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Abstract

Archaea are a domain of prokaryotic organisms with intriguing physiological characteristics and ecological importance. In Microbial Biotechnology, archaea are historically overshadowed by bacteria and eukaryotes in terms of public awareness, industrial application, and scientific studies, although their biochemical and physiological properties show a vast potential for a wide range of biotechnological applications. Today, the majority of microbial cell factories utilized for the production of value-added and high value compounds on an industrial scale are bacterial, fungal or algae based. Nevertheless, archaea are becoming ever more relevant for biotechnology as their cultivation and genetic systems improve. Some of the main advantages of archaeal cell factories are the ability to cultivate many of these often extremophilic strains under non-sterile conditions, and to utilize inexpensive feedstocks often toxic to other microorganisms, thus drastically reducing cultivation costs. Currently, the only commercially available products of archaeal cell factories are bacterioruberin, squalene, bacteriorhodopsin and diether-/tetraether-lipids, all of which are produced utilizing halophiles. Other archaeal products, such as carotenoids and biohydrogen, as well as polyhydroxyalkanoates and methane are in an early to advanced development stage, respectively. The aim of this review is to provide an overview of the current state of Archaea Biotechnology by describing the actual state of research and development as well as the industrial utilization of archaeal cell factories, their role and their potential in the future of sustainable bioprocessing, and to illustrate their physiological and biotechnological potential.

Keywords

Microbial cell factory, prokaryotes, bacteria, eukaryotes, bioprocess, biofuel, bioproduct, biorefinery, bioeconomy, bio-technology readiness level (B-TRL)

1. Introduction

Production of high value bioproducts and commodity chemicals by microbial cell factories or isolated enzymes through biotransformation, biocatalysis or fermentation progressed tremendously in the last decades. As the market demand for biopharmaceuticals, fine and commodity chemicals as substitutes for various petrol-based synthetic products rises, Microbial Biotechnology is becoming ever more relevant. The earliest forms of biotechnology date back several thousand years, before the existence of microbes would be

discovered. Anaerobic beer and wine fermentation for example dates back to about 6000 BC, while the pickling of foods in acetic acid (vinegar) produced though microbial acidification of wine dates back to 5000 BC (Buchholz and Collins, 2013; Lück and Jager, 1997). It was only in 1860, that Pasteur's ground-breaking discovery of the involvement of yeast, acetogenic and lactic acid bacteria in these processes caused a paradigm shift in the perception of microbes. Microbes were now widely accepted as living organisms that could be utilized as chemical reactors (Mir, 2004). Today, microbial cell factories are indispensable. They are involved, among others, in the production of bio-based products such as bioplastics, cosmetics, food additives, high-value chemicals, biopharmaceuticals, and biogenic energy conversion as well as energy storage solutions. As the world progresses towards a sustainable economy, industrial microbiology and biotechnology are appropriate tools to replace petrochemistry-based technologies by biological alternatives (Harwood et al., 2018).

Biotechnology can be defined as: *any technological application using biosystems, organisms, or derivatives thereof, to manufacture or modify bioproducts or to develop and engineer processes for specific application*. It can be subdivided into the following categories: white (industrial), blue (marine and fresh-water), green (plant/agricultural), red (biomedical/pharmaceutical), grey (environment/bioremediation), brown (arid land/dessert), yellow (insect biotechnology) and dark (biological weapons/biowarfare). Because of its diverse of applications, the biotechnology industry is likely to become one of the most important high-tech industry branches within the next few decades, with revenues expected to exceed USD 720 billion by 2025, of which fermentative production is expected to make up USD 86 billion (ResearchAndMarkets.com, 2019). To date, the vast majority of industrialized microbial cell factories are fungal or bacterial based, with *Escherichia coli* and *Saccharomyces cerevisiae* as the predominant systems. However, the recent advances in bioprocessing technologies, bioinformatics, and synthetic biology have shown the potential of archaeal cell factories (Bill, 2014; Pecorari et al., 2015; Singh and Singh, 2017; Straub et al., 2018). In Microbial Biotechnology, Archaea are historically overshadowed by Bacteria and Eukarya in terms of public awareness, industrial application, and scientific studies, although their biochemical and physiological properties show a vast potential for a wide range of biotechnological applications.

Archaea inhabit almost every environment on Earth and some archaea have adapted to survive under extreme environmental conditions (Rampelotto, 2013). Not only have archaea thrived in these extremes, but it has been shown that, methanogenic archaea (methanogens) and Thaumarchaeota are key organisms in the anaerobic and marine food webs, respectively (Pester et al., 2011). Methanogens are a physiologically and phylogenetically diverse group of anaerobic microorganisms, which produce methane (CH_4) as an end product of their carbon and energy metabolism (Thauer et al., 2008). Thaumarchaeota are a metabolically diverse group of microbes found in almost every environment ranging from marine waters and arctic soils to the human skin biome; and representatives have shown to perform ammonium oxidation (Stieglmeier et al., 2014). Other ecologically and biotechnologically important archaea are found among the Sulfolobales (Huber and Prangishvili, 2006), first cultivated from volcanic hot springs at pH values of 2 to 3 and 80°C; the halophilic archaea ("haloarchaea"), which grow in salt solutions up to saturation (Stan-Lotter and Fendrihan, 2015) and the anaerobic, hyperthermophilic Thermococcales cultivated at over 90°C (Bertoldo and Antranikian, 2006). Archaea have been utilized in undefined consortia with Bacteria for biomining, bioleaching, anaerobic digestion, and soil/waste water remediation, and are well known for their extremely stable enzymes, but there are very few industrialized applications of pure cultures (Straub et al., 2018a). Recently an extensive review summarized the bioremediation potential of archaea in the degradation of hydrocarbons, metal remediation, acid mine drainage, and

dehalogenation (Krzmarzick et al., 2018). Therefore, these applications will not be covered in this review. Here, we will focus on the utilization of archaea as microbial cell factories in bioproduction. Archaea have been shown to naturally produce or can be engineered to produce a range of products such as biofuels (e.g., biomethane, biohydrogen, bioethanol, or biobutanol), bioplastics (PHA), compatible solutes, nanobiotechnology components (surface-layer proteins, lipids) and precursor chemicals (e.g., acetate; 2,3-butanediol) needed for the industrial synthesis of high value chemicals. A major progress in facilitating Archaea Biotechnology is the relatively recent development of genetic techniques, methods which are essential for the development of microbial production platforms, but which are not yet reflected by the current TRL system (see below). The progress in genetic methods for archaea, especially thermophiles, have been reviewed recently (Farkas et al., 2013; Straub et al., 2018; Zeldes et al., 2015), and are not subject to this review. Nonetheless, it is obvious and covered by this review that the production of many compounds within the archaea was significantly improved by genetic engineering. To date, only a few companies are working on archaeal cell factories and commercialization is limited to halophilic archaea producing high value products with a growing market demand such as bacterioruberin, ether lipids, and various isoprenoids (Figure 1).

The aim of this review is to provide an overview of the current state of Archaea Biotechnology by analysing the state of research & development in the industrialization of archaeal cell factories as well as their role and potential in the future of sustainable biotechnology, and to highlight their physiological and biotechnological potential. To objectively analyse the progress of the technologies in question, the established Technology Readiness Level (TRL) scale was adapted for specific application in Microbial Biotechnology. The new Bio-Technology Readiness Level (B-TRL) scale was applied throughout this review.

