fig. 12. Performance of common *Sulfolobus* strains on the VD Medium supplemented with Wolin´s Vitamin solution vs. without vitamin supplementation. A (Sso): *Sulfolobus solfataricus* P2; B (Stok): *Sulfolobus islandicus* Rey15A; C (Sisla): *Sulfolobus tokodaii* str. 7; D (Ssh): *Sulfolobus shibatae* B12. Note that here the growth curves with and without vitamin supplements overlap almost completely. All strains were grown at 75 °C due to technical limitations.

cell densities

- Reduced overall concentration of salts and trace elements for lower costs and reduced risk of growth inhibition

Using the described VD-Medium for the cultivation of *S. acidocaldarius* DSM 639 in shake flasks yields 1.1 g/L dry cell weight (OD_{600} = 1.7) after a typical incubation time of 95 h with an overall biomass yield of 0.33 $g_{DCW}/g_{substrate}$.

Author contribution

OS conceived the idea for this study. JQ, HG and MH performed experiments for media development. JQ supervised experiments for media development, performed data evaluation and drafted the manuscript. AA performed gene expression analysis and evaluation and statistical analysis of expression data. OS and JK critically reviewed and corrected the manuscript and gave substantial input.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.04.028.

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At-line viability determination of *Sulfolobus acidocaldarius* for efficient bioprocessing

Contribution to Chapter 4

(Monographic chapter)

At-line viability determination of *Sulfolobus acidocaldarius* for efficient bioprocessing:

A journey from 19th century´s plating techniques to today's state-of-the-art methods for accelerated bioprocess development and monitoring

Goal

Goal of this chapter is the development of reliable assays for timely viability determination of *Sulfolobus acidocaldarius* applicable in industrial bioprocesses. The results should be comparable to a plating assay, which is currently regarded as the gold standard for viability determination of *Sulfolobus* species.

Background

During development and scaling of industrial bioprocesses it is crucial to monitor the impact of critical process parameters on the cultivated organism (Rieseberg et al., 2001; Díaz et al., 2010). A frequent obstacle for process development & optimization is the lack of fast, statistically sound methods to assess cell viability. Members of the crenarchaeal genus *Sulfolobus* harbor great potential for the exploitation as production hosts and biocatalysts in biotechnological applications, nevertheless no industrial processes have been developed yet (Quehenberger et al., 2017). One of the reasons for the absence of industrial processes is, that up to date the viability of *Sulfolobus* cultures has to be determined via laborious, time- and material-intensive plating assays due to a lack of efficient alternatives. Therefore, there is a strong demand for the development of a fast and reliable at-line method for the assessment of cell viability.

Viability is defined as the ability of a microbe to survive under given conditions, which means to have the capacity to form progeny (Cangelosi and Meschke, 2014). Strictly following this definition implies the use of culture dependent methods for viability determination with the major drawback of a significant time delay of the results, of up to several days or even weeks in case of slow growing organisms. On the other hand, cultivation independent methods that compensate this disadvantage can only be indirect measures for the prerequisites for proliferation (e.g. intact cell membrane, physiological membrane potential or high activity of housekeeping enzymes). Consequently, any cultivation independent methods have to be selected cautiously and a careful evaluation of the relevance of the acquired viability parameters is necessary (Netuschil et al., 2014; Stiefel et al., 2015).

In the literature a number of fluorescence based, culture independent detection methods of *Sulfolobus* species can be found (Brock et al., 1972; Bernander and Poplawski, 1997; Hjort and Bernander, 1999; Leuko et al., 2004; Bernander, 2007; Han et al., 2017). Unfortunately, many published protocols suffer from low reproducibility and a lack of transferability between different laboratories. This study aims to develop methods for the simple determination of

viability parameters using fluorescence stains that have already been used for *Sulfolobus* species or that have originally only been employed in Bacteria and mammalian cultures.

Material and Methods

Strains and growth conditions

For biomass generation during development of the presented viability methods *Sulfolobus acidocaldarius* DSM 639 was grown aerobically at 75 °C in a reciprocal shaking oil bath at 100 rpm in 100 mL long neck Erlenmeyer flasks to avoid evaporation. The flasks were filled with 50 mL culture medium and the initial pH was adjusted to 3.0 with 4.8 w% H_2SO_4. Unless stated otherwise, a defined medium ("VD-Medium"), containing sodium glutamate and glucose as carbon sources was used (Quehenberger et al., 2019). For the direct comparison of the viability methods *S. acidocaldarius* DSM 639 was cultivated in a 3.6 L glass bioreactor (Labfors 3, Infors, Germany) implemented as continuous stirred-tank reactor (CSTR) with 2 L working volume. The preculture was grown in a 100 mL shake flask at 75 °C and 100 rpm in 50 mL VD-Medium (Quehenberger et al., 2019) containing 1.75 g/L Na-glutamate (MSG), 3 g/L D-glucose and 0.5 g/L citric acid, adjusted to pH 3.0 with sulfuric acid. At an OD_{600} of 0.75 the preculture was transferred aseptically to the culture vessel yielding an initial OD_{600} of 0.023 and a total starting volume for the initial batch phase of 1.5 L, containing 2 g/L MSG and 1 g/L D-glucose. Batch phase was performed at 75 °C, pH 3.1, followed by a short fed-batch phase to increase the working volume to 2.0 L. After reaching 2 L culture volume the vessel was operated in continuous mode by harvesting at the same rate as medium was being supplied.

Permeabilization of *S. acidocaldarius* cells

To generate "dead" cells as control sample for viability indicating dyes it was necessary to reliably permeabilize and inactivate *Sulfolobus* cells without compromising dye performance. Therefore permeabilization with an ultrasound probe, via microwaving or treatment with isopropanol was tested. Although all methods produced permeabilized cells, treatment with isopropanol was the most reproducible method and yielded the most consistent results. For the procedure an aliquot of culture broth was centrifuged (10 000 rfc, 5 min, room temperature) and the resulting cell pellet was resuspended in phosphate buffered saline (PBS) pH 5.5 to an OD_{600} of 0.3. After addition of two parts isopropanol (100%) and brief vortexing the sample was incubated for 1 h at room temperature with brief vortexing every 15 minutes. After 1 h isopropanol was removed via centrifugation, cells were washed once with PBS and finally resuspended in 1 aliquot PBS.

Investigated fluorescence-active dyes

A total of 10 fluorescence-active dyes were investigated regarding their suitability for viability determination of *S. acidocaldarius* (Table 1).

Table 1: Overview of the investigated fluorescent dyes.

Dye	Ex. λ_{max} [nm]	Em. λ_{max} [nm]	Fluorescence colour	Permeability*	Mode of interaction	For detection of
Acridine orange AO	500	526	green	permeable	DNA/RNA	living and dead cells
SYTO	485	500	green	permeable	DNA/RNA	living and dead cells
Hoechst 33342	350	461	blue	permeable	DNA, A-T rich regions	living and dead cells
RH414	532	716	red	permeable	cell membrane	living and dead cells
Fluorescein diacetate FDA	485	520	green	permeable	enzymatic fluorophore generation	living cells
DAPI	358	461	blue	impermeable	DNA, A-T rich regions	dead cells
Ethidium bromide EB	300	590	orange-red	impermeable	DNA	dead cells
Propidium iodide PI	535	617	red	impermeable	DNA/RNA	dead cells
7-AAD	546	647	red	impermeable	DNA, G-C rich regions, RNA	dead cells
$DiBAC_4(3)$	493	516	green	impermeable	positively charged or hydrophobic regions	dead cells

*here permeability describes the capability of a dye to enter intact (uncompromised) cells
Ex… excitation; Em… emission;

Analytical methods and equipment

Determination of biomass growth: Growth was monitored with a photometer (Genesys 20 photometer (Thermo Scientific, USA)) via determination of the optical density at 600 nm.

Reference method for viability determination: A plating assay for the determination of colony forming units were carried out on plates containing 0.6 w% gelrite, 2 g/L glucose, 1 g/L NZ-amine, 3 mM $CaCl_2$ and 10 mM $MgCl_2$ in addition to salts and trace elements as described for a growth medium by Brock et al (1972). Growth on the plates was assessed after 5 days of incubation at 75 °C.

Fluorescence microscope: A Leica DMI 8 fluorescence microscope (Leica Microsystems, Germany) equipped with a mercury light source was used for screening of fluorescence-active dyes. Three filter configurations were available: Filter 1, excitation (ex.) 532-558 nm / emission (em.) 570-640 nm); Filter 2, ex. 450-490 nm / em. 500-550 nm; Filter 3, ex. 600-660 nm / em. 662-738 nm. Undiluted cell suspensions were applied on glass slides and after addition of fluorescent dyes samples were incubated in the dark over a range of 5 to 50 minutes before investigation with the microscope.

FDA staining with flow cytometry: A Cube 8 flow cytometer (Sysmex, Germany) equipped with a 488 nm laser was used for evaluation of the performance of fluorescence-active viability dyes and the measurement of esterase activity on the cellular and culture level. An aliquot of 300 µL freshly harvested broth was centrifuged (10 000 rcf, 10 min, room temperature) and resuspended in 3 parts of 1:5 diluted PBS pH 5.5. After addition of 4.5 µL of a FDA stock (5 g/L dissolved in acetone) the sample was quickly vortexed and incubated for 10 min at 37 °C in the dark. The incubated samples were washed once with 1:5 diluted PBS pH 5.5 to remove excess FDA and subsequently diluted to a theoretical OD_{600} between 0.001 and 0.0015. FCM measurements were carried out with a FSC gain of 180 V, FL1 gain of 200V, FCS threshold of 0.004 V, flow rate of 2 µL/sec and a total measured volume of 50 µL.

FDA staining with plate reader: An infinite 200 microplate reader (Tecan, Switzerland) was used for fluorescein diacetate (FDA) fluorescence measurements to determine esterase activity in *S. acidocaldarius* culture samples. An aliquot of 300 µL was centrifuged (10 000 rcf, 10 min, room temperature) and resuspended in 1/5 to 1/3 part of PBS pH 5.5 depending on the OD_{600} of the sample. In a 96-well plate 0.5 µL a FDA stock (5 g/L dissolved in acetone) were added to 100 µL of the resuspended cells and immediately vortexed and incubated in the plate reader for 10 min at 37 °C in the dark. Subsequently the fluorescence in the wells was determined (excitation 485 ±4.5 nm, detection 520 ±10 nm).

Results and Discussion

Screening of viability indicating fluorescence-active dyes for *S. acidocaldarius*

An overwhelming number of viability indicating dyes are commercially available for mammalian cells and bacteria (Johnson and Spence, 2010), while for Archaea the complete opposite is the case. Nevertheless, SYTO and acridine orange (AO) have already been tested for the use in Archaea and have been reported to successfully stain *Sulfolobus* cells (Brock et al., 1972; Leuko et al., 2004). In this study the performance of 10 viability dyes, shown in Table 1 originally used for mammalian or bacterial cells were tested regarding their applicability for viability determination of *S. acidocaldarius*. Out of the 10 investigated dyes 4 led to fluorescent cells under the fluorescence microscope: AO, fluorescein diacetate (FDA), SYTO and propidium iodide (PI). Unfortunately, staining efficiency, fluorescence intensity and loss of fluorescence due to photobleaching were strongly influenced by incubation time, dye to cells ratio, as well as excitation time and intensity. Due to this lack of robustness the development of a microscope based viability assay was not pursued. Instead, a transfer to a more reliable system with a higher degree of automatization was pursued. For this purpose flow cytometry was selected as method of choice.

Transfer of stains from fluorescence microscope to flow cytometry

Flow cytometry (FCM) is described as a reproducible method for acquiring optical and fluorescence information of cultures on a single cell level (Rieseberg et al., 2001; Díaz et al.,

2010). With multiple thousand measurements per second statistically reliable datasets can be quickly generated. Out of the 4 dyes that were positively tested with the fluorescence microscope, AO and FDA also resulted in fluorescent signals when stained cells were investigated with the flow cytometer. While AO permeates cells regardless of their (membrane) integrity and subsequently leads to fluorescence emission when bound at (intracellular) nucleic acid, FDA staining is based on an increase of fluorescence upon cleavage of the FDA molecule by cellular esterases. Consequently, compromised cells with a reduced amount of esterases exhibit lower to no fluorescence compared to healthy cells (Jones and Senft, 1985).

For the development of a viability assay it is desirable to employ complementary stains: one dye that stains *all* cells and another dye that specifically stains either viable *or* dead cells. Although complementarity is given in case of AO (stains all cells) and FDA (only living cells) it is not feasible to use the two dyes simultaneously in a single assay, since both dyes have very similar emissions maxima (526 vs. 520 nm) and consequently their signals cannot be discriminated due to spectral overlap. In order to still obtain useful viability information, we adapted the FCM assay so that the population of "all" cells could be detected only based on the optical forward scatter (FSC) signal. In combination with the "viable" cells determined with the fluorescence signal originating from FDA hydrolysis (FL1) it was possible to calculate a ratio of "viable" to "all" cells representing the cultures' overall viability.

Figure 1 shows the results of viability determination during a batch cultivation of *S. acidocaldarius*. Viability was determined with A) plating assay ("VC_{plate}"), expressed as ratio of colony forming units (≙ *viable* cells) to OD_{600} (≙ *all* cells) and compared with B) the viability determined with flow cytometry ("VC_{FCM}"), which is expressed as ratio of FL1 (≙ *viable* cells) to FSC (≙ *all* cells).

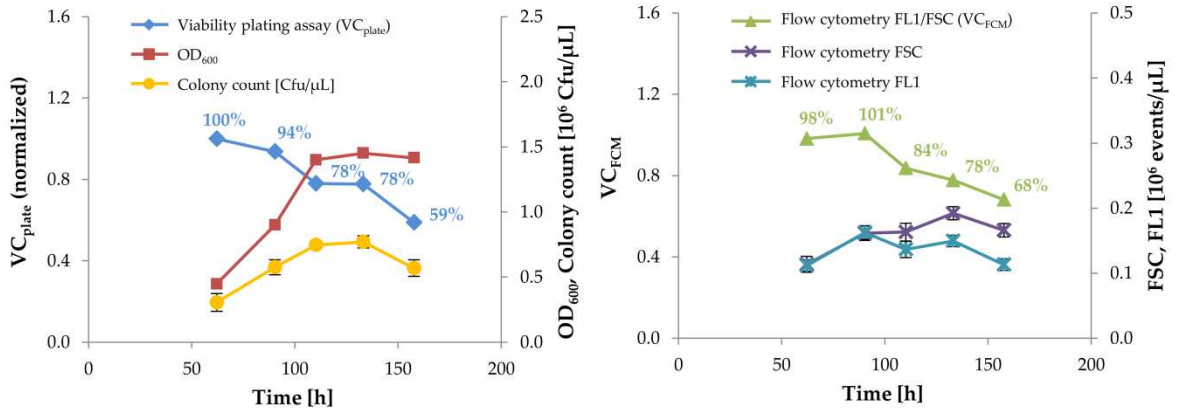

Figure 1: Trend of cell viability and viability related parameters during a cultivation of *Sulfolobus acidocaldarius* determined by plating assay (Figure 1A) vs. FCM (Figure 1B). Upon reaching the stationary phase a drop in both the viability calculated via the ratio of Cfu/µL to OD_{600} (yellow to red curve) and B) the viability calculated via the ratio of FL1 to FSC (teal to violet curve) can be observed. The correlation between VC_{plate} and VC_{FCM} is shown in Figure 2.

Plotting the viability determined via plating on gelrite plates (VC_{plate}), which is considered the gold-standard for classical viability determination of *Sulfolobus* (Lindström and Sehlin, 1989), and the newly developed method for viability determination based on FDA staining combined with flow cytometry (VC_{FCM}) revealed a good correlation between the two methods (Figure 2). These results support the assumption that the developed method is suitable for fast and reliable determination of cell viability of *Sulfolobus* cells.

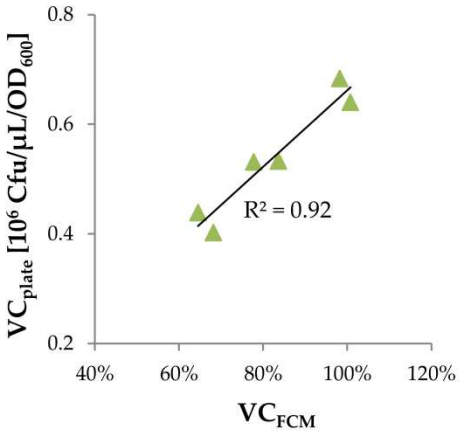

Figure 2: Correlation between viability calculated via the ratio of 10^6 Cfu/µL to OD_{600} (VC_{plate}) and viability calculated via the ratio of FL1 to FSC (VC_{FCM}).

Transfer of the FDA assay from flow cytometry to plate reader

The suitability of FDA as viability indicator was tested with a fluorescence plate reader, in order to show the platform character of FDA as marker, but also to get access to a method that is less dependent on highly specialized equipment that is unavailable in most laboratories. In contrast to FCM the plate reader can only quantify the fluorescence signal across the whole population and not on a cellular level. This means that per sample only a single fluorescence reading is generated that is proportional to the amount of FDA that is hydrolyzed starting from the addition of FDA until the time point of measurement. As a result the potential emergence of subpopulation leading to a culture with inhomogeneous (esterase) activity pose a limitation of this method since a potential serious drop in viability could be overlooked. This lies in contrast to FCM where each single cell is being investigated individually and can be categorized into metabolically active ("viable") or inactive ("dead").

However, the plate reader assay proved to be a fast and simple method yielding reliable quantitative results down to cell densities of approximately OD_{600} = 0.05. The fluorescence response of a stepwise diluted broth sample harvested at the late stationary phase is shown in Figure 3. This experiment demonstrated a linear range of the assay over the whole range of cell densities achieved in common shake flask cultivations using standard batch media like the Brock medium (Brock et al., 1972) or VD Medium (Quehenberger et al., 2019).

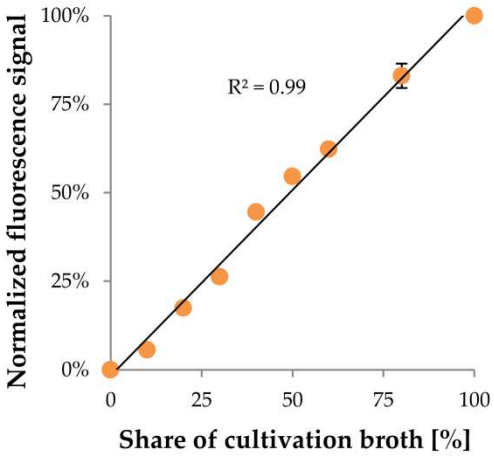

Figure 3: Normalized fluorescence of a stepwise diluted shake flask sample of *S. acidocaldarius* harvested during the late exponential phase. Samples were measured on a fluorescence plate reader. Source for the fluorescence signal is the enzymatic hydrolysis of fluorescein diacetate (FDA).