2. Bio-Technology Readiness Level (B-TRL)

Since its inception at the National Aeronautics and Space Administration (NASA) in the 1970s, the TRL scale has become a globally accepted tool for the assessment of technological progress and potential. The initial 7 TRL system was designed to check if the proposed space crafts could actually be built with the current technology (Héder, 2017). After several internal iterations, a 9 level TRL scale including detailed explanations, examples of technologies and defined work achievements for each of the levels, where integrated into the U.S. Department of Defence technology acquisition program (Héder, 2017; Mankins, 2009; Sadin et al., 1989; United States, 1991). This kick-started the adaptation of the TRLs by civil contractors and other international space agencies and ultimately by public funding agencies for basic and applied research. As such the EU Horizon 2020 Work Program integrated TRLs as one of the criteria with which funding-eligibility was to be determined. Because funding through this program covered a much larger array of disciplines, the TRL definition were generalized to be applicable to various disciplines (Pfeifer et al., 2020). The reduced Horizon 2020 TRLs were lacking discussions and examples for each level as well as other aspects found in the original system, which are vital for the application of TRLs as intended by the NASA (Héder, 2017).

Although the established TRLs (Figure 2) seemed to be the best approach to assess the current state of archaea-based biotechnologies, it became apparent, that the vagueness of TRLs provided by funding agencies made it difficult to analyse and compare the state of various technologies within a highly specialized field objectively and rigorously, whereas the TRLs published by NASA were too engineering specific. Therefore, in efforts to objectively analyse the broad range of biotechnological applications of archaea within the scope of this review, the existing TRLs were redefined (Table 1), and corresponding work achievements for each level were added (Pfeifer et al., 2020) to create the Bio-Technology Readiness Level (B-TRL) scale. The definitions of individual levels were created as a consensus of

various industrial and institutional TRLs (Pfeifer et al., 2020), while the work achievements were adapted from established examples, readily accepted in aviation and space flight (DDR&E(R&T), 2009; ISO, 2013; Mitchell, 2007).

Our new B-TRL system was applied throughout this review to provide a consistent and objective analysis of the current state of each application. The work achievements were supported by publicly available data, published in peer-reviewed journals. The publications were only considered as evidence of a work achievement if the application/product in question was specifically mentioned. Furthermore, the progression through the B-TRLs had to be documented for a specific organism, and not by publications based on different organisms utilized for the same application. The B-TRLs for the application were thus defined as the highest B-TRL achieved by an individual organism. It is noteworthy that often the quantity of a product that is demanded by the market is small enough and/or can be sold at a high enough price that production in small batches is sufficient for commercialization. To accommodate this, we have expanded the B-TRL scale beyond the established 9 levels, to where each Level (B-TRL 2 and above) can be connotated with a "C", identifying it as a technology or product that is commercially available. Furthermore, starting at B-TRL 5 information published in peer-reviewed journals was often no longer sufficient to provide evidence for the work achievements, as this progress is mostly not disclosed by the companies developing the technologies. Therefore, any production that exceeded B-TRL 5 has been assigned the B-TRL ≥ 5or ≥ 5C.

3. Methane

Methanogens represent one of the most widespread groups of microorganisms on Earth. They thrive in habitats from hot vents in the deep oceans (Jeanthon et al., 1999, 1998; Ver Eecke et al., 2012) to ice-cold permafrost soils (Mondav et al., 2014; D. Wagner et al., 2013), in rice field soils (Kitamura et al., 2011), freshwater and marine sediments (Borrel et al., 2012; Lomans et al., 1999), as well as in the intestine and oral cavity of animals (Poulsen et al., 2013; Söllinger and Urich, 2019) and humans (Chaudhary et al., 2018). The energy metabolism of methanogens, which is independent of molecular oxygen (O_2) and often independent of the presence of any organic molecules, is unique and possibly developed on Early Earth (Martin et al., 2008). One of the metabolic products of the methanogenic energy metabolism is the potent greenhouse gas CH_4. Methanogens act as the final consumers of volatile fatty acids (VFAs), alcohols, or gases in the terminal step of the anaerobic food chain (Abdel Azim et al., 2018; Liu and Whitman, 2008; Lyu et al., 2018; Schink and Stams, 2006; Thauer et al., 2008). As such, their estimated contribution to the global carbon cycle is paramount (approx. 1 Gt carbon yr^{-1}) (Lyu et al., 2018; Thauer et al., 2008; Tian et al., 2016).

Methanogens are known to metabolize the following substrates: gases (molecular hydrogen (H_2), carbon dioxide (CO_2) and carbon monoxide (CO)), acetate, methylated compounds (e.g., methanol), methylated compounds plus H_2, and methoxylated compounds (Borrel et al., 2012; Ferry, 2010; Liu and Whitman, 2008; Mayumi et al., 2016; Rother et al., 2007; Rother and Metcalf, 2004; Thauer et al., 2008). This phylogenetically diverse group of organisms (Adam et al., 2017, 2018; Borrel et al., 2013b, 2016) has a growth temperature range spanning from below 0 to up to 122°C (Takai et al., 2008; Taubner et al., 2015). With respect to biotechnological applications of methanogens, bioelectrochemical CO_2 conversion through application of voltage and the application of wild type methanogens for CO_2-based biological CH_4 production (CO_2-BMP) using H_2 are under research and development. Studies have shown that the CO_2-BMP technology holds high potential for demand-oriented and intermittent power storage of excess electric energy (e.g., electricity from wind or solar power plants) in the form of chemical energy (Bernacchi, 2013a, 2013b; Griese et al., 2019).

Several pure cultures of methanogens have been examined with respect to CO_2-BMP prerequisites and characteristics in both fed-batch and continuous cultivations (S. Rittmann et al., 2015; Rittmann, 2015; Rittmann et al., 2018). For CO_2-BMP, there is a trade-off that must be made between the CH_4 evolution rate (MER / mmol L^{-1} h^{-1}) and Vol.-% CH_4 in the offgas. Based on the H_2/CO_2 inflow rate and conversion efficiency, the desired quality of the CH_4 content in offgas may be adjusted to customer needs (S. Rittmann et al., 2015; Rittmann, 2015; Rittmann et al., 2018; Seifert et al., 2014). At a low gas volume flow per unit of working volume per minute (vvm) the CH_4 concentration in the offgas increases as more gaseous substrate is usually transferred into the liquid phase and converted by methanogens, on the other hand the MER is decreased, because the amount of available substrate decreases. When the vvm is increased in CO_2-BMP, the CH_4 content in the offgas decreases. However, MER increases due to a higher H_2/CO_2 availability. Hence, there are two approaches to CO_2-BMP, a high vvm approach with high MER, or a low vvm approach with high Vol.-% CH_4 in the offgas. Currently the research and development route is aiming on increasing MER and Vol.-% CH_4 in the offgas in parallel.

In fed-batch cultivation mode, both the highest MER of 476 mmol L^{-1} h^{-1} and the highest CH_4 content in offgas of 96.6 Vol.-% were achieved with *Methanothermobacter marburgensis* DSM 2133 in a 2.2 L bioreactor and at a vvm of 2 (Abdel Azim et al., 2017). The highest MERs of 954 and 1280 mmol L^{-1} h^{-1} (Nishimura et al., 1992; Seifert et al., 2014) were obtained in continuous culture with *M. marburgensis* and *Methanobacterium* sp. KN-15, respectively, using continuously stirred tank bioreactors. However, also membrane, hollow-fibre fixed-bed and high pressure bioreactors have been investigated (Jee et al., 1988a, 1988b, 1987; Pappenreiter et al., 2019). Furthermore, it has been shown that continuous cultivation and production of CO_2-BMP can be performed from pure gases as well as from any suitable gas of biotic and abiotic origin, with the largest recorded cultivation being achieved in a 42 L bioreactor and a vvm of 0.01 (Hoffarth et al., 2019; Martin et al., 2013; Rittmann et al., 2014; Seifert et al., 2013; Taubner et al., 2018) (Table 2).

There are currently three European companies actively involved in research and development activities on CO_2-BMP (Krajete GmbH, Austria; Electrochaea GmbH, Germany; Micropyrus GmbH, Germany). Therefore, although analysis of the published data shows the B-TRL of CO_2-BMP to be ≥5 (Pfeifer et al., 2020). On the other hand, bioelectrochemical CO_2 methanation is currently at B-TRL 2 (Pfeifer et al., 2020) with MERs in the range of 0.9-4 nmol h^{-1} cm^{-2} in *Methanothermobacter thermoautotrophicus* (Beese-Vasbender et al., 2015; Hara et al., 2013; Sato et al., 2013) and 40-1000 nmol h^{-1} in mutants of *Methanococcus maripaludis* (Deutzmann et al., 2015; Lohner et al., 2014) to 743 nmol h^{-1} in *Methanobacterium palustre* (Cheng et al., 2009; Pfeifer et al., 2020).

4. Molecular hydrogen

Ever since it was used to power the first internal combustion engine in 1807, H_2 has become an integral part of modern industry (Stolten, 2010). The global demand for H_2 has more than tripled since 1975 and is predicted to grow much faster in the coming years as the necessity of CO_2-neutral fuel sources increases (Handelsblatt, 2019; IEA, 2019). However, more than 75% of the global H_2 production relies on natural gas and coal, whereas only a small fraction of global output is produced from electricity and renewable resources. With the rising demand in H_2 and the efforts to reduce CO_2 emissions, there is an obvious and urgent need to rapidly develop alternative renewable energy production systems. Microbial production of H_2, also referred to as biohydrogen production, is an attractive method for sustainable H_2 generation. The essentiality of H_2 for the successful future of a sustainable economy is highlighted by the announcement of the EU commission to invest approx. 430 billion € into the development of a sustainable H_2 infrastructure over the next decade (Handelsblatt, 2020).

H_2 can be produced by a variety of microorganisms, which can be divided into three main groups: oxygenic phototrophs, anoxygenic phototrophs as well as facultative and strict anaerobes with all known archaeal H_2 producing strains belonging to latter. H_2 production from archaea was first reported in the 1980s (Fiala and Stetter, 1986). Phylogenetically, currently known archaeal H_2 producers are among the Euryarchaeaota Methanococci, Thermococci and Thermoprotei, known to metabolize a variety of substrates and use ferredoxin as reducing equivalent for H_2 generation by membrane bound hydrogenases, which results in a high specific H_2 productivity (qH_2) and high H_2 yield ($Y_{(H2/S)}$) (Ergal et al., 2018; Kim et al., 2010; Lim et al., 2010; Rittmann et al., 2012; S. K.-M. R. Rittmann et al., 2015). Furthermore, it has been shown that for biohydrogen production a temperature increase from 37°C to 100°C heavily benefits the production of H_2 (Ergal et al., 2018; S. K.-M. R. Rittmann et al., 2015; Rittmann and Herwig, 2012; Verhaart et al., 2010). Thus, the most studied archaea for H_2 production include the hyperthermophilic *Desulfurococcus amylolyticus*, *Pyrococcus furiosus*, *Thermococcus barophilus*, *Thermococcus kodakarensis*, *Thermococcus litoralis*, *Thermococcus onnurineus* and *Thermococcus paralvinellae* (Bae et al., 2012; Bálint et al., 2005; Hensley et al., 2016; Kanai et al., 2005; Kozhevnikova et al., 2016; Reischl et al., 2018). Since the first observation of H_2 production, efforts have been made to optimize production conditions for these species on various substrates such as CO (Bae et al., 2012; Kim et al., 2013; Kozhevnikova et al., 2016), carboxylate anions of carboxylic acids such as pyruvate and formate (Bae et al., 2015, 2012; Kanai et al., 2005; Kozhevnikova et al., 2016; Lee et al., 2012; Lim et al., 2012; Schäfer and Schönheit, 1991), peptides and amino acids (Bálint et al., 2005; Hensley et al., 2016; Oslowski et al., 2011), mono-, di- and poly-saccharides such as fructose, cellobiose and starch (Bae et al., 2012; Chou et al., 2007; Hensley et al., 2016; Oslowski et al., 2011; Reischl et al., 2018; Schicho et al., 1993), as well as complex carbohydrates such as lignocellulose (Oslowski et al., 2011; Reischl et al., 2018) (Table 3) (Pfeifer et al., 2020).

To date, the highest production values have been achieved by *T. onnurineus* NA1 grown on CO or formate in batch or fed-batch cultivations (Bae et al., 2015; Lim et al., 2012). Once cultivation conditions had been optimized, *T. onnurineus* grown on formate produced H_2 at a H_2 evolution rate (HER) of 236 mmol L^{-1} h^{-1} in a 30 L bioreactor (Bae et al., 2015). In fed-batch, *T. onnurineus* was shown to reach an optical density of 18.6 at 600 nm wavelength (OD_{600}), with an HER of 2829 mmol L^{-1} h^{-1}, which is ten times higher than any other reported archaeal value (Lim et al., 2012). Improvements of genetic systems allowed for the overexpression of *frhAGB* genes conveyed *T. onnurineus* NA1 the ability to overcome O_2 inhibition by changing the transcriptional level of several stress response genes. This strain showed a qH_2 of 323 mmol g^{-1} h^{-1} on formate under oxic conditions and a qH_2 of 365 mmol g^{-1} h^{-1} under anoxic conditions, indicating that the presence of O_2 had almost no effect (S. H. Lee et al., 2019).

Similarly, the CO-dependent H_2 production using *T. onnurineus* NA1 (MCO1) overexpressing the native carbon monoxide dehydrogenase (CODH) and hydrogenase resulted in a HER of 124 mmol L^{-1} h^{-1}, which is a 3-fold increase in H_2 production potential over the wild-type strain (Kim et al., 2013). Furthermore, the study showed that *T. onnurineus* NA1 (MCO1) was able to grow and produce H_2 at a rate of 60 mmol L^{-1} h^{-1} when fed with CO rich off-gas from a steel production plant, showing the potential of this technology to utilize industrial waste products (Kim et al., 2013). Yet another approach to increase H_2 production from CO was to naturally evolve *T. onnurineus* NA1 to withstand higher CO concentrations, by batch cultivating the cells for 20 h with a 100 Vol.-% CO in the headspace, after which they were transferred to a standard medium. The strain 156T is a result of 156 such selective transfers and was shown actively produce H_2 with a HER of 220 mmol L^{-1} h^{-1} when it was continuously fed with CO at a feeding rate of 800 mL min^{-1}; CO concentrations that were lethal to the wild-type and other mutants (Lee et al., 2016). Lastly, cultivation of the wild-type

T. onnurineus NA1 in a pressurized bioreactor at 4 bar increased its HER to 360 mmol L^{-1} h^{-1} (Kim et al., 2017). Improvements in H_2 production by these and related archaea will come with improved understanding of their metabolism to inform metabolic engineering efforts.

As with all biological production systems, the question of the appropriate feed source plays a role in the feasibility of realizing industrial scale productions. It has recently been proposed that formate would be an ideal industrial "intermediate between the physicochemical and biological realms" (Müller, 2019), where it could function as energy storage derived from various industrial processes, as well as a feed source for the production of value-added products, animal feed and fuels by formatotrophic microbes, creating a formate-based bio-economy (Yishai et al., 2016). The advances in utilization of formatotrophic and carboxydotrophic hydrogenogenic archaea show the potential of this technology to become an additional source of H_2 gas as a clean and renewable energy carrier.

Evaluating the current state of H_2 production by hydrogenogenic archaea, we conclude that the technology is currently at a B-TRL of 3 (Pfeifer et al., 2020). Various organisms have been tested on readily available feed sources, and efforts have been made in the scaling and optimization of growth conditions, as well as in enhancing the organism's productivity through genetic engineering. Yield and productivity of the organisms have been drastically improved, but to our knowledge lifecycle- and techno-economical assessments are still pending.

5. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are produced by a variety of archaea and bacteria as intracellular carbon and energy storage. With over 150 characterized monomers, PHA are the most diverse group of known bio-polyesters, making them extremely interesting and promising as a replacement for various fossil based polymers (Chen, 2010; Kim et al., 2007). This structural diversity allows for the formation of biocompatible homo- and/or hetero-polymers with a range of chemical and physical properties, vastly increasing the range potential applications compared to other bioplastics (Cambridge Consultants, 2018; Doi and Steinbüchel, 2002; Koller et al., 2007a; Korsatko-Wabnegg and Korsatko, 1990; Martin and Williams, 2003). PHA can be categorized into short-chain-length (*scl*) stiff thermoplastic polymers and medium-chain-length (*mcl*) polymers with elastic/latex-like properties. However, PHA makes up only 1.4% of the 2.11 million tons of bioplastics produced in 2018, and its market share is only slowly increasing (Rosenheim et al., 2018). The industrially available PHA is largely produced using bacteria such as *Cupriavidus necator, Azohydromonas* sp. and genetically modified *E. coli,* by a small number of companies (Cambridge Consultants, 2018; Kourmentza et al., 2017; Malacara et al., 2015). Although not yet industrialized, there is another group of organisms that has shown to be promising for large scale PHA production: the haloarchaea.

Haloarchaea constitute a class of the Euryarchaeota that are found in hypersaline environments across the globe and require salt concentrations upwards of 15% (w/v) for optimal growth. Research into PHA producing halophilic archaea has been ongoing since the first isolation of *Haloarcula marismortui* from the Dead Sea in 1972 (Kirk and Ginzburg, 1972). Over the following decades, large strides have been made in an effort to improve archaeal PHA production efficiency and scalability (Koller, 2019; Nicolaus et al., 1999). Haloarchaea have been shown to controllably produce the *scl*-PHA like poly(3-hydroxybutyrate) (PHB), poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBHV) naturally, and the terpolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) (PHBHV4HB) when co-fed with γ-butyrolactone (GBL). Especially these PHBHV4HB terpolyesters, together with poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (PHB4HB)

copolyesters are desirable for high value biomedical applications ranging from artificial blood vessels and implants for bone regeneration to surgical tools, dentistry purposes and for controlled delivery of pharmaceutically active substances (Cambridge Consultants, 2018; Doi and Steinbüchel, 2002; Koller et al., 2007a; Korsatko-Wabnegg and Korsatko, 1990; Martin and Williams, 2003). It has been shown that it is possible to control co-polyester compositions through the selection of carbon sources and precursor substrates, thus producing PHA with desired properties such as melting temperature and crystallinity (Ferre-Guell and Winterburn, 2018, 2019; Han et al., 2015; Hermann-Krauss et al., 2013; Koller et al., 2007a; Koller, 2018; Kourmentza et al., 2017). The ability to control polymer composition in an archaeal PHA-production process has been shown to translate 1:1 from a shaking flask scale to a 2 L fed-batch system running for more than 600 h (Ferre-Guell and Winterburn, 2019). To date, the largest production setup for archaea-based PHA biosynthesis has been a 220 L fed-batch cultivation (Koller, 2015a), the longest recorded continuous production was 3 months (Lillo and Rodriguez-Valera, 1990), and the highest production rate and mass fraction of PHA in cell dry mass (PHA/CDM g g^{-1}) has been 0.21 g L^{-1} h^{-1} (Bhattacharyya et al., 2012) and 87.5% (Koller et al., 2007a), respectively. Although the highest recorded PHA yield using halophilic archaea is lower than that achieved with various bacterial strains, halophiles offer other advantages, which make them extremely interesting additions to the industrialized bacterial PHA producers (Koller et al., 2008; Kourmentza et al., 2017; Margesin and Schinner, 2001; Quillaguamán et al., 2010). Nonetheless, there are still obstacles that need to be tackled until PHA production can compete with established bacterial systems or petrochemical polymers (Bhattacharyya et al., 2015, 2014; Choi and Lee, 1997; Koller et al., 2005).

The price of PHA is largely influenced by the price of the raw materials such as the carbon feed source, nitrogen sources and the copious quantities of salt required for the cultivation (Bhattacharyya et al., 2015, 2014; Choi and Lee, 1997; Koller et al., 2005). The carbon source can account for up to 50% of production costs (Bhattacharyya et al., 2015, 2014; Choi and Lee, 1997; Koller et al., 2005), consequently efforts have been made to identify combinations of suitable inexpensive raw materials such as various industrial wastes and the optimal organisms for the bioconversion of each waste stream into value added products. The waste streams that have been tested as substrates for archaeal PHA production include hydrolysed whey from dairy industry (Koller et al., 2007a, 2007b, 2008; Koller, 2015a; Koller et al., 2015; Pais et al., 2016), vinasse and raw stillage from bioethanol production (Bhattacharyya et al., 2015, 2014, 2012; Pramanik et al., 2012), the crude glycerol phase (CGP) from biodiesel production (Hermann-Krauss et al., 2013; Koller et al., 2005), crude oil and petrochemical wastewaters from petrochemical processing plants (Taran, 2011a, 2011b, 2011c), sugarcane and cassava waste from Sago starch production (Salgaonkar et al., 2019; Salgaonkar and Bragança, 2017) and phenol-rich olive mill wastewater, which has shown to be growth inhibiting to *C. necator* (Alsafadi and Al-Mashaqbeh, 2017; Pfeifer et al., 2020) (Table 4). Although industrial waste streams are cheap carbon sources, many of them must be processed before they can be utilized as feed. Whey, for example, must often be hydrolysed either enzymatically of by acidic hydrolysis to make the carbohydrates available to the microorganisms (Amaro et al., 2019; Doi and Steinbüchel, 2002; Korsatko-Wabnegg and Korsatko, 1990; Martin and Williams, 2003). Enzymatic hydrolysis of lactose in whey is performed at low temperatures and is prone to contaminations, whereas acidic hydrolysis, performed at high temperatures (90°C) and low pH (0.7), produces a sterile product, which must be neutralized and subsequently dialyzed. Both hydrolysis approaches are costly, but it has been shown that when working with haloarchaea, the dialysis step after the acidic hydrolysis with hydrochloric acid solution can be omitted, because when neutralized using sodium hydroxide solution, a salt rich carbon source is produced, which is a prerequisite for cultivating halophilic organisms (Koller et al.,

2016). Furthermore, the lactose in whey is hydrolyzed into glucose and galactose, of which galactose is barely utilized by the best-described haloarchaeal PHA producer, *Haloferax mediterranei*, under normal growth conditions. It has been shown, that upon increasing the amount of the trace element solution SL-6 added to the growth medium, galactose uptake was favoured, increasing the storage yield from 0.33 g g^{-1} to 0.78 g g^{-1} compared to whey extract with lower SL-6 concentrations in previous experiments (Pais et al., 2016). As with many other biotechnological productions, sterility is a major cost and risk factor. The highly saline (150-300 g L^{-1} NaCl) cultivation conditions of haloarchaea allow them to be cultivated with minimal to no sterility precautions (Bhattacharyya et al., 2014; Hermann-Krauss et al., 2013; Koller, 2015b; Koller et al., 2016; Maheshwari and Saraf, 2015; Poli et al., 2011). Although these conditions reduce the risk of contamination, the downside is that the large quantities of salt required for cultivation heavily influence production costs not only in acquisition, but also in desalination of spent medium. This issue has been addressed in several studies, which showed that it is possible to recycle up to 99.3% of medium salts and to reutilize spent cultivation broth as a cheap nitrogen source for subsequent cultivations (Bhattacharyya et al., 2015, 2014; Koller, 2015a). With the recycling of salts and the use of industrial waste products as a feed source, the production costs of PHBHV by haloarchaea can be reduced improving the economic feasibility of PHA production by haloarchaea.