Method comparison

For testing in a more versatile setup and for comparison of the viability methods, a bioreactor cultivation comprising of a batch phase, stationary phase, fed-batch phase and continuous phase (Figure 4) was performed. The different operation modes were employed to provoke phases of different *actual* cell viability with the goal to determine whether the fluorescence based viability methods (with FCM and plate reader) are also applicable under realistic process conditions over an extended period of time and if they correlate with the results of the plating assay when a culture is subject to changing environmental conditions.

Both fluorescence based viability methods showed high correlations with the plating assay of $R^2 = 0.93$ (FCM) and $R^2 = 0.87$ (microplate reader) as shown in Figure 5 and consequently appear to be well suited as alternative to the plating assay. While the viability determined with the plate reader (ratio of fluorescence signal to OD_{600}; VC_{96}) largely overlaps with the viability determined by the plating assay (VC_{plate}), a clear offset can be observed for the viability determined via FCM (VC_{FCM}) as pictured in Figure 4. This offset is likely a result of basal, unspecific excitation of the detector, due to technical reasons e.g. fluorescence bleeding or too sensitive detector settings. It should be mentioned that the lag- and early exponential batch phase of the bioreactor cultivation (0 to 50 h after inoculation) were not included in the correlation analysis. Although during this phase of low cell density ($OD_{600} < 1$) the fluorescence based methods correlated well with each other, they rather anticipated the trend of VC_{plate}. These results may reflect the accumulation of host cell proteins (including esterases) during the lag phase that precedes the exponential growth phase and accompanying increase in proliferable cells. A phenomenon that is clearly revealed by all three methods is a significant drop in viability during the unlimited fed-batch phase that succeeds the batch phase. Thereby, the viability methods show their capability to reveal how an unfavorable process mode that induces metabolic burden, and consequently a drop in cellular fitness and eventually a decreased viability.

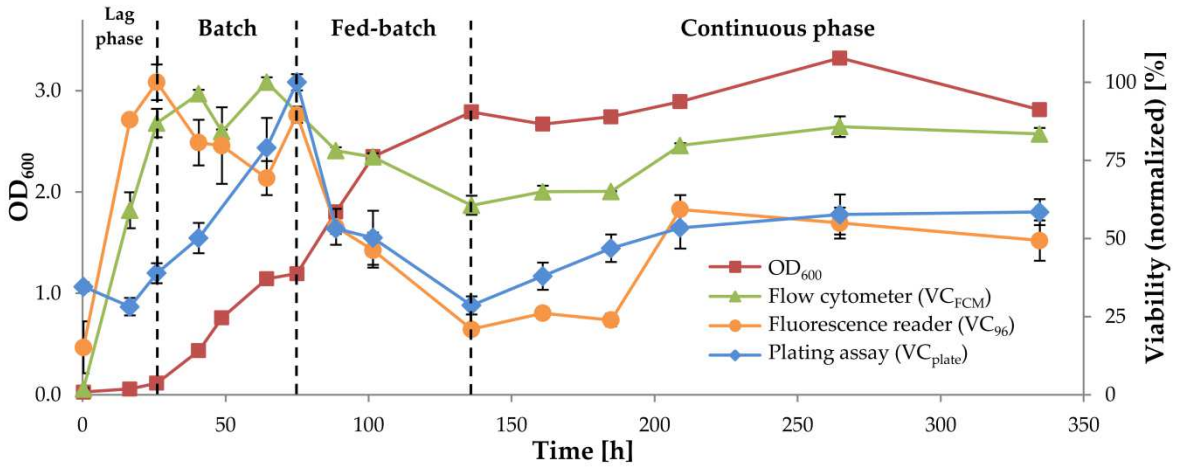

Figure 4: Comparison of viability measurements of *S. acidocaldarius* during different process phases. For method comparison under process conditions the viability was determined with flow cytometry (VC_{FCM}), fluorescence plate reader (VC_{96}) and, as reference, by plating (VC_{plate}). Notably, there is a discrepancy between VC_{plate} and the assays based on esterase activity (VC_{FCM} and VC_{96}) in the lag and early batch phase. During the remaining course of the cultivation all three methods show a high degree of correlation, including a significant drop in viability during the unlimited fed-batch phase following the batch phase.

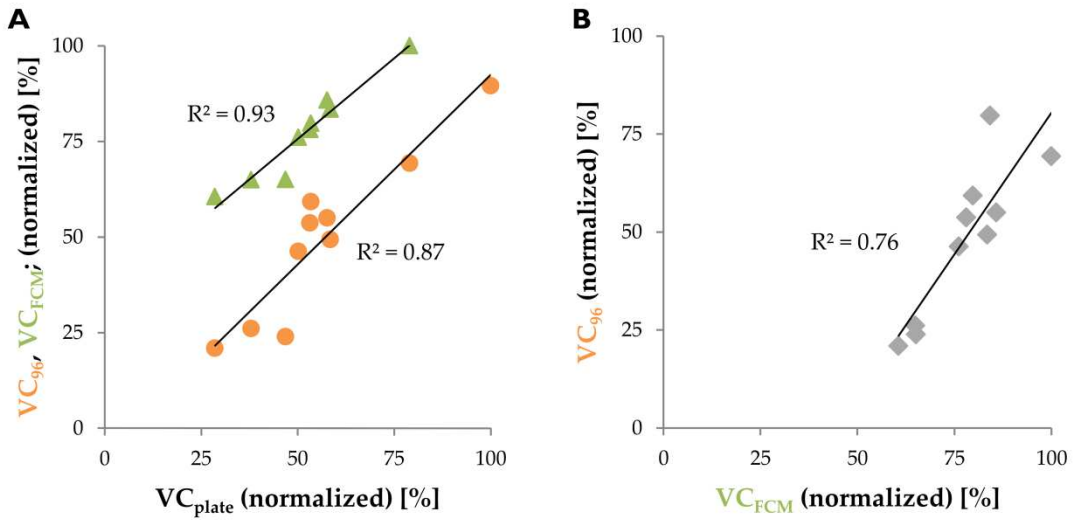

Figure 5: Correlation analysis of viability methods. A: Correlation between viability results determined with fluorescence plate reader (VC_{96}) vs. plating assay (VC_{plate}) and flow cytometry (VC_{FCM}) vs. plating assay (VC_{plate}). B: Correlation between VC_{96} vs. VC_{FCM}. Notice that the early batch phase is excuded from the correlation analysis.

Conclusion

It should be emphasized that culture dependent methods and staining based methods for viability determination measure two fundamentally different properties. The former method determines the amount of proliferable cells (the definition of viability in the classical sense) and the latter determine either the state of the cell membrane or enzymatic activity of the organism (physiological conditions that are only indirectly linked to cell viability). This may lead to discrepancies between the different methods, as shown in this study for the lag and

early exponential batch phase. What, at first sight, looks like a methodological shortcoming is in reality a reflection of a physiological state that has different implications for the parameters VC_{plate} vs. VC_{96} and VC_{FCM} (such states cannot be inferred from the current specific growth rate but have to be considered in the context of the culture history).

Nevertheless, within the investigated scope of this study (shake flask cultivations and a bioreactor cultivation with various different process modes over a continuous cultivation period of 300 h), both the plate reader and FCM based method matched the results of the plating assay which was employed as current standard reference method. Both fluorescence based methods have their merit as shown in Table 2. VC_{96} represents the easier to determine parameter owing to the assays robustness, simple execution due to low demand on sample preparation and equipment set up, as well as low equipment costs. On the other hand, the determination of VC_{FCM} can be potentially combined with other fluorescence dyes for the determination of further physiologic parameters (e.g. membrane integrity, DNA concentration, etc.). Furthermore, FMC allows determination of all those parameters on a single cell level, therefore allowing the revelation of potential sub-population within a culture.

Table 2: Comparison of advantages and disadvantages of flow cytometry and plate reader.

	Flow cytometry (FCM)	**Plate reader**
Statistical relevance	Statistically reliable datasets due to multiple thousand measurements per second	Low information content 1 sample ≙ 1 measurement
Equipment costs	High	Medium
Robustness	Results highly dependent on equipment and settings	Simple setup and transfer between different devices
Sample preparation effort	Medium	Low
Investigation level	Investigation on a cellular level	Detection of subpopulations impossible
Method expandability	Combinable with other fluorescence markers	1 parameter per sample

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Kinetics and Predicted Structure of a Novel Xylose Reductase from *Chaetomium thermophilum*

Contribution to Chapter 5

(Scientific publication)

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Article

Kinetics and Predicted Structure of a Novel Xylose Reductase from *Chaetomium thermophilum*

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Abstract: While in search of an enzyme for the conversion of xylose to xylitol at elevated temperatures, a xylose reductase (XR) gene was identified in the genome of the thermophilic fungus *Chaetomium thermophilum*. The gene was heterologously expressed in *Escherichia coli* as a His6-tagged fusion protein and characterized for function and structure. The enzyme exhibits dual cofactor specificity for NADPH and NADH and prefers D-xylose over other pentoses and investigated hexoses. A homology model based on a XR from *Candida tenuis* was generated and the architecture of the cofactor binding site was investigated in detail. Despite the outstanding thermophilicity of its host the enzyme is, however, not thermostable.

Keywords: xylose reductase; *Chaetomium thermophilum*; kinetics; structure; homology model; cofactor binding; stability

1. Introduction

Chaetomium thermophilum is a thermophilic fungus of the phylum Ascomycota. It is one of the most thermophile Eukaryotes, growing optimally at temperatures of 50–55 °C on rotting organic matter [1]. The organism is a mycelium-forming fungus with multinucleate hyphae, perforated by septa—similar to other filamentous ascomycetes, such as *Neurospora crassa* or *Aspergillus* sp. The complete nuclear and mitochondrial genomes of *C. thermophilum* are sequenced and the nuclear genome is estimated to be 28.3 Mbp with 7227 open reading frames while the mitochondrial genome is 127 kbp with 15 protein coding genes [2].

Due to the host's outstanding thermostability, it is a promising source of stable enzymes. In total, 191 Protein Data Bank (PDB) entries for *C. thermophilum* have been deposited to this date; a number that has been rapidly increasing in recent years compared to only 19 entries until the year 2013 [1]. From an industrial point of view the organism is especially interesting for prospecting thermostable enzymes that are exclusively present in Eukaryotes. These enzymes cannot be found in extreme thermophiles (growth at ≥85 °C), a group of organisms that are only found in the prokaryotic Domains Bacteria and Archaea [3].

The enzyme D-xylose reductase (EC 1.1.1.307) belongs to the aldose reductase family. It is commonly found in filamentous fungi and yeasts, and catalyzes the reduction of D-xylose to the sugar alcohol xylitol. This reaction marks the initial step of the fungal xylose catabolism, eventually leading to the pentose phosphate pathway via oxidation of xylitol to D-xylulose by the enzyme xylitol dehydrogenase. Generally, wild type XRs strongly favor NADPH as cofactor, while dual

cofactor specificity for NADH and NADPH was only observed in some cases [4–7]. Only a single XR was reported that preferred NADH over NADPH [8]. More recently, several XRs have been generated via mutation with increased affinity towards NADH in order to circumvent the problem of cofactor balance in enzyme cascades with NAD^+-dependent xylitol dehydrogenases [9–15]. The core structure of XRs is a $(\beta/\alpha)_8$-barrel fold composed of eight alpha-helices running antiparallel to eight beta sheets in the center of the barrel [16]. Commonly, XRs are found to form dimers in their native state [17].

XRs are of significant industrial interest due to their importance for the utilization of hemicellulosic biomass as an energy and carbon source for ethanol production in yeasts [18,19], as well as for use as a biocatalyst for the conversion of xylose to the value added product xylitol. Xylitol is a naturally occurring, low-caloric sugar alcohol with a relative sweetening power comparable with sucrose [20]. Due to its anticariogenic properties and low glycemic index, it is widely used in chewing gum, food and beverages as well as in the pharmaceutical industry [21,22]. In a recent estimation, the global market for xylitol was expected to reach USD 1.37 billion by 2025 with an annual growth rate of 6.6% [23].

A considerable number of different XRs has been investigated with regard to stability and reactivity and mutated variants have been produced with the aim of increased activity and shift in cofactor preference, with examples in references [9–11,13,15]. Apart from the general desire to improve the stability of industrially relevant enzymes, a more specific benefit of thermostable XRs could be the possibility of direct utilization of heat-treated hemicellulosic biomass for the conversion of liberated xylose to xylitol. Nevertheless, most of the described XRs are mesophilic, whereas the highest described temperature optimum is 45–55 °C for an XR from *N. crassa* [24], and 50 °C for an XR from *Candida tenuis* [6].

In order to find an XR with high activity at elevated temperatures, we chose to investigate the genome of one of the most thermostable Eukaryotes, namely *C. thermophilum*, with the aim to heterologously express, purify and characterize a novel XR.

2. Results and Discussion

2.1. Protein Production

After 34 h total fermentation time, 230 g/L wet cell weight (corresponding to 62 g/L dry cell weight (DCW)) were harvested. Crude extract derived from 230 g wet cell weight contained a total activity of 360 U, resulting in a titer of 360 U/L or 5.8 U/g DCW. The space time yield (volumetric productivity) equals 10.6 U/L/h.

2.2. Protein Purification

Recombinant His6-tagged *Ct*XR was purified to near homogeneity and the apparent molecular mass (Mw), estimated with SDS-PAGE to be approximately 41 kDa, agreed with the theoretical Mw of 39,244 Da. As shown in Table 1, loss of active *Ct*XR during purification was significant. Mass overload during immobilized metal affinity chromatography (IMAC) caused product loss in the flow through. Poor product retention during spin filtration and cutting of product peak during size exclusion chromatography (SEC), to increase purity, led to further product loss. This results in a total recovery of only 3% but a high purification factor of 25. Product size and purity of the various fractions can be seen in the SDS-PAGE image in Figure 1.

Table 1. Summary of the purification process of *Ct*XR. Recovery and purification factor are given relative to the activity in the crude extract and, in brackets, for retained fractions relative to the preceding fraction.

	Total Protein (mg)	Specific Activity (U/mg)	Total Activity (U)	Recovery (%)	Purification Factor ( )
Crude extract	4296	0.08	359	100	1
IMAC Flow through	4158	0.09	392	109	1.1
IMAC Eluate 1 (pre elution peak)	11.85	0.28	3.3	0.9	3.4

IMAC Eluate 2 (post elution peak)	3.96	0.12	0.5	0.1	1.4
IMAC Elution peak	55.7	1.49	83.1	23.1 (23.1)	17.8 (17.8)
Spin filter Flow through	25.1	2.12	53.1	14.8	25.3
Spin filter retentate	34.1	0.76	26.0	7.2 (31.3)	9.1 (0.5)
SEC Pool 1 (discarded fraction)	13.4	0.76	10.1	2.8	9.0
SEC Pool 2 (discarded fraction)	5.20	0.86	4.5	1.2	10.3
SEC Peak (collected fraction)	4.51	2.13	9.6	2.7 (36.9)	25.5 (2.8)

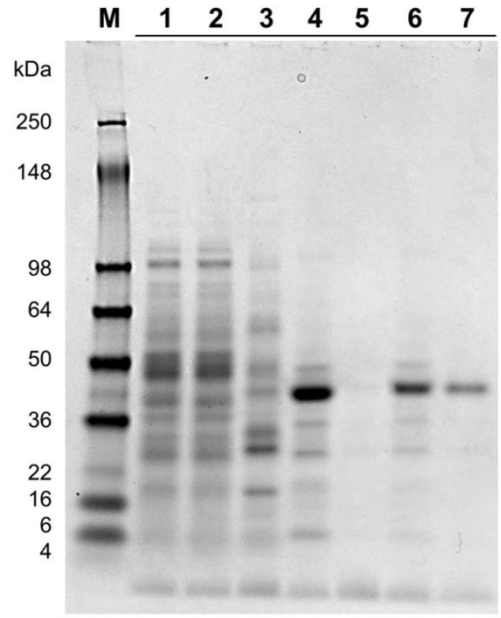

Figure 1. SDS-PAGE image to evaluate the purification process of *Ct*XR. The target protein is located at a height between 36 and 50 kDa with an estimated mass of 41 kDa. M: Protein ladder (SeeBlue Plus2 prestained standard); 1: Crude extract (diluted 1:10); 2: IMAC Flow through (1:10); 3: IMAC Eluate 1 (pre elution peak; 1:1); 4: IMAC Elution peak (determined by a jump in the UV absorption signal in the eluate; 1:10); 5: IMAC Eluate 2 (post elution peak; 1:5); 6: Spin filter Flow through (1:40); 7: SEC Peak (collected fraction based on a jump in the UV absorption signal; 1:20).

2.3. Biochemical Characterization

2.3.1. Enzyme Activity

*Ct*XR was kinetically characterized at 30 and 55 °C with the substrate D-xylose in limiting concentrations while NADPH was provided in non-limiting concentrations (Figure 2). k_{cat} increases from 30 to 55 °C while there is only little change in K_M. (Table 2). This means, that while the turnover rate (k_{cat}) increases, the apparent affinity towards the substrate only decreases slightly with rising temperature.

Table 2. Kinetic parameters of the xylose reductase from *C. thermophilum*. d-Xylose (varied between 0.001–0.5 M).

Temperature	k_{cat} (s^{-1})	$K_{M,(Xylose)}$ (mM)	$k_{cat}/K_{M,(Xylose)}$ ($mM^{-1}\cdot min^{-1}$)
30 °C	11.4 ± 1.1	22.3 ± 6.1	31 ± 9
55 °C	35.2 ± 1.4	25.4 ± 4.0	83 ± 14

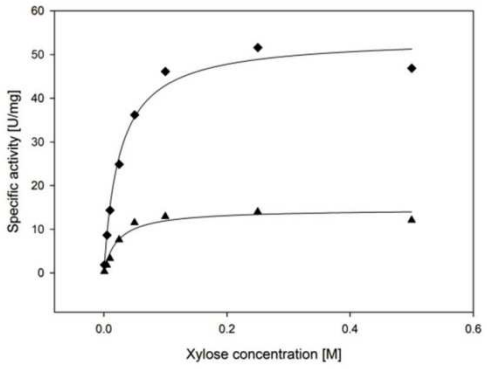

Figure 2. Michaelis-Menten curves at ▲ 30 °C and ◆ 55 °C. The concentration of D-xylose was varied and concentration of NADPH was kept at 0.4 mM.

2.3.2. pH Optimum

The pH optimum of most xylose reductases described in the literature is at pH 6.5. We determined a pH optimum of *Ct*XR in a citrate-disodium phosphate buffer at pH 7.0 (Figure 3). In 50 mM citrate and 50 mM phosphate buffer the pH optimum was found at pH 6.0 and 6.5, respectively (Supplementary Figure S1). These results indicate that increased ionic strength, or, more specifically, a higher phosphate concentration, leads to an apparent shift of the pH optimum towards higher values, as the citrate disodium phosphate buffer contains 165 mM phosphate at pH 7.