Scalability of archaeal PHA production does not only depend on the cost reduction through cheap feed sources but is also determined by the ease of handling. Experimentation with downstream processing has shown that, unlike productions with the bacterial PHA producer *Cupriavidus necator*, production runs with *H. mediterranei* do not have to be interrupted immediately after the depletion of exogenous carbon source, as 70% of the produced PHA remains after 24 h. The organisms initially consume low molecular weight polymers, thus enriching the high molecular weight fraction prior to extraction in a trading off quantity for quality. Furthermore, untreated cultures can be stored at 4°C for up to 262 h postproduction without loss of polymer or at room temperature for the same period if pasteurized. This allows for flexibility and the accumulation of biomass for large scale extraction runs (Koller, 2015b). PHA is extracted from the biomass through cell lysis, but unlike the bacterial PHA producers which must be lysed chemically, haloarchaea can be lysed in hypotonic media due to their extreme intracellular osmotic pressure.

Evaluating the current state of PHA production, we conclude that archaea based PHA production is currently at a B-TRL of 5 (Pfeifer et al., 2020). Various archaeal organisms have been tested to identify the optimal strain for the bioconversion of different carbon sources, after which the cultivation conditions were optimized, and the scalability displayed. Furthermore, up- and down-stream processes have been studied and optimized for handling and cost reduction. Lastly, techno-economic analyses have shown the competitive potential of the technology and process engineering plans have been proposed on integration of archaeal PHA production into existing industrial plants and processes as part of the emerging concept of bioeconomy.

6. Surface-layer proteins

The surface layer (S-layer) proteins are cell wall components almost ubiquitously among the archaea and are found in the cell envelopes of nearly all taxonomic groups of walled bacteria (Albers and Meyer, 2011; Rodrigues-Oliveira et al., 2017; Sleytr and Sara, 1997). The proteins self-assemble into a highly regular paracrystalline monomolecular lattice reminiscent of a chainmail, covering the entire cell surface (Sleytr et al., 2014). While in bacteria, the S-layer is part of a more complex cell envelope structure, where it is often bound to peptidoglycan or lipopolysaccharides, it constitutes the sole cell wall component in most archaea, bound directly to the cell membrane (Albers and Meyer, 2011; Fagan and Fairweather, 2014; Rodrigues-Oliveira et al., 2017). Both archaeal and bacterial S-layer

proteins are, in most cases, highly glycosylated and their glycosylation profiles play a major role in the glycoprotein's function (Abu-Qarn et al., 2008; Eichler, 2020; Jarrell et al., 2014; Schäffer and Messner, 2004). Although the entire functions of the S-layer glycoproteins have not yet been understood, it has been shown that they are involved in the maintenance of cell morphology and cell division and influence the cells resilience to osmotic stress (Engelhardt, 2007; Jarrell et al., 1982; Mohr and Larsen, 1963; Pum et al., 1991; Wildhaber and Baumeister, 1987; C. Zhang et al., 2019; Zink et al., 2019). Furthermore the glycans found on these proteins have shown to be involved in controlling cell-surface hydration and endow the cell surface with antifouling properties (Schuster and Sleytr, 2015). Because the S-layer lattice is consistently exposed to the harsh environmental conditions of the host´s natural habitats, the archaeal S-layer glycoproteins retain their structure and function in a wide range of conditions, ranging from pH of 1 to pH of 12, temperatures up to 120°C and in various organic solvents, as well as often being resistant to proteases.

These properties give S-layers rise to a whole range of potential technological applications (Table 5). Bacterial S-layers have been utilized for the majority of technological studies and bacterial S-layer based filters were developed by the NANO-S Biotechnologie GmbH in the early 2000s, but never reached commercialization. To our knowledge, the only nanotechnological application of archaeal S-layer proteins is the utilization of fragments from *Sulfolobus acidocaldarius* for the nanostructuring of surfaces and nanocluster formation (Douglas et al., 1992, 1986; Winningham et al., 2001, 1998). By this means, a direct replication of the inverse pattern of the S-layer protein lattice in the form of ordered metal nanodots (Ti, Pd, Au) was feasible. However, in principle the applications described for bacterial S-layer proteins (Table 5) can also be achieved with archaeal S-layer proteins. The latter might have the additional advantage to be more stable at high temperature, pH-value and/or salt concentrations (Cai et al., 2013; Debabov, 2004; Singh and Singh, 2017).

S-layer proteins are among the most abundant biopolymers on the planet (Whitman et al., 1998), and the production capacities are only limited by the ability to produce biomass of the desired organisms and the ability to extract the S-layer proteins from the biomass. The S-layer of each archaeon has evolved to function in the environments of the organism it is protecting. Even though the fundamental function of the S-layer is conserved, the molecular properties and structures vastly differ. Therefore, the isolation techniques vary depending on the organism from which the S-layer proteins are extracted (Nußer and König, 1987; Rodrigues-Oliveira et al., 2019; Sumper et al., 1990; Veith et al., 2009). All the extraction methods have been developed for the study of the archaea S-layer on a lab scale and to the best of our knowledge, there are no publications discussing the scaling of productions or S-layer proteins yield parameters. Recently first indication of the *in vitro* self-assembly of the S-layer proteins of *Sulfolobus solfataricus* have been published, indicating the potential of these proteins for applications similar to those achieved with bacterial ones (Gambelli et al., 2019). Archaeal S-layer proteins could be an interesting value-added product to be extracted from spent biomass produced by various other industrial archaeal based productions in the future. The studies published to date show that S-layer production utilizing *Sulfolobus acidocaldarius* at B-TRL 2 is the furthest advanced S-layer cell factory (Pfeifer et al., 2020).

7. Gas vesicles

Gas vesicles (GVs) are naturally occurring protein-based buoyancy organelles that have been found in photosynthetic and mesophilic bacteria, as well as in halophilic archaea (Pfeifer, 2012). Of the four strains of halophilic archaea know to produce GVs, *Halobacterium salinarum, H. mediterranei Halorubrum vacuolatum* and *Haloquadratum walsbyi*, only *H. salinarum* has been used intensively to explore potential biotechnological applications (Childs and Webley, 2012; DasSarma et al., 2014, 2013; Pecher et al., 2016; Sremac and Stuart, 2010, 2008; Stuart et al., 2004, 2001, 2001). GVs are made up of mainly two proteins,

GvpA and GvpC, and about five additional minor proteins. GvpA is the major structural protein, with GvpC being bound to the external surface of the nanoparticles to stabilize the structure. Possible applications of GVs are the utilization as epitope presenting scaffolds in vaccine development and as contrasting agents for medical diagnostics such as ultrasound and MRI (Bulte, 2018; DasSarma and DasSarma, 2015; Farhadi et al., 2018).

The global vaccine market revenue is expected to grow from 49 to 54.2 billion USD in the year 2019 (Matej, 2019), which is driving innovation on new technological approaches for vaccine development and administration. Catering to the multivalent immune system-pathogens interactions and the growing necessity of subunit vaccines, one biotechnical approach to vaccine design encompasses the production of biological nanostructured scaffolds that can display a multitude of peptide copies to the immune system (Foged, 2011). Inspirations for these nano-scaffolds has been drawn among others from outer membrane vesicles and self-assembling protein scaffolds such as viruses and archaeal gas vesicles (Foged, 2011; Frey et al., 2018; DasSarma and DasSarma, 2015).

Recombinant GVs can be used to display a desired antigen on the surface of the GV, by fusing it to the GvpC protein, thus producing a GV-nanoparticle (GVNP). In 2001, an immune response to a recombinant GVNP displaying a 6 amino acid simian immunodeficiency virus (SIV) epitope was observed for the first time (Stuart et al., 2001). Since then, recombinant GVNPs have been shown to elicit a long-term immune response when displaying subunit-epitopes of *Chlamydia* sp. (Childs and Webley, 2012), *Salmonella* sp., various other SIV-subunits (Sremac and Stuart, 2010, 2008; Stuart et al., 2004), *Plasmodium* spp. (Dutta et al., 2015), and have been shown to rescue mice from endotoxic shock by displaying the human bactericidal/permeability-increasing protein (BPI) and thus reducing the interaction of lipopolysaccharides (LPS) with the Toll-like receptor 4 (Balakrishnan et al., 2016). Further research indicates that protective immunity against *Plasmodium* spp. can be induced by administered *Plasmodium falciparum* Circumsporozoite Protein (CSP) lacking the glycosylphosphatidylinositol (GPI) signal region, but it has not been possible to express the full-length protein in conventional expression systems (Kastenmueller et al., 2013). A subsequent study achieved the expression and purification of the full-length CSP, and successfully displayed it on GVNP of *Halobacterium* sp. NRC-1 (Pecher et al., 2016). Furthermore, it has been shown that is possible to display multiple epitopes on GVNPs by expressing different recombinant GvpC fusion proteins within the same organism (DasSarma et al., 2013)

The use of GVNPs offers several advantages in addition to the ease of designing new fusion proteins introduced by a versatile expression cassette (Stuart et al., 2004) and the ability to present multiple epitopes on a single Nano-scaffold. The GVNP system has been shown to be scalable by ease of production and downstream processing, where 0.5 mg L^{-1} of GVs displaying the desired epitope can be harvested by simply washing the cells in MilliQ water and collecting the floating GVNPs after a single low speed centrifugation (Pecher et al., 2016). Furthermore, GVNPs have been shown to remain stable without refrigeration for extended periods of time, suggesting that this platform is a valuable alternative for vaccines targeting diseases in developing countries (DasSarma et al., 2015, 2014, 2010). To the best of our knowledge, the scale-up of production for recombinant GVNPs has remained at lab-scale, and are extracted from cell lawns grown on agarose plates (DasSarma et al., 2014). The current state of *Halobacterium* sp. NRC-1 based recombinant GV production has therefore been determined to be B-TRL 2 (Pfeifer et al., 2020).

8. Bacteriorhodopsin

Composed of covalently bound barcterioopsin and retinal, bacteriorhodopsin (bR) is a 7-helix transmembrane analogue to the visual rhodopsin in mammalian eyes. It was first

observed in *H. salinarum*, a halophilic archaeon isolated in 1971 (Oesterhelt and Stoeckenius, 1971). When *H. salinarum* cells are exposed to O_2-limited conditions and UV-light, bacteriorhodopsin expression is increased to up to 300,000 copies per cell, making up approximately 75% of the total membrane dry mass (25% consists of lipids) (Oesterhelt and Stoeckenius, 1971, 1973; Ventosa and Oren, 1996; Essen et al., 1998; Haupts et al., 1999). The expressed bR forms distinctly coloured patches in the plasma membrane known as purple membrane (PM). Bacteriorhodopsin is expressed from the *bop* gene cluster along with the bat and prb proteins, which induce bR synthesis under low O_2 and light exposure, respectively (Shand and Betlach, 1994, 1991). Both the subunits and the PM retain their functions from pH of 2 to pH of 12 in temperatures up to 80°C (in solution) and 140°C (dry), are stable in non-polar organic solvents and once dried, have shelf life of years (Knoblauch et al., 2014).

Its stability, photoelectric-, photochromic-, and proton transport-properties have in the past been the source of inspiration for numerous technical applications such as artificial retinal prosthesis, optical memories, holographic associated processors, biosensors such as motion sensors, X-ray detectors and immunosensors, enhancement of photoelectrochemical water splitting, bio-camouflage as well as bio-based and bio-enhanced photovoltaic cells (Al-Aribe et al., 2013; Armstrong and Warner, 2003; Bertoncello et al., 2003; Das et al., 2019; Hampp and Oesterhelt, 2008; Karna et al., 2011; Li et al., 2018; Thavasi et al., 2009; Trivedi et al., 2011; Yen et al., 2011). Although many of these ideas are no longer being pursued, because of new research and development in Material Science, they show the vast potential of this protein. It has further been shown that the properties of bR can be enhanced through directed evolution (Wise et al., 2002; N. L. Wagner et al., 2013). Although vastly interesting, the potential and state of development of bioelectronic technologies based on bR are discussed in great detail elsewhere (Al-Aribe et al., 2013; Bertoncello et al., 2003; Hampp and Oesterhelt, 2008; Karna et al., 2011; Thavasi et al., 2009; Trivedi et al., 2011; Yen et al., 2011) and are outside the scope of this review. The sole focus of this review is the analysis of the biotechnological production capacity of bacteriorhodopsin by cultivation of halophilic archaea, specifically *H. salinarum*.

Although several halophilic archaea can produce bR, *H. salinarum* has become the model organism for industrialization (Table 6) (Kanekar et al., 2016; Pfeifer et al., 2020; Shakuri et al., 2016). *H. salinarum* shares both the cultivation advantages and disadvantages of halophilic archaea that have been discussed above, but unlike the PHA producing strains, *H. salinarum* does not utilize simple carbon sources such as glucose or sucrose. Instead it thrives in amino acid rich concentrated brine, where it synthesises bR as a mode of shifting energy production from oxidative phosphorylation to phototrophy (Oesterhelt and Stoeckenius, 1973; Shand and Betlach, 1991).

Commercially, bR extracted from lab-scale cultivations of *H. salinarum* can readily be purchased from various suppliers including Merck AG, BOCSCI Inc. and Halotek at a price point of 553 € mg^{-1} (CAS: 53026-44-1) (Halotek UG, 2017; Sigma-Aldrich, 2020). Increased research into its technological potential is driving the demand for bR, but only small amounts are available due to the difficulty of cultivation and poor yield. Therefore, it has become increasingly important to improve production capacities and reduce production costs (Ghasemi et al., 2008; Hampp and Oesterhelt, 2008; Kalenov et al., 2016; Seyedkarimi et al., 2015).

Over the last decades, there have been various approaches to increase the bR yield from *H. salinarum*. Cultivation conditions optimized for high yield productions include media composition, nitrogen source, agitation, light intensity, O_2 availability and the removal of inhibitory metabolites. These optimizations have been performed in batch (Ghasemi et al., 2008; Helgerson et al., 1992; Jeganathan et al., 2019; Kahaki et al., 2014; Kushner, 1966;

Rajab et al., 2019; Seyedkarimi et al., 2015), fed-batch (Kalenov et al., 2016) and repetitive batch (Shiu et al., 2015) and were in part based on *Design of Experiment* optimizations (Ghasemi et al., 2008; Manikandan et al., 2009; Seyedkarimi et al., 2015). In 1998, Lee *et al*. was the first to show that the CDM and bR yield of a 1.5 L culture grown on carbon/nitrogen limited substrate (0.2 g L^{-1} yeast extract) can be increased by multiples of 16 and 23 to final concentrations of 30.3 g L^{-1} and 282 mg L^{-1} (1.2 g L^{-1} h^{-1}) respectively, by removal of inhibitory metabolites from the culture through cell-cycle cultivation (Lee et al., 1998). Ghasemi *et al.* later showed that a batch cultivation (4 L) on a more complex carbon/nitrogen source (casamino acids 3.75 g L^{-1}, meat extract 10 g L^{-1} and corn steep liquor powder 50 g L^{-1}) yielded similar quantities (234.6 mg L^{-1}) (Ghasemi et al., 2008). Although this approach doubled the volumetric productivity (2.4 g L^{-1} h^{-1}), it required a much higher quantity of substrate, thus drastically increasing the production cost. The highest production to date was achieved using a mutated strain (KSK 03307) that was produced utilizing UV-mediated directed evolution selecting for elevated levels of bR and reduced levels of carotenoids (Kalenov et al., 2016). Using this strain, a yield of 1750 mg L^{-1} from 45 g L^{-1} CDM with a productivity of 0.6 g L^{-1} h^{-1} was achieved in a 3 L fed-batch cultivation over 6 days. During the cultivation, illumination was steadily increased and inhibitory metabolites were removed utilizing activated charcoal. This bioprocess not only produced the highest recorded yield of bR, but also decreased the expression of carotenoids, thus facilitating the downstream processing of bR and PM.