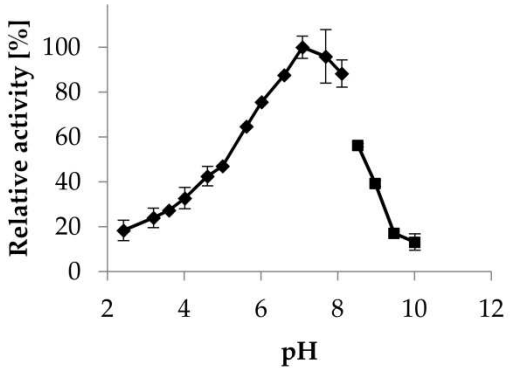

Figure 3. Xylose reductase activity at different pH values. Measurements were taken at 30 °C in a citric acid-disodium phosphate buffer (McIlvaine buffer) [25] (◆ pH 2.4–8.1) and 50 mM glycine buffer (■ pH 8.5–10.0).

2.3.3. Cofactor Preference

To test whether *Ct*XR preferred NADPH or NADH as cofactor, K_M, k_{cat} and the catalytic efficiency were determined (Figure 4). The enzyme shows dual cofactor specificity and similar K_M values for NADPH and NADH at 30 °C. Nevertheless, k_{cat} and the catalytic efficiency are significantly higher for NADPH (Table 3).

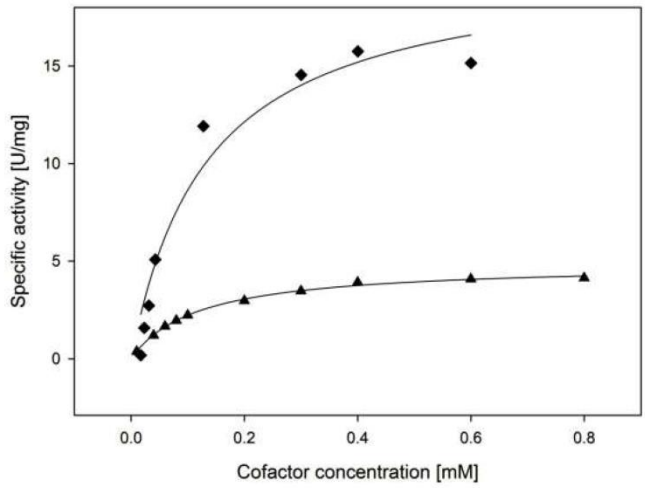

Figure 4. Michaelis-Menten curves for determination of cofactor preference: ◆ 0.5 M D-xylose, variation of NADPH; ▲ 0.5 M D-xylose, variation of NADH. Measurements were taken at 30 °C.

Table 3. Cofactor preference of the investigated xylose reductase. Measurements were taken at 30 °C.

Cofactor	k_{cat} (s^{-1})	$K_{M,(Cofactor)}$ (μM)	$k_{cat}/K_{M,(Cofactor)}$ ($\mu M^{-1} \cdot min^{-1}$)
NADPH	11.4 ± 1.1	135 ± 44	5.1 ± 1.7
NADH	3.2 ± 0.1	119 ± 6	1.6 ± 0.1

2.3.4. Substrate Specificity

Relative activity compared to D-xylose was determined for all D-pentoses, L-xylose, L-arabinose, as well as for D-glucose, D-galactose and D-mannose (Figure 5). The enzyme shows promiscuity at high substrate concentrations, readily converting some pentoses and hexoses (L-arabinose, D-ribose, D-galactose, D-glucose), while others are converted slightly or to no extent (D-lyxose, D-mannose, L-xylose, D-arabinose).

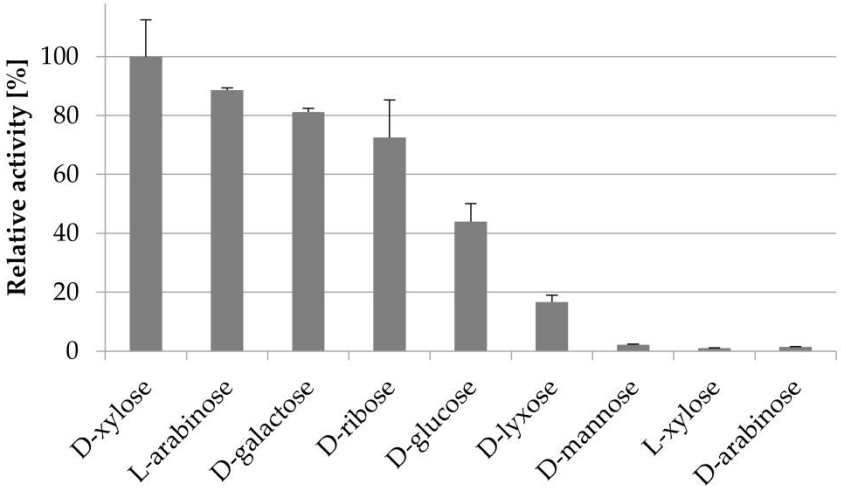

Figure 5. Relative activity of *Ct*XR at 0.5 M substrate concentration. 0.4 mM NADPH was used as cofactor in a 50 mM bis-tris buffer, pH 6.5, at 30 °C.

To further investigate the XR´s apparent affinity towards substrates of industrial relevance, we determined K_M, k_{cat} and catalytic efficiency for D-xylose, L-arabinose, D-galactose and D-glucose

(Figure 6). These sugars represent major constituents of hemicellulose and are converted with reasonably high relative activity. A clear difference between the conversion of the pentose L-arabinose and the hexoses D-glucose and D-galactose can be seen. L-arabinose, being structurally similar to D-xylose, has a k_{cat}/K_M value of still 33% of the value for D-xylose, whereas D-galactose and D-glucose result in only 6.3% and 1.3% of the k_{cat}/K_M for D-xylose. For the observed cases the difference in the catalytic efficiency is mainly a result from different K_M values rather than differences in k_{cat}, as shown in Table 4. This indicates that the reduction of the carbonyl carbon is influenced to a lesser degree, compared to the binding of the substrate itself.

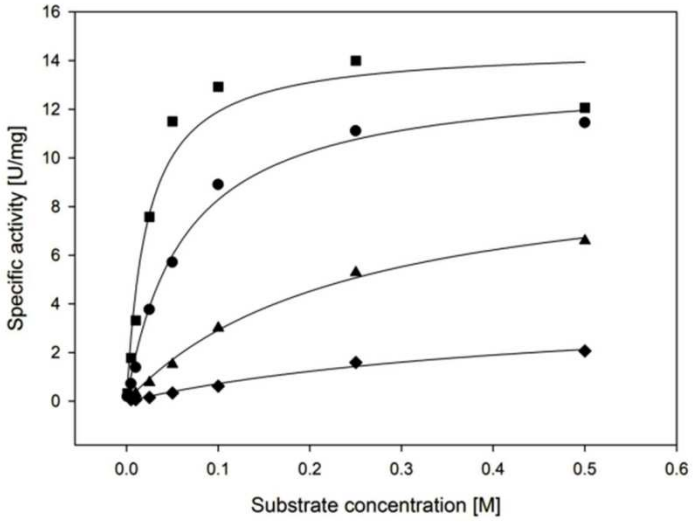

Figure 6. Michaelis-Menten curves with the substrates ◆ D-glucose, ▲ D-galactose, ● L-arabinose and ■ D-xylose. The concentration of sugars was varied and concentration of NADPH kept at 0.4 mM, measurements were taken at 30 °C.

Table 4. Substrate specificity of the investigated xylose reductase. The enzyme showed a clear preference for D-xylose (k_{cat}/K_M). Measurements were taken at 30 °C.

Substrate	k_{cat} (s^{-1})	$K_{M,\,(Substrate)}$ (mM)	$k_{cat}/K_{M,\,(Substrate)}$ ($mM^{-1}\cdot min^{-1}$)
D-xylose	9.2 ± 0.6	22.3 ± 6.1	26 ± 7
L-arabinose	8.8 ± 0.4	62.5 ± 7.7	8.4 ± 1.1
D-galactose	6.5 ± 0.4	241 ± 29	1.6 ± 0.2
D-glucose	2.7 ± 0.5	471 ± 141	0.3 ± 0.1

2.3.5. Temperature Stability

The half-life ($T_{1/2}$) of *Ct*XR was determined at 30, 55, 65 and 75 °C. Enzyme inactivation was assumed to follow first order kinetics. After plotting the natural logarithm of the specific activity against the incubation time, the decay rate λ was determined as the slope of a linear fit during the degradation phase (Figure 7). When incubated at 30 °C no loss in activity could be observed over the course of 3 h incubation time. However, stability dropped significantly when *Ct*XR was incubated at 55 °C, resulting in a half-life of 1.8 min, which decreased even further to 4.2 and 2.8 s at 65 and 75 °C, respectively (Table 5). Due to the low number of sample points caused by the fast decrease of activity, the latter two values should be considered as estimations. The comparatively low temperature stability of the enzyme is surprising, since its original host *C. thermophilum* is among the most thermophile Eukaryotes with an optimal growth temperature of 50–55 °C [1]; also a xylanase derived from *C. thermophilum* has been shown to be stable at 60 °C [26]. Possible explanations for this phenomenon could be that the pathway of xylose degradation in *C. thermophilum* is only active at temperatures significantly below the optimal growth temperature, or that the enzyme is greatly

stabilized in the native environment of the host´s cytosol, making the in vitro results not transferable to natural conditions. Nevertheless, a stabilizing effect due to presence of cofactor (NADPH) or substrate (D-xylose) could not be confirmed in vitro (data not shown).

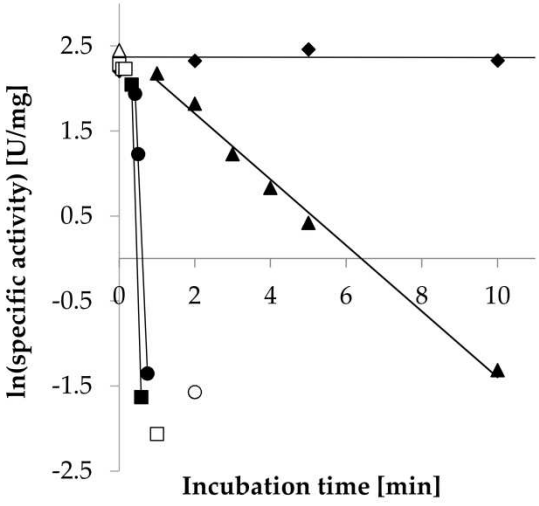

Figure 7. Plot of residual activity against incubation time; the decay rate λ was determined as the slope of a linear fit during the degradation phase (see Table 5). ◆ 30 °C, ▲ 55 °C, ● 65 °C and ■ 75 °C incubation temperature. Data points with empty symbols were not taken into account for the calculation of the decay rate.

Table 5. Decay rate λ and half-life $T_{1/2}$ at 30 °C, 55 °C, 65 °C and 75 °C.

Temperature	Decay Rate (λ) (s^{-1})	Half-Life ($T_{1/2}$)
◆ 30 °C	n.a.	n.a.
▲ 55 °C	$-6.5 \cdot 10^{-3}$	1.8 min
● 65 °C	−0.17	4.2 s
■ 75 °C	−0.25	2.8 s

n.a. not applicable (no loss of activity over 3 h).

2.4. Structural Analysis

The initial homology model generated by SWISS-MODEL [27] with *Ca*XR as template (PDB code 1SM9, [28]; 52.7% sequence identity to *Ct*XR) provides a reliable prediction of the *Ct*XR with a global model quality estimation (GMQE) score of 0.80, and QMEAN Z-score (reliability) of −0.73.

The modeled nicotinamide, ribose and diphosphate regions of the cofactor-binding site in *Ct*XR are identical in sequence to that of *Ca*XR (PDB code 1MI3). Structural discrepancies are confined to the adenosine-binding site (adenine ring and ribose moieties) where amino-acid replacements in the loop comprising residues 276–283 (same numbering in *Ct*XR and *Ca*XR) are predicted to affect the precise details of adenosine binding, redox properties, and the relative preference for NAD(H) versus NADP(H). Notable *Ca*XR-to-*Ct*XR replacements include N276T, L277R, R280I and Q283S (Figure 8).

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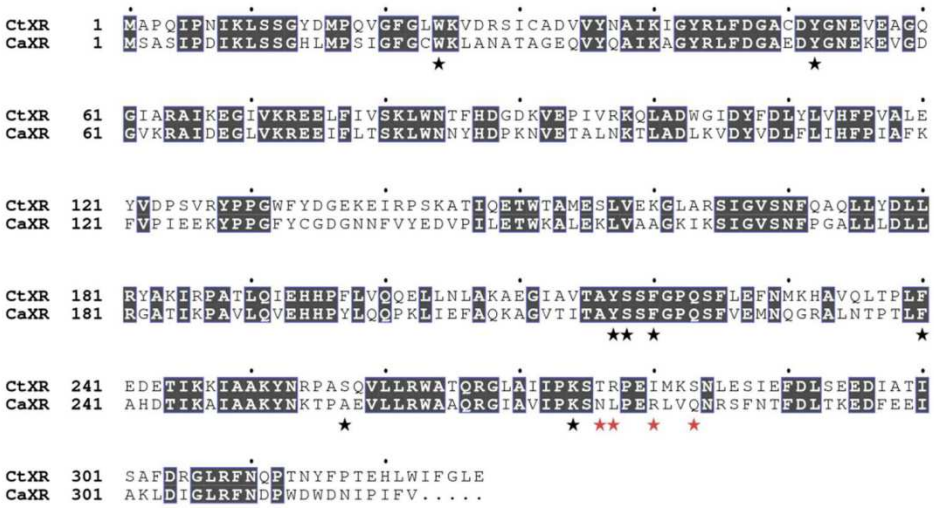

Figure 8. Sequence alignment for *Ct*XR and *Ca*XR (UniProt O74237). Shaded boxes represent identities. Black asterisks are residues highlighted in Figure 9 and red asterisks represent notable amino-acid replacements in the loop involved in binding the adenosine moiety of the cofactor.

Comparing the modeled NAD(H) complex of *Ct*XR (Figure 9A) with the corresponding crystal complex of *Ca*XR (Figure 9B) indicates that the shorter threonine side chain at position 276 would be unable to provide stabilization of the adenosine ribose O2B atom to the same extent as Asn276 in *Ca*XR. Furthermore, the cation-π-stacking interaction of the Arg280 guanido group with the pyrimidine ring of the adenine-purine system in *Ca*XR is abolished by the isoleucine side chain in *CtXR*. The effects on NAD(H) binding due to the replacements of Leu277 and Gln280 by Arg and Ser in *Ct*XR, respectively, are less obvious from the homology model.

The model of *Ct*XR with bound NADP(H) was based on details of NADP(H) binding in the crystal structure of pig aldehyde reductase *Ss*ADH (PDB code 1HQT). While *Ct*XR and *Ss*ADH display more pronounced overall differences at the sequence level, the cofactor-binding site appears sufficiently similar to justify using *Ss*ADH as template for prediction of NADP(H) binding in *Ct*XR. In *Ct*XR, the ribose 2′-phosphate group of NADP(H) would receive stabilization through the donation of a hydrogen bond from Thr276 Og1 and ionic interactions with the guanido group of Arg277, and possibly Lys274 depending on its precise side-chain conformation (Figure 9C). The overall number of interactions provided for NAD(H) and NADP(H) would appear to favor NADP(H) over NAD(H) binding (Figure 9D). However, we did not observe this preference in our biochemical data (Table 2).

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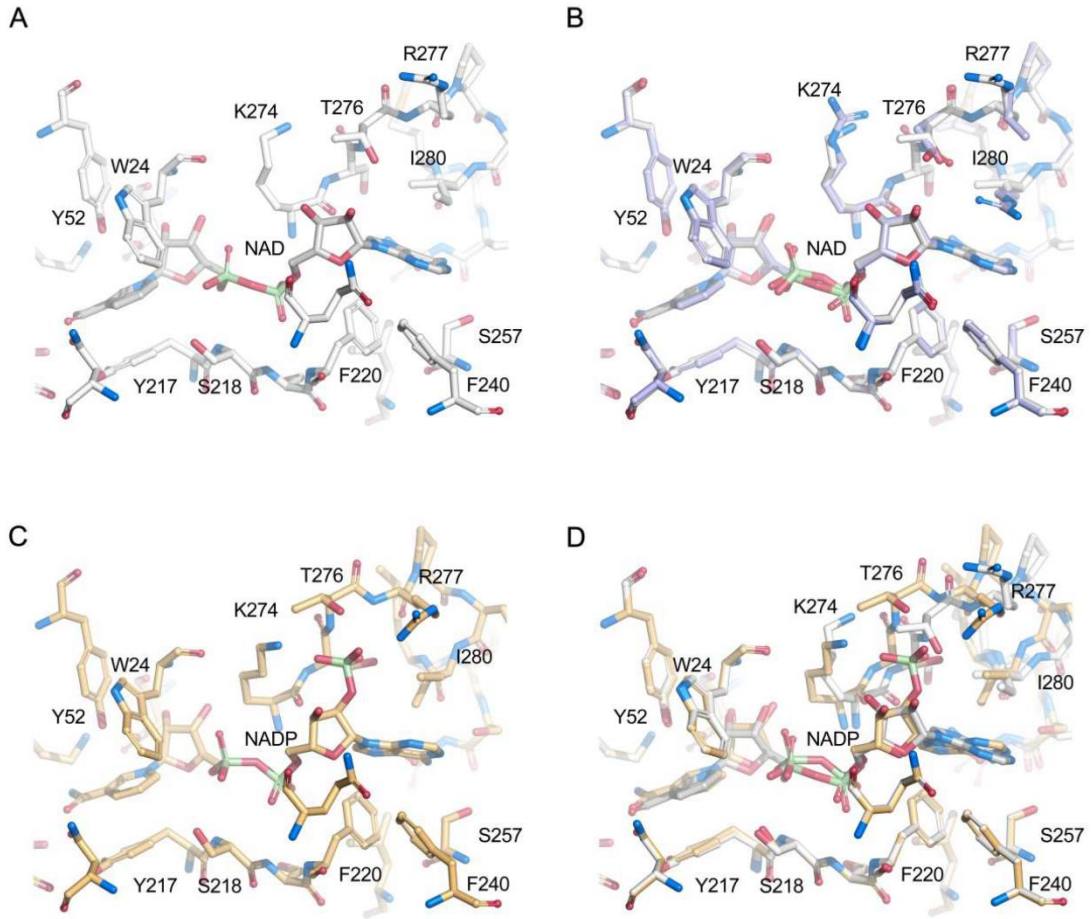

Figure 9. Representation of the modeled cofactor-binding site in *Ct*XR. (**A**) Details of the *Ct*XR-NAD homology model (gray). (**B**) Overlay of the *Ct*XR homology model (gray) and the *Ca*XR crystal structure (light blue). (**C**) Details of the *Ct*XR-NADP homology model (orange). (**D**) Overlay of the *Ct*XR-NAD (gray) and *Ct*XR-NADP (orange) homology models. All side-chain notations are according to the *Ct*XR sequence.

2.5. Comparison with Xylose Reductases in the Literature

Comparison with other XRs in the literature shows that k_{cat} and also $k_{cat}/K_{M,\,(Xylose)}$ of *Ct*XR are about average, whereas $K_{M,\,(Xylose)}$ is the lowest of the reported XRs (Table 6). The pH optimum of 6.0 to 7.0 is similar to other XRs (generally between pH 5.5–6.5). Contrary to what could be expected from the thermophilicity of its natural host, the temperature stability of *Ct*XR is comparatively low and more thermostable XRs have been described (e.g., from *N. crassa* [24] or *C. tropicalis* [29]). Like many other XRs, the enzyme shows a preference for NADPH, nevertheless dual cofactor specificity can be observed: k_{cat}/K_M is approximately 3.2-times higher when NADPH is used as cofactor instead of NADH. A ratio of 13.6 can be found for *N. crassa* [24], while an XR from *C. tenuis* shows a ratio of 1.4 [4,6] or 1.8 [28] and an XR from *Candida parapsilosis* was even reported to strongly favor NADH over NADPH as cofactor with a $k_{cat}/K_{M,\,(Xylose)}$ ratio of 0.013 [8].

Table 6. Comparison of xylose reductases from various hosts: If possible, missing data were complemented with calculation based on the accessible values. In case of publication of multiple values for identical kinetic parameters, the mean value is shown in the table.

Organism and Source	Cofactor	k_{cat} (s^{-1})	$K_{M,(Xylose)}$ (mM)	$k_{cat}/K_{M,(Xylose)}$ ($mM^{-1}\cdot min^{-1}$)	$K_{M,(Cofactor)}$ (μM)	$k_{cat}/K_{M,(Cofactor)}$ ($\mu M^{-1}\cdot min^{-1}$)	Assay Conditions		Optimum		Temp. Stability
							Temp. (°C)	pH	Temp. (°C)	pH	
Chaetomium thermophilum (this work)	NADPH	35.2	25.4	83			55	6.5	55	6.5	No loss of activity after 3 h at 30 °C $T_{1/2}$ = 1.8 min (55 °C)
	NADH	3.2			119	1.6	30				
Neurospora crassa [24]	NADPH	60	34	106	1.8	2000	25	6.3	45-55	5.5	$T_{1/2}$ = 49 min (40 °C)
	NADH	5.2	37	8.4	16	19.5					
Candida tenuis [4,6]	NADPH	21.7	72	18.1	4.8	271	25	7.0	50	6.0	Start of decrease at 30-35 °C
	NADH	18.2	87	12.6	25.4	43					
Candida tropicalis [30]	NADPH	49	30	99	18	329	RT	7.0	-	6.0	Complete loss after 1 h at 60 °C
Candida intermedia [5]	NADH	16.5	30	31.8	10	101	25	7.0	-	-	-
Pichia stipitis [7]	NADPH	25.2	42	36	9	167	30	6.0	-	6.0	-
Candida parapsilosis [8]	NADPH	4.6	244	1.14	36.5	7.6	37	6.0	-	6.0	$T_{1/2}$ = 4.5 h (45 °C); 2 min (50 °C)
	NADH	49	32	87.6	3.3	939					
Candida tenuis [28]	NADPH	13	96	8.1	3	260	25	7.0	-	-	-
	NADH	11	142	4.6	38	17					
Candida tropicalis [29]	NADPH	240	31.5	457	45.5	316	45	5.5	45	5.5	$T_{1/2}$ = 15 min (60 °C)
	NADH	-	-	-	162	-					

RT room temperature; - not determined.

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4. Materials and Methods

4.1. Materials and Reagents

NADH, as disodium salt hydrate, L-xylose and Bradford reagent were ordered from Sigma-Aldrich (St. Louis, MO, USA); D-lyxose from Carbosynth (San Diego, CA, USA); D-arabinose from TCI Europe (Antwerp, Belgium); NADPH, as tetrasodium salt, and all other chemicals were ordered from Carl Roth (Karlsruhe, Germany).

4.2. Construction of the Expression Plasmid

The XR gene was identified in the genome of *C. thermophilum* DSM 1495 by homology search and the construct was ordered from genscript (Piscataway, NJ, USA), codon optimized for *Escherichia coli* (wild type and codon optimized nucleotide sequences as well as the amino acid sequence of *Ct*XR can be found as Supplementary Material). The open reading frame was amplified and restriction sites. NcoI and XhoI were added by polymerase chain reaction on the 5′ and 3′ end, respectively. For the construction of the expression plasmid, the amplicon was integrated in a pET28a vector containing a C-terminal hexahistidin (His6) tag. The expression plasmid was transformed into chemically competent cells *E. coli* BL21(DE3) using a heat shock protocol: 2 µL of plasmid (160 ng/µL) were added to 50 µL cell suspension. After 30 min incubation on ice, heat shock was performed at 42 °C for 45 s. Cells were transferred back on ice for 2 min and subsequently diluted with 950 µL SOC medium. After 1 h at 37 °C cells were plated on LB-agar plates containing 100 mg/L ampicillin for picking transformants after overnight incubation at 37 °C (10, 50 and 200 µL per plate). Correct insertion and integration were verified by sequencing (Microsynth, Balgach, Switzerland).

4.3. Protein Production

The protein was produced via fed-batch fermentation in a 15 L Sartorius Cplus bioreactor with 8 L working volume (Satorius, Göttingen, Germany). The preculture was grown in a shake flask for 20 h at 37 °C and 230 rpm in 500 mL of a medium according to DeLisa et al. [31] containing 8 g/L glucose and 100 mg/L ampicillin, adjusted to pH 7.2. The preculture was transferred aseptically to the culture vessel yielding a total starting volume for the batch phase of 5 L, containing 20 g/L glucose and 100 mg/L ampicillin. Batch phase was performed at 37 °C, followed by an uninduced fed-batch phase of 14 hours (exponential feed, $\mu = 0.1\ h^{-1}$) to increase cell density to 35 g/L DCW and finally an induced fed-batch phase (exponential feed, $\mu = 0.04\ h^{-1}$), performed at 25 °C to increase protein solubility. Production of the recombinant protein was induced by aseptic addition of 0.1 mM IPTG (final concentration).

Cells were harvested by centrifugation (4500× *g*, 4 °C, 30 min) 14 h after induction at a final cell density of approximately 62 g/L DCW. The cell pellet was stored at −20 °C for later use.

Dissolved oxygen (dO_2) was measured with a dissolved oxygen electrode Visiferm DO425 (Hamilton, Reno, NV, USA) and dO_2 levels were maintained above 30% by aerating with 7.5 L/min pressurized air. Air was substituted with pure oxygen if necessary. pH was monitored with an Easyferm electrode (Hamilton) and kept at 7.2 by addition of NH_4OH (12.5% v/v) via the pump module of the bioreactor. Feed was supplied via a Preciflow peristaltic pump (Lambda, Switzerland) following a feed-forward controlled exponential feeding strategy. Mixing was performed at 1400 rpm. CO_2 and O_2 content in the offgas were analyzed with a DASGIP GA, (Eppendorf, Hamburg, Germany). Process parameters were adjusted and recorded via the process information management system Lucullus (Securecell, Schlieren, Switzerland).

4.4. Protein Purification

230 g frozen biomass were thawed and resuspended in a loading buffer (50 mM bis-tris, 30 mM imidazole, 5% *w/v* glycerol, pH 6.5) to a total volume of 500 mL. Cell disruption was carried out with a homogenizer (4 passages, 1500 bar; PandaPLUS 2000, GEA Mechanical Equipment, Parma, Italy).

Cell debris was removed via tangential flow filtration (0.2 µL cutoff, max. transmembrane pressure 1.5 bar, max. flow rate 0.9 mL/min), resulting in 400 mL cell free crude extract.

For protein purification an Äkta Pure25 (GE Healthcare, Solingen, Germany) was used. IMAC was used as the first purification step, followed by size exclusion chromatography. 365 mL crude extract was loaded on a His Trap FF Crude column (5 mL column volume (CV); GE Healthcare) with a flow rate of 15 cm/h. After washing with 2 CVs with loading buffer, elution was carried out with a linear gradient over 6 CVs (0–100% elution buffer; 50 mM bis-tris, 500 mM imidazole, 5% *w*/*v* glycerol, pH 6.5). Protein concentration was monitored in the flowthrough at 280 nm and the target protein was collected manually in a single fraction of 8 mL.

After concentration to a volume of 2 mL with centrifugal filters with a 10 kDa cut off (Amicon Ultra 15, Merck, Darmstadt, Germany), the protein was purified via size exclusion chromatography (SEC). 2 mL protein solution were loaded on a high load superdex 75 pg column (120 mL CV; GE Healthcare, Germany) with a flow rate of 30 cm/h (running buffer: 25 mM tris-HCl, 150 mM NaCl, 5% *w*/*v* glycerol, pH 7.4). Fractions of 1 mL were collected.

Protein purity was assessed with SDS-PAGE. Samples of all stages of the purification process were diluted appropriately. After dilution with 2x Laemmli buffer samples were incubated at 95 °C for 5 min. 10 µL of each sample and 4 µL SeeBlue Plus 2 protein standard (Thermo Scientific, Waltham, MA, USA) were loaded onto 4–15% Mini-PROTEAN TGX precast gels (Bio-Rad, Hercules, CA, USA). Gels were run for 45 min at 160 V and stained with Coomassie Sensitive stain (50 g/L aluminum sulfate (14–18 hydrate), 100 mL/L ethanol, 23.5 mL/L orthophosphoric acid, 0.2 g/L Coomassie blue G250) over night, washed with water and analyzed with Gel Doc XR system and ImageLab software (Bio-Rad, Hercules, CA, USA).

4.5. Data Analysis

For evaluation of purification steps, enzyme activity (U/mL) and protein concentration (mg/mL) in crude extract, IMAC eluate, spin filter retentate and collected SEC fraction were determined and the respective specific activities (U/mg) were determined. By comparing the specific activity of the crude extract to those of the fractions the purification factor (PF) was calculated Equation (1).

$$Purification\ factor\ (PF) = \frac{specific\ activity_{fraction}}{specific\ activity_{crude\ extract}} \quad (1)$$

To quantify the loss of target protein during the purification, the recovery of the catalytic activity was calculated according to Equation (2).

$$Recovery\ (\%) = \frac{total\ activity_{fraction}}{total\ activity_{crude\ extract}} \times 100 \quad (2)$$

4.6. Protein Quantification

Protein concentration was measured photometrically (Genesys 20, Thermo Scientific) using the Bradford Coomassie Blue assay with bovine serum albumin as standard. When necessary, samples were diluted with their respective buffers.

4.7. Biochemical Characterization

4.7.1. Enzyme Activity

Enzyme activity, proportional to the conversion of the cofactors NADPH or NADH, was determined photometrically following the decrease of absorption at 340 nm (point of max. absorption of NADPH and NADH; ε_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$) in duplicates. The activity was calculated according to Equations 3 and 4 (volumetric and specific activity, respectively). The mean specific activity was plotted against the substrate concentration (7 to 8 data pairs) and by fitting a Michaelis-Menten curve to the data points, V_{max} and K_M were determined using the program

SigmaPlot 12.5 (Systat Software, San Jose, CA, USA). The standard errors for K_M and k_{cat} specified in the Results section were calculated per default, with SigmaPlot describing the quality of the fit. k_{cat} and the catalytic efficiency (k_{cat}/K_M) were calculated according to Equations 5 and 6. For the calculation of the confidence interval for k_{cat}/K_M both the errors from k_{cat} and K_M were considered using the formula for the propagation of uncertainty, as shown in Equation (7).

$$\text{volumetric activity} = \frac{\Delta\text{Abs} \cdot V_{\text{total}}}{\varepsilon \cdot V_{\text{enzyme}} \cdot d} \quad (3)$$

$$\text{specific activity} = \frac{\text{volumetric activity}}{c_{\text{enzyme}}} \quad (4)$$

$$k_{cat} = V_{max} \cdot M_{\text{enzyme}} \quad (5)$$

$$\text{catalytic efficiency} = \frac{k_{cat}}{K_M} \quad (6)$$

$$\sigma_{catalytic\ efficiency} = \sqrt{\left(\frac{1}{K_M}\right)^2 \cdot SE_{k_{cat}}{}^2 + \left(-\frac{k_{cat}}{K_M{}^2}\right)^2 \cdot SE_{K_M}{}^2} \quad (7)$$

ΔAbs: Change in absorption (min^{-1}); V_{total}: Assay volume (mL); ε: Extinction coefficient of NAD(P)H at 340 nm (6.22 $L \cdot mmol_{NAD(P)H}^{-1} \cdot cm^{-1}$); V_{enzyme}: Volume of enzyme solution (mL_{XR}); d: Path length (1 cm); Volumetric activity: Activity per mL of enzyme solution ($\mu mol_{NAD(P)H} \cdot min^{-1} \cdot mL_{XR}^{-1}$ = U/mL); c_{enzyme}: Concentration of enzyme solution (mg_{XR}/mL_{XR}); Specific activity: Activity per mg of enzyme ($\mu mol_{NAD(P)H} \cdot min^{-1} \cdot mg_{XR}^{-1}$ = U/mg); V_{max}: Maximum enzyme activity ($mol_{NAD(P)H} \cdot s^{-1} \cdot mg_{XR}^{-1}$); M_{enzyme}: Molecular mass of enzyme (39,244 Da); k_{cat}: Turnover number ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot s^{-1}$); K_M: Apparent affinity constant ($M_{substrate}$); k_{cat}/K_M: Catalytic efficiency ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot M_{substrate}^{-1} \cdot s^{-1}$); $\sigma_{catalytic\ efficiency}$: confidence interval of k_{cat}/K_M ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot M_{substrate}^{-1} \cdot s^{-1}$); SE: standard error (of k_{cat} ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot s^{-1}$) or K_M ($M_{substrate}$)).

The purified enzyme was incubated in 50 mM bis-tris buffer pH 6.5 containing different amounts of cofactor and D-xylose or alternative substrates and analyzed in a thermoregulated photometer (V-630 Spectrophotometer, Jasco, Pfungstadt, Germany). Depending on the investigated cofactor and substrate the enzyme concentration in the assay ranged from 0.564 to 45.2 µg/mL. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol NAD(P)H/min. The kinetic parameters K_M, k_{cat} and catalytic efficiency were determined at 30 and at 55 °C (the highest temperature at which a characterization was justified due to the enzyme´s moderate thermostability). D-Xylose (0.001–0.5 M) was used as substrate and NADPH as cofactor (0.4 mM). To ensure that the change in absorption was not caused by thermal degradation of NAD(P)H, activity assays containing no *Ct*XR were performed as blanks. In accordance with the literature [32], thermal cofactor degradation was insignificant under assay conditions compared to enzymatic activity.

Activity of samples derived during protein purification was measured at 30 °C in 50 mM bis-tris buffer pH 6.5 containing 0.5 M D-xylose and 0.4 mM NADPH.

4.7.2. pH Optimum

pH range was determined by measuring the enzymatic activity at 30 °C in a citric acid-disodium phosphate buffer (McIlvaine buffer) [33] (pH 2.4–8.1) and 50 mM glycine buffer (pH 8.5–10.0). NADPH (0.4 mM) was used as cofactor and D-xylose (0.5 M) as substrate. Measurements were performed in triplicates. Additionally, the pH optimum was determined in 50 mM citrate and phosphate buffers.

4.7.3. Cofactor Preference

Kinetic parameters K_M, k_{cat} and catalytic efficiency were determined for the cofactors NADPH (0.01–0.6 mM) and NADH (0.01–0.8 mM) in a 50 mM bis-tris buffer, pH 6.5, at 65 °C.

4.7.4. Substrate Specificity

We determined relative activities for the substrates D-xylose, L-xylose, D-arabinose, L-arabinose, D-ribose, D-lyxose, D-glucose, D-galactose and D-mannose. Measurements for the determination of the relative activity were performed in triplicates using 0.5 M substrate concentration and 0.4 mM NADPH as cofactor in a 50 mM bis-tris buffer, pH 6.5, at 30 °C. Additionally, kinetic parameters K_M, k_{cat} and catalytic efficiency were determined for the substrates D-xylose, L-arabinose, D-galactose and D-glucose (each 0.001–0.5 M) using NADPH as cofactor (0.4 mM).

4.7.5. Temperature Stability

The enzyme solution (0.2825 mg/mL; without substrate or cofactor) was incubated at 30, 55, 65 and 75 °C. At different time points aliquots were drawn and the catalytic activity was immediately determined at 30 °C. NADPH (0.4 mM) was used as cofactor and D-xylose (0.5 M) as substrate. For the calculation of the temperature stability of the enzyme, data acquired during the first ~15 s of incubation were omitted, since this was the time required to fully heat the test tubes (including 0.5 mL sample volume) on the thermoblock. The half-life ($T_{1/2}$) of *Ct*XR was determined at 30, 55, 65 and 75 °C according to Equation (8).

$$T_{1/2} = -\frac{ln(2)}{\lambda} \quad (8)$$

λ: Decay rate (min^{-1}); $T_{1/2}$: Half-life (min)

4.8. Homology Modeling

An initial homology model of *Ct*XR was produced using the SWISS-MODEL server (https://swissmodel.expasy.org) [26] that was further optimized manually using *COOT* (version 0.8.9.1) [34]. Based on the optimized model, homology models of *Ct*XR in complex with either NAD or NADP were generated manually by careful inspection of NAD binding in xylose reductase from *Candida tenuis* (*Ca*XR; PDB code 1MI3, [35]; 1SM9, [28]) and NADP binding in aldehyde reductase from *Sus scrofa* (*Ss*ADH; PDB code 1HQT; [35]).

5. Conclusions

The particular xylose reductase from *C. thermophilum* was chosen for this study, since we were looking for a thermostable enzyme for the conversion of D-xylose to xylitol for the utilization in high temperature processes operating at around 75 °C. The enzyme exhibits dual cofactor specificity for NADPH (K_M = 35.8 ± 7.6 mM) and NADH (K_M = 121 ± 12 mM), has reduced activity on L-arabinose and residual activity on D-galactose and D-glucose, and optimal activity between pH 6.0 and 7.0. Unfortunately, with a half-life of only several seconds at 75 °C, the investigated *Ct*XR cannot be used in the desired high temperature environments. Under thermophilic conditions XRs from *N. crassa and C. tropicalis*, described by Woodyer et al. [24] and Zhang et a. [29], respectively, have similar or even higher k_{cat}/K_M values compared to *Ct*XR and can potentially be deployed in more thermophilic processes due to higher stability.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/1422-0067/20/1/185/s1.