The downstream processing to extract bR for technical applications has until recently been based on a tedious and lengthy sucrose density gradient ultracentrifugation, prior to which cells were lysed in deionized water and PM enriched through differential centrifugation (Oesterhelt, 1974). Optimization of the downstream extraction process has been performed by Shiu *et al*., who managed to reduce the processing time for bR extraction 10-fold from 27 h to 2.5 h (Shiu et al., 2013). This was achieved by extracting the bR using an aqueous two-phase separation (ATPS), where the bR could be recovered from the PEG-phosphate interface, which eliminated the need for a sucrose gradient. This new process propels the PM technology closer to large scale applications. This method was later improved upon to create the one-step-three-phase extraction system (A3PS). By the addition of CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and polypropyleneglycol (PPG) to the PEG (Poly(ethylene glycol) and potassium phosphate based ATPS, the PM fragments were delipidated during the extraction, and could be collected from the PEG-phosphate interface. This approach yielded a 89.7% bR recovery rate and a threefold reduction of lipid content (Shiu et al., 2014). Post both ATPS and A3PS extraction, bR had to be further purified by removing any residual PEG by ultrafiltration. The purified, A3PS extracted bR was cleaner than, and showed a 60% higher photocurrent generation than, the bR extracted utilizing the ATPS method.

Bacteriorhodopsin is in demand for both research and technological application, but due to the difficulty of *H. salinarum* cultivation and low bR yield, currently only small amounts at large price points are available. Although advancements in understanding the physiology of the organism has allowed for the optimization of cultivation conditions to improve bR production, the commercialization of the bR based technologies remains limited due to the high production cost (Kalenov et al., 2016; Patil et al., 2012; Seyedkarimi et al., 2015; Shand and Betlach, 1991; Shiu et al., 2014). Based on the data presented and the fact that *H. salinarum* bR is commercially produced by Halotek, we have defined the archaeal production of Bacteriorhodopsin to be at B-TRL 3C (Pfeifer et al., 2020).

9. Isoprenoids

Isoprenoids are key precursors of archaeal cell envelope compounds such as tetraether lipids, carotenoids and methanophenazine (Matsumi et al., 2011). Archaea have therefore

been suggested as platform organisms for the synthesis of a range of isoprenoids (Liman et al., 2019).

9.1. Ether- and Tetraether-Lipids

Archaea share many features with Bacteria and Eukarya, but the composition and structure of their lipids are one of several characteristic that uniquely sets them apart (Sprott, 1992). While bacterial lipids are characterized by an unbranched and often unsaturated fatty acid core bound to a sn-glycerol-3-phosphate backbone by ester-linkage, archaeal lipids are composed of a saturated isoprenoid based core bound to a sn-glycerol-1-phosphate backbone by ether-linkage (Benvegnu et al., 2008). The archaeal ether lipids come in a wide range of variations, each with unique physiochemical properties. The most abundant being the archaeol (diether) and its dimer caldarchaeol (tetraether) with all other core lipids being derivatives of these (Jain et al., 2014; Nishihara et al., 1987). The polar archaeol is ubiquitous to all archaea and forms a bilayer similar to that formed by bacterial phospholipids, whereas the bi-polar caldarchaeols are found in extreme thermophiles, acidothermophils and methanogens (Baumann et al., 2018; Taubner et al., 2019) forming de-facto monolayers (De Rosa, 1996; Sprott, 2011). However, the term caldarchaeol summarizes a variety of different ether-lipids. Therefore, the different caldarchaeols must be more unambiguously named when it comes to its identification of within an archaeon. Compared to phospholipids, the physiochemical properties of the archaeal lipids have higher resistances to oxidative stress, phospholipases as well as a wide range of pH-values and temperatures, thus making them highly interesting as additions to, or replacements for, phospholipids in liposome-based commercial applications (Sprott, 2011).

Liposomes are in their simplest form, synthetic uni- or bi-lamellar lipid vesicles produced from phospholipids. Their physical properties can be customized with the addition of various amphiphilic compounds or sterols such as cholesterol (Gregoriadis and Perrie, 2010). The ability of liposomes to both entrap pharmaceutical compounds in the aqueous phase and incorporate lipid-soluble compounds in the lipid phase has led to the development of an array of commercially available and clinically applied products for skin-care, drug-delivery and as adjuvant additives and antigen presenting scaffolds for vaccines (Bozzuto and Molinari, 2015; Bulbake et al., 2017). Additionally, proposed applications include the use of liposomes for gene delivery, as contrasting agents in diagnostics such as nuclear imaging and ultrasound, and for the encapsulation of additives and ingredients in the food processing industry (Nkanga et al., 2019). Liposomes composed entirely of, or including archaeal di-and tetra-ether lipids, are referred to as archaeosomes (Kaur et al., 2016; Sprott et al., 1996). It has been shown that the physiochemical properties of the archaeal lipids are transferred to archaeosomes, which have an increased resistance to phospholipases, bile salts, wider pH ranges and temperatures. These properties endow the liposomes with increased stability, allowing them to be autoclaved without leakage of entrapped compounds and for an increase the shelf-life of archaeosome-based products. (Sprott, 2011).

The first archaeosomes were produced from extracted and purified total polar lipids (TPL) from halophiles, methanogens, and thermophiles. The *Methanobrevibacter smithii* based TPL archaeasomes, delivering entrapped antigens, showed an improved immune response compared to that triggered by phospholipids. Their robust and long-lasting immune response was shown to be attributed to the adjuvating properties of the caldarchaeol content (Krishnan et al., 2003, 2000), but the batch-to-batch dependent lipid composition of *M. smithii* made it difficult to consistently produce archaesomes with identical compositions (Stark et al., 2019). To overcome these limitations, semi-synthetic lipids were developed by covalently linking a sulphated saccharide group to the free sn-1 hydroxyl backbone of archaeal core lipids extracted from *H. salinarum (*containing 100% archaeol), to create sulphated lactosyl archaeol (SLA) (Kates et al., 1993; McCluskie et al., 2017). Interestingly, the biocompatibility

of *H. salinarum* lipids has been known since 1991, when the *H. salinarum* strain ORE was isolated from the Thai fish sauce *Nnam Pla* (Thongthai and Suntinanalert, 1991). Simplified antigen-encapsulating SLA-archaeosomes have shown to induce an immune response similar to that produced by the traditionally formulated MS-archaeosomes, while improving productions consistency and reducing production costs (Akache et al., 2018). The ease of synthesis and production efficiency of archaeosome-based adjuvants was further improved, when it was shown, that the immune response retained the same robustness and longevity whether the antigens were tediously encapsulated in the SLP archaeosomes or were simply admixed with the SLP archaeosomes (Jia et al., 2019; Stark et al., 2019). Although pre-clinical studies have shown that SLP archaeosomes admixed with antigens are equal or even superior to commercial adjuvants such as aluminium hydroxide, they have not yet been tested clinically (Akache et al., 2019, 2018).

Another application of archaeal lipids, which has been shown to work in mice, is the oral delivery of antigens and drugs utilizing *Sulfolobus*-based archaeosomes. While conventional liposomes lost up to 61% of their payload within 90 min of exposure to the acidic GI environment, archaeosomes based on the polar lipid fraction E (PLFE) of *S. acidocaldarius* tetraether lipids only lost 30% (Li et al., 2011). This 2-fold increase in delivery efficiency has led to an increased immune response in mice, orally immunized with archaeosomes encapsulating IgG antibodies. Other studies have shown the same improvements in stability and reduction of leakage in peptide base therapeutics such as insulin, and in the controlled drug release (Li et al., 2010).