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Abbreviations

XR	xylose reductase
His6	hexahistidine
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NADH	nicotinamide adenine dinucleotide (reduced)
PDB	Protein Data Bank
EC	Enzyme Commission (number)
NAD^+	nicotinamide adenine dinucleotide (oxidized)
*Ct*XR	xylose reductase from *Chaetomium thermophilum*
DCW	dry cell weight (g/L)
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
IMAC	immobilized metal affinity chromatography
SEC	size exclusion chromatography
V_{max}	maximum enzyme activity ($mol_{NAD(P)H} \cdot s^{-1} \cdot mg_{XR}^{-1}$)
k_{cat}	turnover number ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot s^{-1}$)
K_M	substrate affinity ($M_{substrate}$)
k_{cat}/K_M	catalytic efficiency ($mol_{NAD(P)H} \cdot mol_{XR}^{-1} \cdot M_{Substrate.}^{-1 \cdot s-1}$)
SE	standard error
*Ca*XR	xylose reductase from *Candida tenuis*
GMQE	global model quality estimation
*Ss*ADH	aldehyde reductase from *Sus scrofa*
CV	column volume (mL)
NAD(H)	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate

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Heterologous expression of a xylose reductase in *Sulfolobus acidocaldarius*

Contribution to Chapter 5

(Monographic chapter)

Recombinant protein production and whole-cell catalysis:

Taking up the challenge of microbial production of xylitol using a recombinant strain of *Sulfolobus acidocaldarius*

Goal

The gene of a characterized xylose reductase from the filamentous fungus *Chaetomium thermophilum* (Gene number: XP_006696018) should be integrated in *Sulfolobus acidocaldarius* via an *E. coli-S. acidocaldarius* shuttle vector. After expression of the xylose reductase gene the heterologous host should be able to intercellularily convert xylose to xylitol as whole-cell biocatalyst at acidic pH and elevated temperature.

Background

Xylitol is an important C5-sugar alcohol which is in strong demand for applications in food, pharmaceutics, cosmetics and dental care. For the year 2017 its market value was estimated to account for over USD 860 million with an expected annual growth rate of 7.44 % (Mordor Intelligence, 2018). However, the conventional, chemical production of xylitol via Raney-nickel catalysts is expensive and comes with a high energy demand due to high temperatures and pressure required for the catalytic step (Mikkola et al., 1999). The biotechnological production of xylitol is a widely favored alternative as it is considered to be more environmentally friendly due to its milder reaction conditions. The environmental aspect of xylitol production has to be valued since the sugar substitute is partly marketed in the food market segment where targeted consumers are sensitive towards the origin of their products. On an overall scale, economics and production costs are the most important factors for any xylitol production process to prevail. A significant cost factor in the conventional xylitol production is the necessity of H_2, a fossil fuel based reducing agent, that demands high pressure and/or organic solvents to reach required solubility and overcome mass transfer limitations (Mikkola et al., 2000). A similar problem is encountered in the cell free, enzymatic xylitol production since cofactor recycling has to be carried out employing a second set of enzyme and substrate to acquire the reducing power. A task that is by no means trivial. On the other hand, during microbial production of xylitol the required reduction equivalents are directly supplied by the biocatalyst. In addition to the benefit of cofactor replenishment, lower purity requirements regarding the feedstock represent an advantage of the microbial production compared to chemical catalysts. This catalytic robustness enables the use of cheap and sustainable hemicellulosic hydrolysates as substrates derived from industrial waste streams (e.g. from the pulp and paper industry) without the need of substrate purification (Fehér et al., 2018).

In order to efficiently utilize hemicellulose in microbial fermentation processes and to achieve high yields of fermentable sugars, the feedstock has to be subjected to a pretreatment step at low pH values and/or high temperature (Mosier et al., 2005). A way to safe costs of

subsequent neutralization and cooling steps is to utilize extremophilic microorganisms as production hosts (Zeldes et al., 2015). Not many organisms can fulfill the temperature and pH requirements, but the thermo-acidophilic Archaeon *S. acidocaldarius*, growing optimally at 75 °C and pH 3, is one of them and due to its genetic amenability a highly promising candidate for the task of microbial xylitol production.

Although *S. acidocaldarius* is optimally adapted to the aspired process conditions, the conversion step from xylose to xylitol cannot be performed with a wild-type strain since xylose reductase, the enzyme necessary for the conversion, naturally only occurs in Eukaryotes, for example in fungi. While Bacteria employ a xylose isomerase to feed xylose into their metabolism, Archaea pursue an oxidative pathway via a dehydrogenase to metabolize xylose and other pentoses (Karhumaa et al., 2007; Nunn et al., 2010). This implies the need for genetic manipulation of the thermo-acidophilic host *S. acidocaldarius* for the introduction of a heterologous xylose reductase preferentially derived from a thermophilic fungal organism.

We decided to take on this challenge by introducing the gene of a characterized xylose reductase (Quehenberger et al., 2019) into xylose negative *S. acidocaldarius* mutants (harboring a gene knock-out in downstream steps of the xylose degradation pathway) with the ultimate goal to convert xylose into xylitol via whole-cell catalysis.

Material and Methods

Xylose reductase gene and enzyme

The xylose reductase gene XP_006696018 from *Chaetomium thermophilum* (from here on abbreviated as *ctXR*) was codon optimized for *S. acidocaldarius*, purchased from Genscript (USA). The respective enzyme (from here on abbreviated as *Ct*XR) was characterized elsewhere (Quehenberger et al., 2019).

Strains and plasmids

Used *S. acidocaldarius* strains and expression plasmids are described in Table 1.

Table 1: Strains of *S. acidocaldarius* and expression plasmids used in this chapter.

Strain	Description
S. acidocaldarius DSM 639	parental wild-type strain
S. acidocaldarius MW001	Δ*pyrE* mutant with dysfunctional uracil synthesis for the generation of knock-out mutants, efficient plasmid uptake and maintenance using a uracil/5-FOA selection system (Wagner et al., 2012)
S. acidocaldarius MW001 Δ1079	knock-out of the promiscuous glucose dehydrogenase, isoenzyme 1 (GDH-1|Saci_1079; Wagner et al., 2018)
S. acidocaldarius MW001 Δ0885	knock-out of xylonate/arabinoate dehydratase (D-XAD/ L-AraD|Saci_0885; Wagner et al., 2018)

S. acidocaldarius MW001 Δ1939	knock-out of, 2-keto-3-deoxy-xylonate/arabinoate dehydratase (D-KDXD/L-KDAD| Saci_1939; Wagner et al., 2018)
S. acidocaldarius MW2000	Δ*pyrE* mutant with dysfunctional uracil synthesis for the generation of knock-out mutants, efficient plasmid uptake and maintenance using a uracil/5-FOA selection system with improved growth on glucose (unpublished)

Plasmid	Description
pSVAaraFX cH6 +XR	Expression plasmid with xylose and arabinose inducible promotor, multiple cloning site, C-terminal 6-His tag, *pyrEF* cassette, *amp* resistance, *Sulfolobus* and *Escherichia coli ori* (Wagner et al., 2018)
pSVAaraFX nH6 +XR	Expression plasmid with xylose and arabinose inducible promotor, multiple cloning site, N-terminal 6-His tag, *pyrEF* cassette, *amp* resistance, *Sulfolobus* and *E. coli ori* (Wagner et al., 2018)
pSVAmZ-SH10+XR	Expression plasmid with maltose inducible promotor, multiple cloning site, C-terminal 10-His tag, *pyrEF* cassette, *amp* resistance, *Sulfolobus* and *E. coli ori* (Wagner et al., 2014)

Growth conditions

Unless stated otherwise *S. acidocaldarius* strains were grown aerobically at 75 °C in a reciprocal shaking oil bath at 100 rpm in 100 mL long neck Erlenmeyer flasks to avoid evaporation. The flasks were filled with 50 mL culture medium as described elsewhere (Brock et al., 1972). The initial pH was adjusted to 3.0 with 4.8 w% H_2SO_4 and unless stated otherwise 1 g/L glucose and 1 g/L NZ-amine were used as carbon source.

Transformation of *S. acidocaldarius* strains

Preparation of competent cells and transformation was performed as described by Wagner et al. (2012).

Analytical methods

Monitoring of microbial growth: Growth of *S. acidocaldarius* was monitored with a photometer (Genesys 20 photometer (Thermo Scientific, USA) via determination of the optical density at 600 nm.

Determination of xylose and xylitol: Supernatant for determination of xylose and xylitol concentrations was prepared by centrifugation (12 000 rcf, 10 min, 4 °C). Samples for the determination of cytosolic xylitol were prepared by homogenization of 10 mL fermentation broth with a ultrasound probe (6×1 minute with 30 sec cooling periods between pulses, duty cycle 50%, output control 7; Branson Sonifier 250, Thermo Scientific, USA) followed by centrifugation (12 000 rcf, 10 min, 4 °C). Detection of xylose and xylitol in the supernatants

was performed with HPLC equipped with a refraction index detector (Agilent G1362A, USA) using an Aminex HPX-87H column (300x7.8 mm) at 60 °C. 4 mM H_2SO_4 was used as eluent with an isocratic flow of 0.6 mL/min for 30 min.

Detection of the *Ct*XR gene: The presence of *ctXR* and its correct insertion in the expression vectors was investigated by sequencing of plasmid preparations (prepared with Monarch Gel extraction kit according to the manufacturer (NEB, USA)) of the transformed *S. acidocaldarius* strains by the company Microsynth (Switzerland) and subsequent sequence analysis.

Detection of *ctXR* cDNA: RNA was isolated with Trizol (Thermo Scientific, USA) as described elsewhere (Hottes et al., 2004). The obtained RNA samples were treated with RNase-free DNase (Qiagen) according to the protocol and afterwards purified via ethanol precipitation. Total RNA was routinely checked using an Agilent RNA 6000 Nano Kit on a Bioanalyzer (Agilent, Germany). Afterwards, ribosomal RNA was depleted using a RiboZero magnetic kit for bacteria (Illumina, USA) with a modified protocol. Only 90 µl instead of 225 µl magnetic beads were used and for the rRNA removal reaction and only 1 µg RNA was mixed with 4 µl removal solution in a total volume of 20 µl instead of 40 µl. Subsequently, reverse transcription was performed using superscript II reverse transcriptase (Thermo Scientific, USA) according to the protocol. Then PCR was performed with Phusion polymerase (Thermo Scientific, USA) using 35 cycles with an annealing temperature of 62 °C (primer 1: 5′-gtaatgaagtagaagcaggacaag-3′; primer 2: 5′-aaaccatccaggtggatatctaac-3′) and amplicons were visualized on a agarose gel. The gene Saci_0685 (elongation factor 1-alpha) was used as positive control (primer 1: 5′-ccagttgacaaaccgttaagg-3′; primer 2: 5′-gctgagtctccagctttaatg-3′).

Detection of *Ct*XR in *S. acidocaldarius*: Western blot analysis was performed using an anti-His-horseradish-peroxidase antibody (Miltenyi Biotec, USA) and SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, USA) according to the manufacturers′ recommendations. Visualization was carried out using a ChemiDoc Imaging System (Bio-Rad, USA).

Results and Discussion

Selection of the expression host

Generation of knock-out mutants of *S. acidocaldarius*, efficient plasmid uptake and maintenance is commonly ensured by using Δ*pyrE* mutants with dysfunctional uracil synthesis that can be complemented via a pyrEF cassette from *Sulfolobus solfataricus* on the introduced plasmid. In this study we used the Δ*pyrE* mutants *S. acidocaldarius* MW001 (Wagner et al., 2012) and MW2000 (from the same research group as MW001, but unpublished). Because *S. acidocaldarius* naturally harbors a xylose catabolic pathway we had to disable this pathway to ensure that after uptake the organism would not metabolize the

supplied xylose. Thereby, draining the flux of educt via *Ct*XR to xylitol by withdrawing all xylose for the use as carbon source. As the xylose degradation pathway of *S. acidocaldarius* is known and well described (Nunn et al., 2010; Wagner et al., 2018) we integrated *Ct*XR expression plasmids in the MW001 and MW2000 parent strains as well as in a total of 3 different knock-outs strains, as described in Table 1, with deletions at three different positions in the xylose degradation pathway (shown in Figure 1) with the aim of investigating the impact of the different genotypes on the production of xylitol.

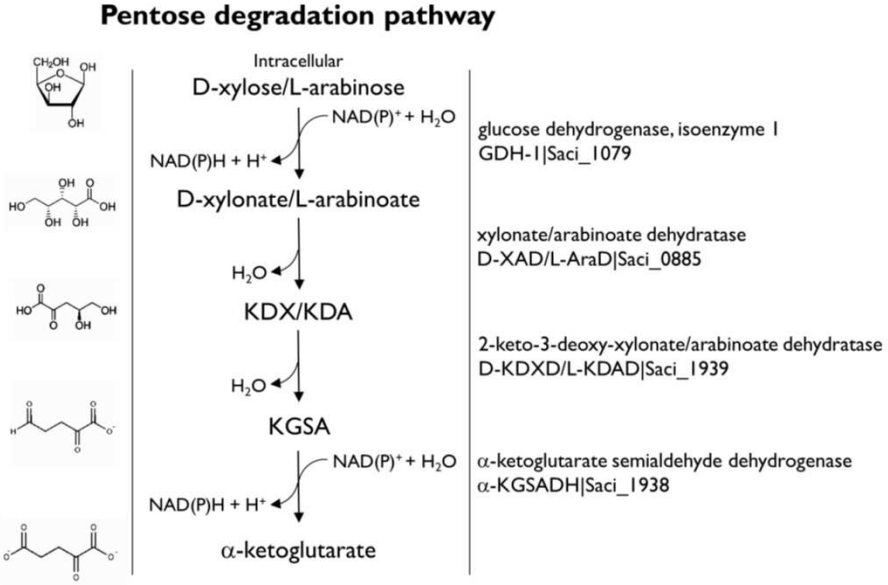

Figure 1: Overview of the pentose degradation pathway in *S. acidocaldarius*. Adapted from (Wagner et al., 2018). Intermediate degradation products: KDX/KDA, 2-keto-3-deoxy-xylonate/arabinoate; KGSA, α-ketoglutarate semialdehyde.

Whole-cell catalysis of xylose to xylitol

Recombinant *S. acidocaldarius* MW001 strains carrying the *Ct*XR gene were cultivated on a medium containing 1 g/L NZ-amine, 1 g/L glucose. Induction was started simultaneously with addition of 2 g/L xylose as educt during the early exponential phase (OD600 ~0.25) by using the 2 g/L xylose (in case of plasmids pSVAaraFX cH6 +XR and pSVAaraFX nH6 +XR) or 3 g/L maltose (in case of plasmid pSVAmz-SH10+XR) as inducers. Although a large set of combination of strains, expression plasmids and cultivation conditions was investigated (Table 2), in neither experiment xylitol could be detected in the culture supernatant or in the cytosol using HPLC. Consequently we investigated all steps on the way from the gene *ctXR* to the product *Ct*XR in order to determine the cause for the failed xylitol production.

Table 2: Investigated combination of *S. acidocaldarius* (Saci) strains, expression plasmids and expression conditions, as well as sampling regime.

Host	Plasmid	Inducer	Expression Temperature	Sampling regime
Saci MW001	pSVAaraFX cT +XR	xylose	75 °C	periodically until 48 h after induction
		arabinose	65 °C	40 h after induction
	pSVAmz SH10 +XR	maltose	75 °C	periodically until 48 h after induction
		maltose	65 °C	periodically until 48 h after induction
Saci MW001 Δ0885	pSVAaraFX cT +XR	xylose	75 °C	periodically until 48 h after induction
Saci MW001 Δ1979	pSVAaraFX nT +XR	xylose	75 °C	periodically until 48 h after induction
Saci MW001 Δ1939	pSVAmz SH10 +XR	maltose	75 °C	periodically until 48 h after induction
		maltose	65 °C	40 h after induction
		maltose	55 °C	40 h after induction
Saci MW2000	pSVAaraFX cT +XR	xylose	75 °C	periodically until 48 h after induction
	pSVAmz SH10 +XR	maltose	75 °C	periodically until 48 h after induction
		maltose	65 °C	40 h after induction

Transformation and plasmid integrity

All investigated strains were cultivated on plates and liquid media without the addition of uracil meaning that the uracil auxotrophy of the MW001 or MW2000 parent strains was successfully complemented via the introduced plasmid. The correct and complete insertion of *ctXR* in the transformed vectors was confirmed via plasmid preparation and subsequent sequencing with primers binding in the respective upstream and downstream regions on the plasmid. No mutations were present and the gene was correctly inserted.

Transcript analysis

PCR with *ctXR* specific primers using generated cDNA as template yielded a band of expected length on the agarose gel. Likewise, PCR with primers specific for Saci_0685, serving as positive control, yielded a band of expected length. Both PCR reactions also yielded the respective products when DNA was used as template. No band was visible when the isolated RNA (before reverse transcriptase treatment) was used as template (negative control). These results are evidence for the correct transcription of the gene and the presence of corresponding mRNA.

Western blot analysis

Staining with an anti-His-horseradish-peroxidase antibody revealed no *Ct*XR in *S. acidocaldarius* strains, while purified *Ct*XR produced in *Escherichia coli* yielded a positive band, revealing that either translation or maturation of the *Ct*XR are the defective steps that prevent the conversion of xylose to xylitol.

Conclusion

We could show that integration and transcription of the *Ct*XR gene was successful in the investigated strains. Nevertheless, translation and protein folding or maturation of the heterologous, eukaryotic protein proved to be very challenging even when the temperature during induction was lowered to 55 °C. Although numerous *Sulfolobus* genes have been successfully expressed in heterologous hosts, mainly in Bacteria (Lin et al., 1992; Manabe et al., 2009; Pisani et al., 1990 ect.) but also in Eukaryotes like *Saccharomyces cerevisiae* or *Pichia pastoris* (Han et al., 2017; Moracci et al., 1992), and though numerous heterologous archaeal genes have been expressed in *Sulfolobus* (Albers et al., 2006; Contursi et al., 2003; Wagner et al., 2012), to my knowledge, no eukaryotic genes have been expressed in *S. acidocaldarius* and described in the literature. This can be taken as hint towards fundamental difficulties in the production of foreign proteins in *S. acidocaldarius* and the here presented results show that the crucial step of translation and protein maturation are worthy to be investigated in much higher detail for the elucidation of the until now unsuccessful production of eukaryotic proteins in *S. acidocaldarius*. With regard to the initially stated problem of biotechnological production of xylitol, I propose engineering of the protein for higher thermostability in order to allow maturation of the protein during process conditions of at least ≥ 55 °C. An alternative option would be using a fungal or eukaryotic expression host with the tradeoff that milder process conditions regarding temperature and pH value have to be employed.

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The influence of growth rate on the lipid composition of *Sulfolobus acidocaldarius* determined with MALDI-MS

Contribution to Chapter 6

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The influence of the specific growth rate on the lipid composition of *Sulfolobus acidocaldarius*

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Abstract

Archaeal lipids are constituted of two isoprenoid chains connected via ether bonds to glycerol in the *sn*-2,3 position. Due to these unique properties archaeal lipids are significantly more stable against high temperature, low pH, oxidation and enzymatic degradation than conventional lipids. Additionally, in members of the phylum Crenarchaeota condensation of two (monopolar) archaeal diether lipids to a single (bipolar) tetraether lipid as well as formation of cyclopentane rings in the isoprenoid core strongly reduce permeability of the crenarchaeal membranes. In this work we show that the Crenarchaeum *Sulfolobus acidocaldarius* changes its lipid composition as reaction to a shift in growth rate caused by nutrient limitation. We thereby identified a novel influencing factor for the lipid composition of *S. acidocaldarius* and were able to determine the effect of this factor on the lipid composition by using MALDI-MS for the quantification of an archaeal lipidome: A shift in the specific growth rate during a controlled continuous cultivation of *S. acidocaldarius* from 0.011 h^{-1} to 0.035 h^{-1} led to a change in the ratio of diether to tetraether lipids from 1:3 to 1:5 and a decrease of the average number of cyclopentane rings from 5.1 to 4.6.

Introduction

Although today Archaea are known to ubiquitously thrive in habitats all over the world, they have long been considered classical model organisms for “extreme” environments. This historic misconception might be a reason for the extensive interest in the archaeal membrane composition from very early on in archaeal research, as the membrane is the main protective barrier of a cell against its surroundings (van de Vossenberg et al., 1998). Indeed, the fundamental difference in membrane architecture and chemistry of membrane lipids between Archaea on the one hand and Bacteria and Eukarya on the other hand is a characteristic feature of distinction and probably the most obvious biochemical trait unique to the archaeal Domain. Archaeal membranes are composed of ether lipids: glycerol-1-

phosphate backbones are linked via ether bonds to two isoprenoid chains (Koga and Morii, 2007; Jain et al., 2014). Two main classes of these ether lipids dominate the archaeal lipid spectrum: polar diether lipids (DELs) commonly containing 20 or 25 carbon atoms per isoprenoid chain, and monolayer forming bipolar tetraether lipids (TELs) commonly containing 40 carbon atoms per isoprenoid chain. The biosynthesis of DELs via the mevalonate pathway is well understood (Jain et al., 2014), while the mechanism of TEL synthesis is not resolved yet. Head-to-head condensation of two DELs and consecutive introduction of cyclic ring structures is the most popular hypothesis (Jain et al., 2014), while also a more complex, stepwise pathway has been proposed (Villanueva et al., 2014). Independent of the actual mechanism, two proteins, Saci_0421 and Saci_1201, responsible for the formation of cyclopentane in *S. acidocaldarius* have been identified by Guan et al. (2017).
Described variations of the two main lipid structures include: (1) differences in head groups – a range of polar compounds and sugar residues were observed as head groups (e.g. phospholipids containing glycerol, serine, ethanolamine, myo-inositol or in case of glycolipids: glucose, galactose, mannose, gulose, N-acetylglucosamine or combinations thereof (Shimada et al., 2008; Oger and Cario, 2013)); (2) chain length of the isoprenoid core (e.g. Matsuno et al., 2009); (3) unsaturation (Dawson et al., 2012); (4) methylation of the isoprenoid chain (Knappy et al., 2009; Yoshinaga et al., 2015); (5) linkage between the isoprenoid chains (Morii et al., 1998); and (6) introduction of ring systems – generally ranging from 1-8 cyclopentane rings (De Rosa and Gambacorta, 1988; Lai et al., 2008; Jensen et al., 2015a), but also the incorporation of a hexane ring has been reported in multiple crenarchaeal species (Damsté et al., 2002, 2018).
In addition to determination of the lipid structures it was discovered, that, just like Eukarya and Bacteria (Sinensky, 1974), Archaea too employ the concept of homeoviscous adaption. This means that their membrane composition is highly flexible and can be modified by the organism in response to a changing environment with the goal to maintain (1) a physiologic state of membrane fluidity and (2) regulate permeability as well as (3) proton and ion gradients across the membrane and (4) lipid-protein interactions (Albers et al., 2000; Oger and Cario, 2013). In Archaea several unusual adaption mechanisms were found, owing to the fundamental differences in membrane architecture and lipid composition. While Bacteria and Eukarya rely on regulation of lipid chain length, changes in the polar head groups or modifications of lipid saturation levels, adaption strategies specific to Archaea also include regulation of the number of cyclopentane moieties in the isoprenoid chains and the adaption of the ratio of mono- to bipolar lipids in the membrane (DEL:TEL ratio, (Kramer and Sauer, 1991)) or a combination of the latter two.

As of now, most studies involving Archaea investigated the effect of growth temperature on the lipid composition. In general, Archaea react to increased temperatures with condensation of two monopolar to one single membrane-spanning bipolar lipid (e.g. Lai et al., 2008; Matsuno et al., 2009). As a further adaption strategy Crenarchaeota incorporate higher numbers of cyclopentane rings in their monopolar lipids (Chong, 2010; Jensen et al., 2015b).

Although no general correlation between the temperature optimum of a Crenarchaeum and the number of cyclopentane rings can be made, it is evident that a rise of the growth temperature stimulates the organisms to increase the number of rings in their lipids. Additionally to the temperature, the influence of hydrostatic pressure, pH and salinity were investigated. Kaneshiro and Clark (1995) reported a decrease of the proportion of TELs by over 20% in *Methanocaldococcus jannaschii* when grown under 50 MPa compared to cells grown at atmospheric pressure. Shimada et al. (2008) observed an increase of the average number of cyclopentane rings in caldarchaeol of *Thermoplasma acidophilum* from 4.0 to 5.1 when pH was increased from 1.2 to 3.0. A change in salinity (15 to 30% w/v) was confirmed to alter the lipid composition of halophilic Archaea by Dawson et al. (2012) causing a change of saturation levels of DELs lipids of various Halobacteriaceae. Clearly, when investigating the adaption of the lipid composition the focus in the scientific literature lies (1) on variations of these external, environmental factors (temperature, hydrostatic pressure, pH and salinity) and (2) on the comparison of exponential growth (log phase) vs. stationary phase which was done by many different groups with different organisms (e.g. Elling et al., 2014; Jensen et al., 2015b; Kramer and Sauer, 1991; Matsuno et al., 2009; Meador et al., 2014; Morii and Koga, 1993). A third factor with supposedly high potential to impact the lipid composition is (3) the level of energy and nutrient supply of the culture. Interestingly, the literature is quite scarce on this topic. A study performed by Manquin et al. (2004), showed that the lipid composition of the methanogen *Methanococcus jannaschii* was influenced by H_2 availability while Yoshinaga et al. (2015) confirmed an effect of H_2 and also reported an influence of potassium and phosphate limitations on the lipid composition of *Methanothermobacter thermautotrophicus*.

A factor that has never been tested for its influence on the lipid composition of Archaea is the *specific growth rate (μ)*. In this study, we cultivated *Sulfolobus acidocaldarius,* a member of the phylum Crenarchaeota and widely popular archaeal model organism, under nutrient limited conditions. We maintained two different substrate uptake rates (q_s), and consequently two distinct μs, by employing different feeding rates during a continuous cultivation. Our goal was to determine the ratio of mono- to bipolar lipids (DEL:TEL ratio) and the number of cyclopentane rings in the isoprenoid lipid core in dependence of μ under controlled nutrient limiting conditions.

Materials and Methods

Cultivation and Bioreactor setup

S. acidocaldarius DSM 639 was cultivated in a 3.6 L glass bioreactor (Labfors 3, Infors, Germany) implemented as continuous stirred-tank reactor (CSTR) with 2 L working volume. The preculture was grown in a 100 mL shake flask at 75 °C and 100 rpm in 50 mL VD-Medium (Quehenberger et al., 2019) containing 1.75 g/L Na-glutamate (MSG), 3 g/L D-glucose and 0.5 g/L citric acid, adjusted to pH 3.0 with sulfuric acid. At an OD_{600} of 0.75 the

preculture was transferred aseptically to the culture vessel yielding an initial OD_{600} of 0.023 and a total starting volume for the initial batch phase of 1.5 L, containing 2 g/L MSG and 1 g/L D-glucose. Batch phase was performed at 75 °C, pH 3.1, followed by a short fed-batch phase to increase the working volume to 2.0 L. After reaching 2 L culture volume the vessel was operated in continuous mode by harvesting at the same rate as medium was being supplied via the substrate feed. To ensure the absence of time effects and to ensure reproducibility of the results, after performing a shift from low to high growth rate (μ = 0.011 to 0.035 h^{-1}) we employed another phase of high dilution rate replicating the conditions of the initial phase (μ = 0.035 h^{-1}). To ensure enough time for the organism to adapt to the different growth rates sampling was performed at least 2 dwell times (~2.5 days and ~8 days, respectively) after changing the dilution rate.

Per sampling point 45 mL aliquots were withdrawn and cells were harvested via centrifugation (14 000 g, 4 °C, 10 min). Sampling points and respective dry cell weights are shown in Table 1. The cell pellet was then stored at -20 °C for later use.

Dissolved oxygen (dO_2) was measured with a dissolved oxygen electrode Visiferm DO425 (Hamilton, USA) and dO_2 levels were maintained above 30% by aerating with up to 0.6 L/min pressurized air. pH was monitored with an Easyferm electrode (Hamilton, USA) and kept at 3.1 by addition of H_2SO_4 (9.6% v/v) via the pump module of the bioreactor. Feed was supplied via a Preciflow peristaltic pump (Lambda, Switzerland) following a feed-forward controlled exponential feeding strategy, while broth was continuously withdrawn via a dip pipe and a Preciflow peristaltic pump (Lambda, Switzerland). Mixing was performed at 350 rpm. CO_2 and O_2 content in the offgas were analyzed with BCP-O2 and BCP-CO2 gas analyzers (Bluesens, Germany). Process parameters were adjusted and recorded via the process information management system Lucullus (Securecell, Switzerland).

Biomass determination

Dry cell weight, DCW [g/L], was determined gravimetrically in triplicates. 5 mL fermentation broth were centrifuged (4000 g, 4°C, 15 min) and subsequently dried at 95 °C for at least 72 h.

Optical density, OD_{600} [ ], was determined photometrically at 600 nm. Samples were diluted with deionized water to stay in the linear range of the photometer (Genesys 20, Thermo Fisher Scientific, USA).

Calculation of dilution rate, specific growth rate and dwell time

Dilution rate, D [1/h], in a CSTR was calculated as the quotient of volume flow [L/h] by the reactor volume [L].

Specific growth rate, μ [h^{-1}], in an equilibrated CSTR can be assumed equal to D. Therefore, during the experimental phases μ was controlled and set to 0.11 and 0.35 h^{-1}, respectively, via the volume flow, which is proportional to the feed rate [g/h], at a constant reactor volume.

Dwell time, τ [h], was calculated as the reciprocal value of the dilution rate D.

Lipid extraction

Frozen cell pellets of 45 mL culture broth per sample (dry cell weight at the time of harvest is shown in Table 1) were thawed and resuspended in 5 mL ammonium acetate (155 mM). After sonication on ice (6×1 minute with 30 sec cooling periods between pulses, duty cycle 50%, output control 7; Branson Sonifier 250, Thermo Fisher Scientific, USA) 20 mL of a chloroform/methanol solution (2:1) were added. Lipids were extracted via vigorous shaking on a vibrating panel at 2200 rpm (ika-vibrax-vxr Typ VX7, Janke&Kunkel, Germany) for 30 minutes at 4°C. For phase separation the mixture was centrifuged (1000 g, 4 °C, 2 min). Subsequently the lower organic phase was removed carefully and the solvent was evaporated to dryness under a nitrogen steam (0.2 L/min) at room temperature. The remaining solid lipid extract was stored at -20 °C for further analysis. All steps following the addition of chloroform/methanol were performed in glassware.

Structural analysis of lipids

All measurements were performed using a Shimadzu MALDI 7090 tandem time-of-flight (TOF) mass spectrometer (Shimadzu-Kratos Analytical, Manchester, UK) described in detail elsewhere (Belgacem et al., 2016). Briefly, the instrument is fitted with a frequency-tripled 2 kHz Nd-YAG laser (λ = 355 nm) operated for sensitivity reasons at 200 Hz, a linear TOF analyzer as MS1 and a wide energy-acceptance curved field RTOF as MS2 for product ion analysis (close to 95% of the selected m/z range), a grounded gas collision cell with differential pumping of the collision region, a double Bradbury-Nielsen wire ion gate for precursor ion selection, an axial spatial distribution focusing (ASDF) lens for obtaining product ion resolutions up to 10 000 (FWHH) and two secondary electron multipliers for linear and reflectron TOF-MS, respectively, for ion detection.

All archaeal lipid samples were dissolved at roughly 1 mg per 1000 µL solvent (methanol:chloroform = 7:3 vol/vol). 2,4,6-Trihydroxyacetophenone was selected as MALDI-matrix for all experiments (positive- and negative-ion detection) by dissolving 15 mg matrix in 1000 µL methanol. Alternatively, the matrix solution was saturated with sodium chloride to improve mono- and disodiated molecules for positive-ion detection. For final sample preparation, analyte solution and matrix solution were mixed 1:1 (vol/vol) immediately before applying 1 µL of the resulting mixture onto the standard stainless steel target surface (dried droplet preparation).

All displayed positive- as well as negative-ion mass spectra represent an average of 10 000 unselected spectra (1 spectrum represents the average of 5 individual laser pulses). This number of shots was acquired in order to obtain satisfactory ion statistics across the mass range measured.

For all positive- and negative-ion CID-experiments helium was introduced into the grounded gas collision cell (E_{LAB} = 20 keV) at a pressure of 1 × 10^{-6} mbar to attenuate the selected precursor ion signal to roughly 50% of its original value. Precursor ion selection was done by selecting a 4-6 mass unit-window centered symmetrically around the monoisotopic ^{12}C-precursor ion ($[M-H]^-$, $[M+Na]^+$ and $[M+2Na-H]^+$) of interest by the ion gate. Again, a total

of 10 000 unselected spectra (1 spectrum represent the average of 5 individual laser pulses) were averaged at slightly elevated laser irradiance in order to avoid detection of unwanted post source decay of the analyte precursor ions using the grounded gas collision cell.

Nomenclature of lipids

In brief, like characteristic for members of the phylum Crenarchaeota, the lipids of *Sulfolobus* species consist of an isoprenoid core structure either in form of two diterpenoides or two tetraterpenoides connected with one or two glycerols, respectively, via ether bonds in the *sn*-2,3 positions. Polar head groups are connected in the *sn*-1 position and commonly consist of (poly-)hexoses (Hex), inositolphosphate (IP) or sulfonated trihexose (sulfono-Hex3). In the literature "archaeol", "diether lipid" (DEL) or more specifically "dialkyl glycerol diether" (DGD) are common terms for the diterpenoide containing C_{40} core structures, while the tetraterpenoide containing C_{80} core structures are known as "caldarchaeol", "tetraether lipid" (TEL) or, more specifically, "glycerol dialkyl glycerol tetraether" (GDGT). These lipid classes which can be distinguished based on their lipid core and head groups can be further divided into lipid species. The latter describe lipids with the same head groups and number of carbon atoms, but with different number of cylopentane moieties. In this study for the notation of the lipid classes we follow the nomenclature described by Jensen et al. (2015a), using the terms DGD and GDGT for the description of the core structures preceded or followed with the above described abbreviations for the polar head groups. The most abundant lipid classes of *S. acidocaldarius* found in this study are shown in Figure 1.

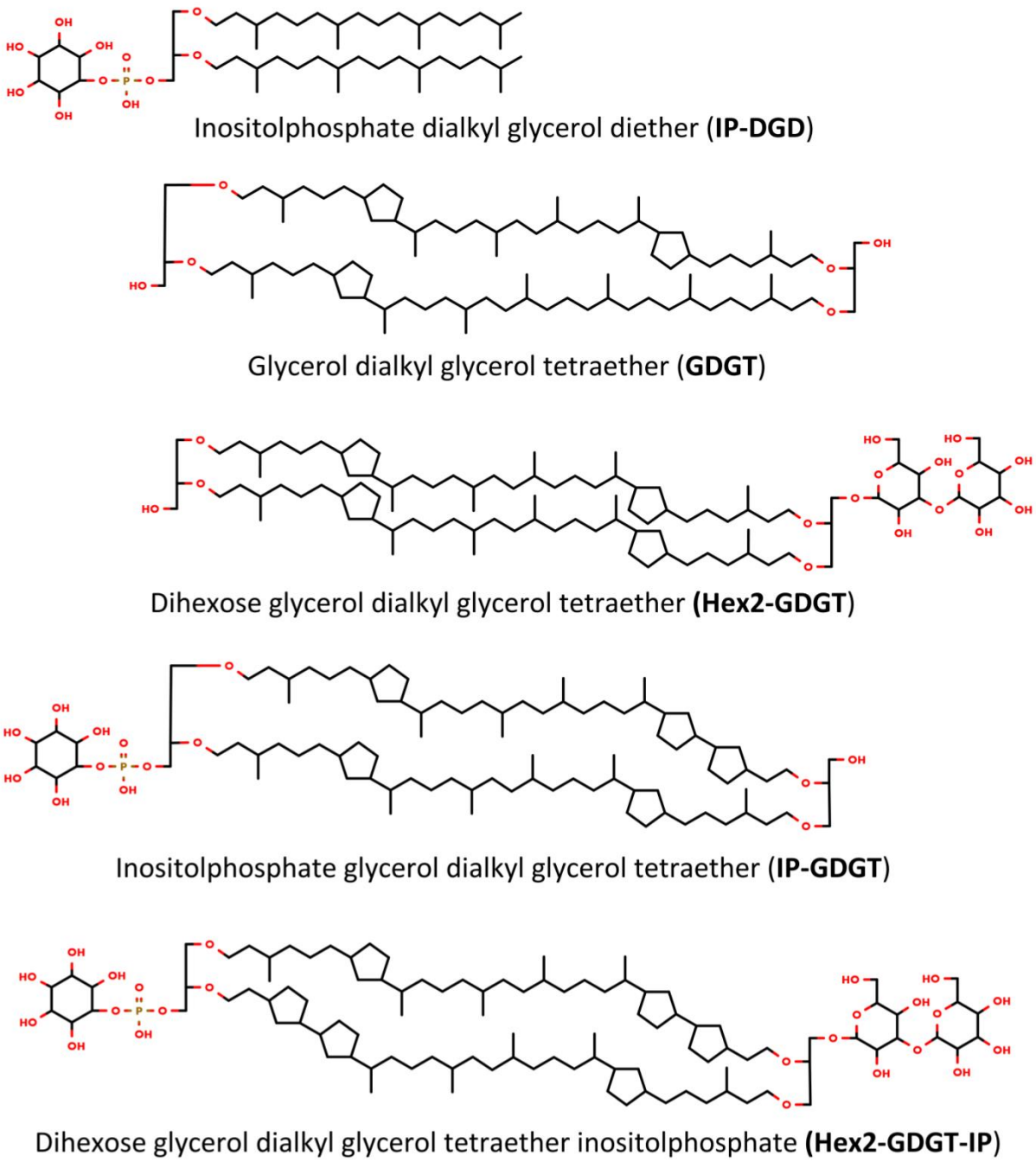

Figure 1: Structures of the most abundant lipid classes in *Sulfolobus acidocaldarius*. The number of cyclopentane rings depicted is exemplarily and generally vary for all glycerol dialkyl glycerol tetraethers (GDGTs) from 3 to 6 per lipid. The position of cyclopentane rings was not determined in this study. Also note that with the used MS method we were not able to distinguish between GDGT and GDNT. Structures were drawn with MarvinSketch 19.3.0, 2019, ChemAxon.

Results

By employing different feed rates during a continuous cultivation we aimed to test the hypothesis whether the lipid composition of the membrane of *S. acidocaldarius* is influenced by the culture´s specific growth rate (μ) under controlled nutrient limiting conditions.

Bioreactor cultivation

Figure 2 shows the time course of the CO_2 signal in the offgas which corresponds to the metabolic activity of the culture and the course of DCW which converges towards its equilibrium state. Dilution rate, calculated as the quotient of the feedrate by the reactor volume, is also shown in Figure 2. DCW, μ, and τ at the end of each phase of constant D, as well as phase duration, are shown in Table 1. Owing to the increased dilution, the DCW is lower in the phases of higher μ (1.9 vs 2.1 g/L).

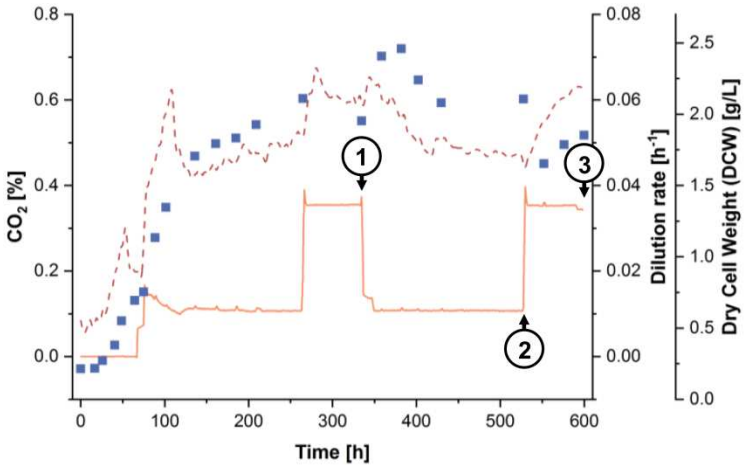

Figure 2: Continuous cultivation of *S. acidocaldarius* in a 3.6 L bioreactor (2.0 L working volume). Samples for determination of the lipid composition were drawn at the end of phases of constant dilution rate at the indicated time points 1, 2 and 3 (where 1 and 3 are replicates of the same biological state). Dry cell weight (DCW) [▪], CO_2 concentration [- -] and dilution rate (D) [–] are shown.

Table 1: End-conditions of the distinct phases of constant dilution rates.

	Time at sampling [h]	Phase duration [h]	Dilution rate (D) & specific growth rate (μ) [h^{-1}]	Dwell time (τ) [h]	Dry cell weight (DCW) [g/L]
Constant Phase 1	334.4	70.0	0.035	28.6	1.95 ±0.05
Constant Phase 2	527.3	192.9	0.011	90.9	2.11 ±0.12
Constant Phase 3	599.5	72.2	0.035	28.6	1.85 ±0.13

Lipid composition and structure

Relative quantification of the lipid classes was performed based on peak height in the MS spectra acquired in positive mode with NaCl spiking. Spiking with a surplus of NaCl guarantees uniform ionization of lipids and allows the detection of neutral lipids (Pittenauer and Allmaier, 2009). For the comparison of the abundances we focused on the most abundant peaks in the lipid spectrum, exemplarily shown in Figure 3. These were identified as the lipid classes Hex2-GDGT, IP-DGD, GDGT, IP-GDGT and Hex2-GDGT-IP.

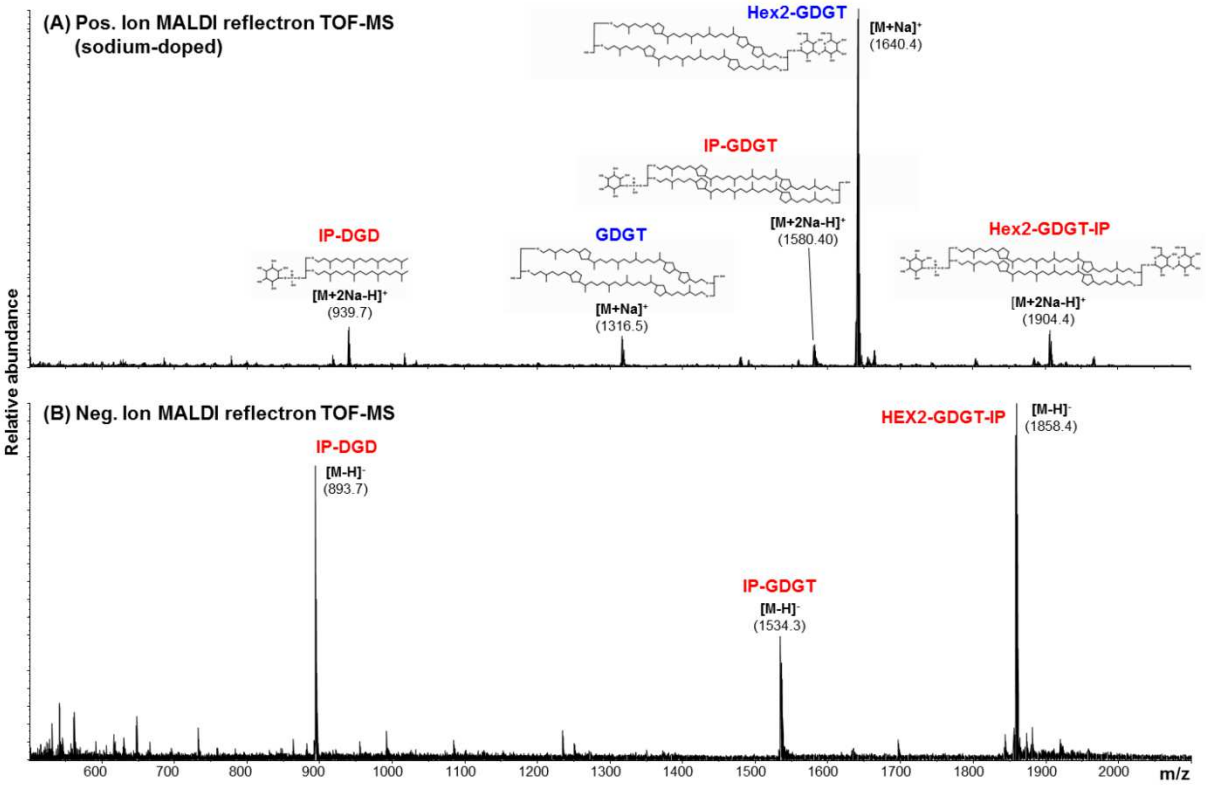

Figure 3: A) Positive-ion MALDI reflectron TOF-MS spectrum of a *S. acidocaldarius* lipid extract spiked with NaCl. The spectrum shows the 5 most abundant lipid species in *S. acidocaldarius*. m/z values and structure of the dominant lipid species are given. **B) Negative-ion MALDI reflectron TOF-MS spectrum of the same sample.** Noteworthy, in the negative-ion mode phosphate-free lipids (highlighted in blue in Figure 3A) cannot be detected.

The most abundant lipid class observed in *S. acidocaldarius* was Hex2-GDGT. Independent of the growth rate 58% of the total amount of lipids were Hex2-GDGT. As second most abundant class followed the diether IP-DGD with its share being highly dependent on the employed growth rate (25% at μ = 0.011 h^{-1} and 16% at μ = 0.035 h^{-1}). IP-DGD was the only class of DELs that we found in significant amounts. GDGT, IP-GDGT and Hex2-GDGT-IP were present in amounts of 3-11% of total lipids. While the abundance of GDGT decreased from 9 to 7% when μ was increased, classes containing inositol phosphate were considerably more abundant at the higher μ (4% at μ = 0.011 h^{-1} and 9% at μ = 0.035 h^{-1}). A summary of the results can be found in Figure 4.

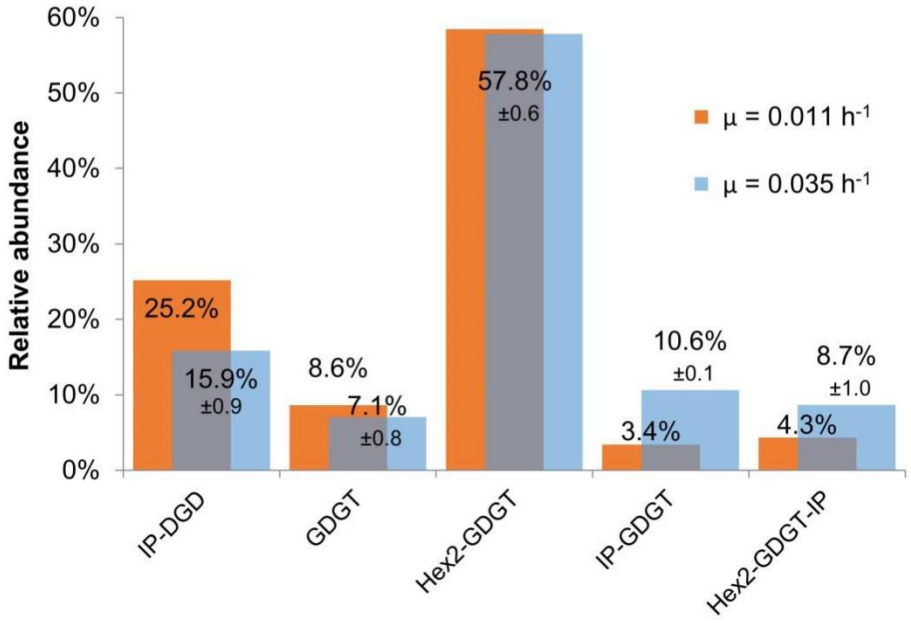

Figure 4: Distribution of the major lipid classes in *S. acidocaldarius* in dependence of the growth rate (μ). Measurements were taken in positive MS mode with addition of NaCl. Values are given as % of the sum of major lipid classes. Errors indicated for the blue bars represent the divergence between the replicates of the two biological states (1 and 3) as shown in Figure 2.

We could show that the average cyclization number (De Rosa et al., 1980) was dependent on the employed μ. At the higher μ of 0.035 h^{-1} a minor fraction of the lipid species harbored six rings, while this share rose to approximately 36% when μ was decreased to 0.011 h^{-1}. These distributions implicate average cyclization numbers of 5.1 at the lower μ and 4.6 at the higher μ. This inverse correlation between μ and average cyclization numbers was identified for all classes. Figure 5 gives an overview of the distribution of cyclopentane rings in the GDGT classes in dependence of μ.

Cyclo-pentane moieties	μ = 0.011 h^{-1}				μ = 0.035 h^{-1}			
	GDGT	Hex2-GDGT	IP-GDGT	Hex2-GDGT-IP	GDGT	Hex2-GDGT	IP-GDGT	Hex2-GDGT-IP
7	-	-	-	4%	-	-	-	3% ±0.7
6	48%	63%	19%	28%	23% ±2.3	28% ±2.5	5% ±1.1	9% ±4.1
5	36%	29%	43%	34%	49% ±0.5	54% ±0.3	34% ±0.6	10% ±0.6
4	16%	8%	26%	23%	28% ±2.8	18% ±2.8	57% ±0.6	35% ±0.5
3	-	-	12%	11%	-	-	4% ±0.1	43% ±4.9
Average cyclization number	5.3	5.6	4.7	4.9	5.0	5.1	4.4	3.9

Figure 5: Distribution of cyclopentane rings and average cyclization number in the investigated GDGT classes of *S. acidocaldarius* in dependence of μ. Errors indicated for the blue bars represent the divergence between the replicates of the two biological states (1 and 3) as shown in Figure 2.

Discussion

In the most recent investigation of the lipidomes of two *Sulfolobus* species, namely *S. islandicus* and *S. tokodaii*, Jensen et al. (2015b) used hybrid ion-trap orbitrap mass spectrometry in negative-ion mode to determine the lipid classes and lipid species in dependence of temperature (cultivation at optimal growth temperature and at ±5 °C thereof) and growth phase (lag-, exponential- and stationary phase). These two species are by far the closest relatives to *S. acidocaldarius* that were investigated in terms of their membrane lipid composition. Based on 16S rDNA analysis *S. acidocaldarius* is phylogenetically more closely related to *S. tokodaii* (Quehenberger et al., 2017), while it is more similar to *S. islandicus* in regards of optimal growth temperature (75 °C for *S. acidocaldarius* and *S. islandicus* (Zhang et al., 2013) vs. 80 °C for *S. tokodaii* (Suzuki et al., 2002)). Table 2 shows an overview of our findings compared to the outcome of the study by Jensen et al. followed by a discussion and direct comparison of the results.

Table 2: Overview of the results from this study and Jensen et al. (2015b). Values are taken at optimal growth temperature (*S. acidocaldarius* and *S. islandicus*: 75 °C; *S. tokodaii*: 80 °C).

	Sulfolobus acidocaldarius		*Sulfolobus islandicus*/ *Sulfolobus tokoadii*	
	this study		Jensen et al. 2015	
	$\mu = 0.011\ h^{-1}$	**$\mu = 0.035\ h^{-1}$**	**lag- or stationary phase**	**exponential phase**
Most abundant lipid classes	Hex2-GDGT-IP, Hex2-GDGT, IP-GDGT and IP-DGD, GDGT		Hex2-GDGT-IP, sulfono-Hex3-GDGT-IP, IP-GDGT and IP-DGD	
DEL:TEL	1:3	1:5.5	1:3/1:2	1:8/1:5
Average cyclization number	5.1	4.6	4.2/5.5	3.9/4.5

Comparison of the results from this work to the literature

Abundance of different lipid classes

The results of the two studies are very similar, with Hex2-GDGT-IP, IP-GDGT and IP-DGD being identified as major lipid classes in both studies. Additionally, we identified significant amounts of Hex2-GDGT or Hex-GDNT, two compounds that we were not able to distinguish with the used MS method. Jensen et al. also found significant amounts of sulfono-Hex3-GDGT-IP, while we detected this lipid class only in minor quantities in our samples. Possibly, this is a result of a sample preparation or measurement bias, causing an over- or underestimation of the compound. Also the fact that we investigated the lipid composition using positive-ion mode has an influence on the detected lipid pattern, since neutral lipids are easily overlooked when only spectra acquired in negative-ion mode are considered (see Figure 3).

Ratio of DEL:TEL among the different *Sulfolobus* species

Jensen et al. found DEL:TEL ratios in the exponential phase of 1:8 and 1:5 for *S. islandicus* (75 °C) and *S. tokodaii* (80 °C), respectively. For the lag phase ratios of 1:3 and 1:2 were calculated. It should be noted that the ratios calculated in the study of Jensen et al. are strongly fluctuating with culture conditions and oftentimes it is hard to find a clear trend or pattern in the DEL:TEL ratios. Based on our results, the DEL:TEL ratio of *S. acidocaldarius* at 75 °C is 1:3 for the lower μ of 0.011 h^{-1} and 1:5.5 for the higher μ of 0.035 h^{-1}. These values lie within the ratios determined for the two related species.

Number of cyclopentane moieties

According to the results of Jensen et al. when grown at the same temperature the lipids of *S. islandicus* have, on average, approximately one ring less compared to *S. tokodaii* (4 vs. 5 rings). A temperature shift of 5 °C results in a difference of ~0.5 rings for both species and since the optimal growth temperature of *S. islandicus* is 5 °C lower than for *S. tokodaii*, the two species actually differ by ~1.5 cyclopentane rings during the exponential phase at their respective optimal growth conditions. For *S. acidocaldarius* we found the average number of rings for the higher growth rate to be 4.6 at the optimal growth temperature of 75 °C.

Effect of growth phase/growth rate

Jensen et al. showed for both organisms that the ring number dropped during the exponential phase and rose again in the stationary phase. They hypothesized that rather than the growth *phase* the specific growth *rate* might be the reason for the change in the lipid pattern. Indeed, while the correlation between average cyclization number and calculated growth rate was rather low for *S. islandicus* (0.43-0.15), in the case of *S. tokodaii* they observed a very high correlation (≥-0.97). We found the same relationship for *S. acidocaldarius*. Jensen et al. "suspected the reason behind the observation in a lacking speed of lipid cyclization during biosynthesis at high growth rates". Interestingly, in all three *Sulfolobus* species the number of rings decreases at the higher growth rate, while at the same time the share of TELs increases. A possible explanation for this behavior is, that caused by the lack of nutrients the organism produces the chemically simpler archaeoles at the expense of the much more complex cyclopentane ring-bearing caldarchaeoles (Jain et al., 2014; Villanueva et al., 2014). Following this assumption, a putative interlinking between average ring number and DEL:TEL ratio could be drawn: during phases of nutrient limitation and therefore lower TEL numbers (like the lag- and stationary phase, or at low growth rates in general), the enzymes responsible for cyclopentane ring formation would, due to the lower amount of substrate present, have the capacity to introduce more rings in the fewer TELs that are available and the connection between DEL:TEL ratio and average cyclization number would have been established again.

Summarizing, the initial hypothesis applies that *S. acidocaldarius* reacts to changes in μ with a change in its lipid pattern. More precisely, at a higher μ the share of IP-GDGT and Hex2-

GDGT-IP increases at the expense of IP-DGD, while the average number of cyclopentane rings decreases.

These findings complement the observations made by others (e.g. Elling et al., 2014; Jensen et al., 2015b; Kramer and Sauer, 1991; Matsuno et al., 2009; Meador et al., 2014; Morii and Koga, 1993) that different growth *phases* influence the lipid pattern, by clearly showing that the changes in the lipid pattern also take place when the culture is in equilibrium state with no difference in the culture´s growth phase. Therefore, based on these results it is clear that the changes in the lipid pattern are rather truly consequences of a different growth *rate* and also happen in absence of the influence of different physiological states, as present in case of lag, exponential and stationary phase.

Why is the lipid composition of *S. acidocaldarius* influenced by μ?

We show that *S. acidocaldarius* is subject to significant changes regarding its DEL:TEL ratio and number of cyclopentane moieties when cultivated at different μs. However, this raises the question, whether the higher amount of DELs at the lower μ is caused by the lack of nutrients that leads to the organism producing the chemically simpler archaeoles at the expense of the much more complex (Jain et al., 2014; Villanueva et al., 2014) cyclopentane ring-bearing caldarchaeoles (Jain et al., 2014; Villanueva et al., 2014; Jensen et al., 2015b), or, alternatively, if the flexibility poses a purposeful adaption to better cope with a changed environment (Manquin et al., 2004; Yoshinaga et al., 2015).

The alternative explanation, that the organism actively modulates its membrane composition to improve its fitness under nutrient limitation is based on the assumption that by increasing the DEL ratio and thereby shifting its membrane to a more fluid and permeable state (Gliozzi et al., 2002), the uptake of the scarcely available nutrients is facilitated and the organism can better cope with the experienced starvations conditions. Manquin et al. speculated that either the change in the lipid compositions serves as hydrogen sink, or as their favored explanation, to increase the membrane fluidity and therefore substrate uptake. Yoshinaga et al. hypothesized that the reason for the change in the lipid head groups under substrate limited conditions (increase of glycolipids, which face the outer leaflet of the membrane bilayer as opposed to phospholipids in the non-limited control experiment) could be “attributed to regulatory mechanisms of cell membrane permeability” or “implicate regulation of lipid-protein interactions, causing protein conformation changes, with crucial consequences for the balance between energy production and conservation”. So again, like Manquin et al., they propose that the organism reacts to increase its efficiency of nutrient uptake and substrate utilization/energy recovery.

A way to test the hypothesis of increased *in-vivo* membrane permeability at a lower TEL ratio is to create knock-out strains with a compromised TEL synthesis pathway and then compare the substrate affinity with the wild-type strain. Following the hypothesis the affinity should be higher for the knock-out with the higher share of DELs. As a side effect, this strain could

be expected to utilize substrate more efficiently, a convenient trait especially for strains utilized in continuous cultures, where substrate affinity is of critical economic importance, since unused substrate is lost via the bleed. Of course, this approach is not trivial because the mechanism of TEL synthesis and the involved enzymes still remain to be described in detail.

Conclusion

The lipid membrane of Archaea is a flexible and highly adaptive system. In this study we show that for the Crenarchaeum *S. acidocaldarius* this flexibility is not only a reaction of homeoviscous adaption to compensate for environmental factors, like shifts in pH or temperature, in order to maintain a constant state of membrane fluidity. Rather, *S. acidocaldarius* reacts with a change in the lipid composition to cope with nutrient limitation.

Summarizing, in this study we identified a novel factor (the controlled specific growth rate) for influencing the lipid composition of *S. acidocaldarius*. We were able to determine the effect of this factor on the lipid composition by for the first time using MALDI-MS for the semi-quantification of an archaeal lipidome. A shift in the specific growth rate during a controlled continuous cultivation of *S. acidocaldarius* led to a change in the ratio of diether to tetraether lipids and in the average number of cyclopentane rings. Limitation of the growth rate led to an increase of the share of diethers, while the remaining tetraether lipids featured a higher average cyclization number.

Author contribution

OS and GA conceived the idea for this study. JQ designed and performed the experiments for cultivation of *S. acidocaldarius* and lipid extraction. EP performed lipid analysis and evaluation of mass spectrometry data. JQ drafted the manuscript. OS critically reviewed and corrected the manuscript and gave substantial input.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Method for producing a composition comprising archaeal lipids from a *Sulfolobus* cell culture

Contribution to Chapter 6

(Filed patent)

Patent number: EP19163020.1 (March 2019)

Erfindungsbeschreibung:

Method for producing a composition comprising archaeal lipids from a *Sulfolobus* cell culture

Für den Einsatz von Archaeosomen (Lipidvesikel die teilweise oder vollständig aus archaealen Lipiden aufgebaut sind) als Carrier von pharmazeutischen Wirkstoffen ist es nötig, je nach gewünschtem Wirkort und Pharmakodynamik eine unterschiedliche Freisetzungskinetik zu gewährleisten. Diese Freisetzungskinetik kann im Wesentlichen über die Stabilität der Archaeosomen beeinflusst werden.

Für die Stabilität ist neben der grundsätzlichen Art der Lipide vor allem das Verhältnis aus kurz- und langkettigen Lipiden ausschlaggebend; sowie das mögliche Vorhandensein von Ringstrukturen in den Lipidketten, welche durch Einschränkung der Kettenbeweglichkeit eine dichtere Packung und Reduktion der Membranpermeabilität bewirken. Die Kombination von Archaeol (auch bezeichnet als „Dialkylglyceroldiether", DGD; mit in Summe 40 Kohlenstoffatome in den Phytanylketten) und Caldarchaeol (auch bezeichnet als „Glyceroldialkylglyceroltetraether", GDGT; mit in Summe 80 Kohlenstoffatome in den Phytanylketten) stellt hier einen geeigneten Hebel dar. Beide Substanzen sind natürliche Membranbestandteile von *Sulfolobus acidocaldarius* und weiteren archaealen Species, wobei insbesondere von Caldarchaeol mehrere verschiedene Varianten vorkommen: Einerseits gibt es Caldarchaeolvarianten mit verschiedenen Modifikationen der endständigen Glycerolreste (keine Modifikation, Phosphatidylinositol (IP), Dihexose (Hex2), sowie Kombinationen daraus: GDGT, IP-GDGT, Hex2-GDGT, IP-GDGT-Hex2). Weiters kommen GDGT-Varianten mit einer unterschiedlichen Anzahl an Cyclopentanringen in den Phytanylketten (Im Falle von *S. acidocaldarius* bevorzugt 4, 5 oder 6 Ringe) vor. Archaeol kommt vor allem als IP-DGD vor.

Aus mehreren Gründen stellt eine Mischung aus Hex2-GDGT und IP-DGD eine besonders interessante Kombination für die Herstellung von Archaeosomen zur oralen Applikation dar:

- Caldarchaeole mit unterschiedlicher Ringanzahl können eingesetzt werden
- Die Mischung aus kurz und langkettigen Lipiden gewährleistet Einflussnahme auf die Stabilität der gebildeten Archaeosomen
- Beide Lipide besitzen eine ausreichende Anzahl an funktionellen Gruppen in den Kopfgruppen zur effizienten Modifikation mit Linkern für die Verwendung zur targeted drug delivery (z.B. Anhängen von Antikörperfragmenten, Aptamere, ect.)
- Diese Modifikation kann selektiv an einer der beiden Lipidklassen durchgeführt werden, da sich die Kopfgruppen in Struktur und chemischen Eigenschaften unterscheiden

In unserem Produktionsprozess ist es möglich über die Stellschrauben Temperatur und Wachstumsrate ein im physiologischen Rahmen beliebiges Verhältnis von DGD:GDGT, sowie eine gewünschte Anzahl an Cyclopentanringen einzustellen. Dadurch wird eine Modifizierbarkeit der Freisetzungskinetik von archaeosomalen Formulierungen erhalten. Arzneien, die in Archaeosomen, aus unseren maßgeschneiderten Lipidmischungen, verpackt werden, können beispielsweise, nach oraler Gabe im Darm eine unterschiedliche Wirkdauer aufweisen.

Ziel der Erfindung:

Herstellung einer Lipidmischung mit möglichst hoher Anzahl an Ringen in den Phytanylketten der Caldarchaeolspezies bei gleichzeitiger Beibehaltung der Einstellbarkeit des Verhältnisses DGD:GDGT (im Rahmen der physiologischen Grenzen).

Aufgrund ihrer besonderen chemischen Eignung (siehe Aufzählung oben) stellen Hex2-GDGT und IP-DGD unsere Zielspezies dar.

Umsetzung:

Hohe Ringanzahl können durch erhöhte Wachstumstemperatur, bei gleichzeitiger Einstellung des DGD:GDGT Verhältnisses über die Wachstumsrate, erreicht werden.

In Kombination mit kontrollierten Prozessbedingungen und definiertem Medium ist es durch unsere Erfindung möglich einen Lipidextrakt zu erhalten, der direkt für die Produktion von Archaeosomen mit hoher Magensaftresistenz und maßgeschneiderter Freisetzungskinetik für unterschiedliche pharmazeutische Wirkstoffe eingesetzt werden kann.

Conclusions

Key achievements of this Thesis are 1) the development of a pretreatment step for the utilization of biomass hydrolysates for waste-to-value applications, 2) the development of a method for biomass determination in turbid media, 3) the development and optimization of a cheap and defined cultivation medium for the production of high value components in *S. acidocaldarius* bioprocesses, 4) development of novel and improved methods for monitoring of viable biomass during a fermentation process as well as 5) the description of critical process parameters for steerage of the lipid pattern of *S. acidocaldarius*.

Following the review on the biotechnological potential of *Sulfolobus* in the first chapter, **the second chapter** of this Thesis showed once again that complex natural substrates are unpredictable in their nature and their utilization as carbon source is challenging even for an extremophile like *S. acidocaldarius*. For the organism to thrive on the tested substrates (enzymatically hydrolyzed beech wood and hot water treated wheat straw) removal of inhibitors via activated carbon and acetate removal via distillation proved to be necessary. Such an additional process step for substrate preparation adds extra costs to any envisioned industrial application. Therefore, I conclude that at the current state the use of biomass hydrolysates cannot be considered advantageous for *S. acidocaldarius* based bioprocesses due to the organism´s sensibility to inhibitors that are formed during the pretreatment processes and the necessity of an additional detoxification step that strongly burdens process efficiency. Consequently these limitations render the use of lignocellulose hydrolysates economically infeasible, certainly for the production of low value bulk chemicals. An option that remains to be investigated is the utilization of lignocellulosic hydrolysates or similar substrates (with high inhibitor concentrations and low amounts of fermentable sugars) in continuous perfusion processes, where unwanted inhibitors are continuously removed while high cell concentrations can be maintained. Additionally, fermentable sugars could be supplied in sufficient quantity by employing feed rates greater than the critical dilution rate. Meanwhile *S. acidocaldarius* should be employed in a more controlled biotechnological environment with reproducible feedstock used in defined media. Thereby crucial knowledge about large scale processes can be acquired and the extremophile can be exploited for its unique enzymes, pathways and metabolites as well as for high value building blocks that are not present is mesophiles.

An economically more attractive option for an *S. acidocaldarius* bioprocess would be the production of high value chemicals according to QbD guidelines. Among other requirements, these guidelines demand the utilization of educts and products with little variations, and the employment of defined media as described in **the third chapter** of this Thesis. To reduce medium costs I was able to eliminate the most expensive component, a mix of polypeptides known as NZ-amine, or similar protein hydrolysate based components, from the *Sulfolobus* standard medium ("Brock Medium") by substituting it with the relatively inexpensive bulk chemical MSG and coined the term "VD Medium" for the newly developed formulation.

Bioprocesses in compliance with QbD guidelines require not only defined media but also monitoring tools that can delivery timely responses for efficient bioprocess development and control. Therefore, in **the fourth chapter** this Thesis includes the adaption of monitoring tools established in Bacteria and Eukarya for at-line monitoring of the viability of *S. acidocaldarius* cultures. Due to the unusual nature of the archaeal cell envelope, many techniques that are successfully used in Bacteria or Eukarya cannot be used for *S. acidocaldarius*. Thus, I propose that in the future there is potential and demand alike for the adaption of novel concepts with unconventional approaches which are orthologous to existing monitoring tools established in conventional bioprocess development. Although not investigated in this Thesis, this could be achieved for example by soft sensors based on online data acquired during the fermentation process.

The fifth chapter demonstrates that on the field of potential applications of *S. acidocaldarius* in production processes it is critical to note that although the molecular basis for genetic manipulation is well founded and gene insertions or deletions can be carried out in a straight forward way, recombinant production of eukaryotic proteins can be difficult due to their different nature regarding protein structure and stability. Possibly, the much more straight forward way of exploiting *S. acidocaldarius* as source of a high value component presented in **the final (sixth) chapter** of this Thesis might, in the end, pose the more successful application in future biotechnology: In order to exploit the unique nature of *S. acidocaldarius* in the most direct way I propose the utilization of the membrane of the Archaeon for the production of tailor-made archaeal lipids. Based on the presented results, steerage of the production pattern regarding lipid classes and lipid species might be possible in future applications via regulation of critical process parameters like temperature and growth rate in a controlled bioreactor environment.

Answers to Scientific Questions

1. **Can lignocellulosic hydrolysates be used as carbon source for *S. acidocaldarius*?**
   Yes. The tested lignocellulosic hydrolysates could be used as growth promoting substrates thereby substituting a significant amount of carbon source, but detoxification of the hydrolysates and addition of minor amounts of supplementary substrate like monosodium glutamate or NZ-amine were necessary for efficient growth.

2. **Can we find a composition for a defined cultivation medium that supports growth of *S. acidocaldarius* with biomass yields and specific growth rates comparable to a complex medium based on protein hydrolysates?**
   Yes. A novel media formulation with improved buffer capacity, reduced number of component, reduction of most salts and trace elements by 50 to 70% and lower raw material cost was developed. The medium is completely defined, less prone to precipitation, easier in its preparation and capable of providing a similar average growth rate and final cell density compared to conventional complex media.

3. **Are the results of fluorescence based, at-line methods for the determination of the viability of *S. acidocaldarius* comparable with a plating assay that is considered the gold standard of viability assays?**
   Yes. Method comparison over the course of a bioreactor cultivation lasting for more than 300 h revealed a high correlation between the methods and showed that stress inducing events are reflected in a drop in viability also in all methods. Nevertheless, results have to be interpreted carefully, since in some incidences, although the assays reveal the same phenomena, the response can be chronologically shifted, as shown for the viability increase during the lag/early exponential phase.

4. **Can *S. acidocaldarius* be used for the expression of a eukaryotic gene with the goal of intracellular conversion of xylose to xylitol?**
   No. Although the gene of interest was successfully introduced and transcribed in *S. acidocaldarius*, neither the protein could be detected via western blot analysis, nor was any xylitol present in the culture broth or cytosol.

5. **Do growth temperature and specific growth rate affect the lipid pattern of *S. acidocaldarius* regarding distribution of lipid classes and lipid species and if yes, how?**
   Yes. An increase in growth temperature also leads to an increase of the average cyclization number of tetraether lipids. The specific growth rate influences both the ratio of diether to tetraether lipids as well as the average cyclization number, where a limitation of the growth rate leads to an increase of the share of diethers, while the remaining tetraether lipids feature a higher average cyclization number.

Take-home Message

In this Thesis I investigated fundamental topics for the utilization of *S. acidocaldarius* as biotechnological production host. The majority of the presented achievements serve to satisfy the requirements of regulatory authorities as stated in the ICH Guideline Q8 on Pharmaceutical Development. I envision that by pursuing these QbD principles skepticism towards extremophilic bioprocesses, which is still present in the biotechnological and specifically pharmaceutical industry, will be overcome more easily. In combination with an approach of integrated bioprocess development this will facilitate applications of continuous biomanufacturing with *S. acidocaldarius*. The utilization of complex natural substrates has to be evaluated critically and on an individual basis for each possible substrate, since no generalizations can be made owing to the diverse properties of the raw materials. In general, due to slow growth, relatively high requirements on substrate composition and susceptibility towards growth inhibiting components *S. acidocaldarius'* biotechnological potential should be valued highest in the field of the production of unique high value chemicals.

In the future, besides exploitation of specialized metabolic pathways and prospection of unique biological compounds it will be necessary to further investigate the impact of critical process parameters in the upstream process on host physiology, downstream processing and critical quality attributes.

Appendix

Curriculum Vitae

Curriculum Vitae

Dipl.-Ing. Julian Quehenberger

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Education

Since 03/16 **Doctoral program in Technical Sciences in the field of biochemical engineering, TU Wien**

- PhD Thesis: "*Sulfolobus acidocaldarius*: establishing a novel host for cutting-edge biotechnology at its extremes"

10/13 – 03/16 **Master´s program Technical Chemistry with focus on Biotechnology and Bioanalytics, TU Wien**

- Diploma Thesis: "Expression tuning in *Escherichia coli* via a glucose/lactose mixed feed system"
- 2014: Merit-based scholarship
- Graduation with distinction

10/10 - 09/13 **Bachelor´s program Chemistry, KFU & TU Graz**

- Bachelor´s Thesis: "Interactions of the lettuce microbiome in the rhizosphere"
- 2011: Merit-based scholarship

Professional experiences and Internships

Since 10/19 **Project assistant** at TU Wien, Group of Integrated Bioprocess Development

- Fellow at FFG-Spin-off fellowship "NovoSome"
- Project management
- Mentoring of BSc, MSc and PhD students

03/16 - 09/19 **Project assistant** at TU Wien, Group of Integrated Bioprocess Development

- Research work on the project "CrossCat" (ERA-IB-15-029) for sustainable valorization of hemicellulosic waste streams with extremophilic organisms
- Mentoring of BSc and MSc Theses and interns
- Tutoring of lab courses "Biochemical Engineering" and "Orientation course for beginners in chemistry I"

11/14 - 12/14 **Research internship** at the Institute of Chemical Engineering, TU Wien and the Department for Agrobiotechnology IFA-Tulln, Austria

- Optimization and validation of microbial source tracking (MST) assays
- Transfer of qPCR based MST assays to isothermal amplification

07/14 - 08/14 **Research internship** at Epidermolysis Bullosa Laboratories, Salzburg, Austria
07/13 - 08/13
- Cultivation and transfection of HEK cells
- Vector construction and gene expression analysis and reporter gene assays

10/09 - 07/10 **Civilian service** at the Austrian Red Cross

List of Publications and scientific contributions

Beisl, S., **Quehenberger, J.**, Kamravamanesh, D., Spadiut, O., & Friedl, A. (2019). Exploitation of wheat straw biorefinery side streams for microorganisms – a feasibility study. Submitted to *Processes*.

Quehenberger, J., Steudler, S., & Spadiut, O. (2019). Evaluation of the potential of an enzymatically treated beech wood hydrolysate as carbon source for *Sulfolobus acidocaldarius*. Submitted to *Bioresource Technology Reports*.

Quehenberger, J., Pittenauer, E., Allmaier G., & Spadiut, O. (2019). The influence of the specific growth rate on the lipid composition of *Sulfolobus acidocaldarius*. Submitted to *Extremophiles*.

Quehenberger, J., Albersmeier, A., Glatzel, H., Hackl, M., Siebers, B., & Spadiut, O. (2019). A defined cultivation medium for *Sulfolobus acidocaldarius. J. of Biotechnology*, 301, 56-67.

Quehenberger, J., Reichenbach, T., Rettenbacher, L., Baumann, N., Divne, C., & Spadiut, O. (2019). Kinetics and predicted structure of a novel xylose reductase from *Chaetomium thermophilum*. *International Journal of Molecular Science*, 20, 1, 185.

Poster presentation at the 12th International Congress of Extremophiles 2018, Ischia, Italy. Biomass determination of *Sulfolobus* spp. using flow cytometry.

Scientific article for GIT Labor journal. Symbiose von biologischen und chemischen Katalysatoren zur Umsetzung von Hemicellulose. http://www.git-labor.de, 13.02.2018.

Quehenberger, J., Shen, L., Albers, S. V., Siebers, B., & Spadiut, O. (2017). *Sulfolobus* – a potential key organism in future biotechnology. *Frontiers in Microbiology*, 8, 2474.

Wurm, D. J., **Quehenberger, J.**, Mildner, J., Eggenreich, B., Slouka, C., Schwaighofer, A., ... & Spadiut, O. (2017). Teaching an old pET new tricks: tuning of inclusion body formation and properties by a mixed feed system in *E. coli*. *Applied microbiology and biotechnology*, 1-10.

Cardinale, M., Grube, M., Erlacher, A., **Quehenberger, J.**, & Berg, G. (2015). Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environmental microbiology*, 17 (1), 239-252.

Skills and Qualification

Computer literacy	Lucullus (PIMS) SnapGene Imaris ImageJ Matlab MODDE Photoshop SigmaPlot inCyght
Languages	German (native) English (fluent) Spanish (intermediate)
Techniques	Molecular cloning Cell culture technique Bioreactor cultivation Protein purification Western blot Enzyme kinetics Confocal microscopy Flow cytometry Bioprocess monitoring and control Design of experiments Statistical data analysis