To date, the *H. salinarum* archaeol and *S. acidocaldarius* PLFE needed for the production of various archaeosomes are produced by the authors themselves or by collaborators (Bligh and Dyer, 1959; Jia et al., 2019). Furthermore, a di-ether lipid mix, offering an alternative to synthetic phospholipids to manufacture highly stable liposomes, can be purchased from Halotek, which cultures *H. salinarum* in 0.4 m^3 bioreactors. Tetraether lipids from *Thermoplasma acidophilum* are extracted according to a patented methods and can be purchased through Matreya LLC (Catalog #:1303) (Bakowsky et al., 2004) and several synthetic lipids are available through Avanti Polar Lipids. The production of lipids is only limited by the fermentation scale and biomass concentrations, as well as the downstream processing of the biomass, but not much work has been published on scaling-up or optimizing this process. According to the analysed publications, we have determined the bi- and tetra-ether production to be at B-TRL 3C (Pfeifer et al., 2020).

9.2. Squalene

Squalene is an unsaturated triterpene intermediate of cholesterol biosynthesis produced via both the methylerythritol 4-phosphate (MEP) and mevalonate (MVA) pathways by plants, animals, and microorganism. Since its first isolation from a shark liver in 1916, it has, among others, been used in cosmetics, food processing, drug delivery, adjuvants in vaccine development and cancer treatment (Gohil et al., 2019; Lozano-Grande et al., 2018; Tsujimoto, 1916). Although annual global market demands have risen to over 2.7 kt, unfortunately shark liver has remained one of the main sources of squalene. It is estimated that 100 million sharks are killed annually, with 3000 shark livers producing 1 ton of squalene (Ciriminna et al., 2014; Gohil et al., 2019; Worm et al., 2013). The ever-rising demand for squalene and decimating shark populations have driven research to more sustainable production, but to date no other commercially viable source with similar squalene concentrations has been identified. Currently commercialized plant sources including olive oil, amaranth, rice-bran- and wheat germ-oils account for >50% production, whereas synthetic squalene produced through yeast fermentation of plant feed by Amyris Inc. accounts for >10% of the global production (Global Market Insights, Inc., 2016).

While yeast-based squalene productions are commercially established, and much has been published on improving bacterial biosynthesis, relatively little work has been published on advancing archaea for commercial squalene production (Xu et al., 2016). The highest production of 230 g L^{-1} achieved with bacteria was accomplished using a genetically engineered *E. coli* strain (Katabami et al., 2015). Among the archaea, squalene production has been reported for a wide range of methanogens, e.g., *Methanocaldococcus villosus* and *Methanothermococcus okinawensis*, *Sulfolobus* sp. and *H. salinarum* (Baumann et al., 2018; Fuke et al., 2018; Gilmore et al., 2013; Kushwaha et al., 1975; Tornabene et al., 1979, 1978). To our knowledge, the only archaeal squalene commercially available is produced by Halotek using *H. salinarum*. According to the analysed publications, research into squalene production in archaea is in B-TRL 2C (Pfeifer et al., 2020).

9.3. Geraniol

Geraniol is an acyclic isoprenoid monoterpene found in aromatic plants, has a rose-like odor and sweet rose-like citrus taste. It is widely used in deodorants, household products, cosmetics, insect-repellents and has shown promise in medical applications as an anti-inflammatory, antimicrobial and antitumor compound (Chen and Viljoen, 2010). Geraniol for industrial applications is usually extracted from essential oils of a variety of plants, but the rising market demand and limited production capacities of geraniol from plants has been driving research to develop alternative sources (Lee et al., 2019). The highest microbial production of 182.5 mg L^{-1} and 1.68 g L^{-1} have been achieved in *E. coli* and *S. cerevisiae*, respectively (Jiang et al., 2017; Zhou et al., 2014). After optimization in fed-batch mode, a genetically-engineered *E. coli* produced up to 2 g L^{-1} geraniol in complex medium from carbohydrates (Liu et al., 2016). Archaeal-based production of geraniol was achieved in *M. maripaludis* S0027, through the heterologous expression of a geraniol synthase (GES) derived from *Ocimum basilicum*. The engineered *M. maripaludis* strain produced a mere 4.6 mg geraniol g^{-1} of CDM when grown on H_2/CO_2 or formate in a two phase culture overlain with decane (Lyu et al., 2016). The advantage of using methanogens for geraniol production is that they are capable of producing geraniol by CO_2 reduction and not from carbohydrates. The production of geraniol is thus at B-TRL 2 (Pfeifer et al., 2020).

9.4. Carotenoids

Carotenoids, a subfamily of isoprenoids, are one of the most diverse group of secondary metabolites. They are yellow, orange, and red pigments produced by a variety of bacteria, algae, fungi, plants and archaea, where they perform functions such as photosynthesis, photoprotection and protection from oxidative stress. Animals require carotenoids as retinoid precursors, as antioxidants, and as colorants. They acquire these through the consumption of carotenoid-rich foods. Industrially, carotenoids are used as feed and food additives, colorants, in cosmetics and have shown to have wide range of health benefits (Maoka, 2020). To date, over 1,100 carotenoids have been identified, and more are continuously being discovered (Yabuzaki, 2017). The global carotenoid market is expected to be USD 1.5 billion by 2021 with ß-carotene (26%), astaxanthin (25%), lutein (18%), canthaxanthin (10%) and lycopene (6%), making up the majority of demanded products, while the demand for bacterioruberin is growing, as the first bacterioruberin containing products are commercialized (Rammuni et al., 2019). Many of the most demanded carotenoids can be produced synthetically with a high yield and purity. However, the synthesis of certain carotenoids is very complex and expensive, and some stereoisomer by-products may not be active or have unwanted side effects. This, and the fact that regulatory agencies and consumers prefer natural products, have increased research interests into biological sources (Rodrigo-Baños et al., 2015). Natural carotenoids are produced largely by extraction from vegetables and plants, but biotechnological productions utilizing microalgae (prime examples: *Dunaliella salina* for β-carotene production, *Haematococcus pluvialis* as

astaxanthin producer), fungi and bacteria are becoming more relevant. Although archaea have been described to produce relevant amounts of lycopene, canthaxanthin and bacterioruberin, the studies mostly focus on pigment characterization, whereas publications on production rates and improvements are scarce, limited to laboratory scale, and the different quantification methods throughout the studies make it difficult to compare the data (Table 7) (Calegari-Santos et al., 2016; Giani et al., 2019; Pfeifer et al., 2020; Vega et al., 2016).

9.4.1. Lycopene

Lycopene is a precursor of most carotenoids such as ß-carotene and bacterioruberin; it is widely used in food supplements (E 160d), pharmaceuticals, and cosmetic products, because of its anti-carcinogenic and anti-oxidative properties; and as colorant in the food industry (Gajowik and Dobrzyńska, 2014; Sumper et al., 1976). Currently, commercially available lycopene is sourced either through chemical synthesis from petrochemicals or from natural sources such as tomatoes, algae, carrots and fermentation processes utilizing *Blakeslea trispora* and *Phycomyces blakesleeanus*, depending on the quality and purity necessary for the desired applications (Chandi and Gill, 2011; Ciriminna et al., 2016). Conventional extraction methods from vegetables require food grade organic solvents or pressures over 400 bar and are time consuming, thus much work is being done to improve the fermentative production (Ciriminna et al., 2016; Wang et al., 2019). While the lycopene content in tomatoes can reach 6.7 mg g^{-1}, the highest fermentative yield has been achieved utilizing engineered *E. coli* (448 mg g^{-1} in a microtiter plate and 67 mg g^{-1} in 50 mL culture) and *S. cerevisiae* (55.6 mg g^{-1} in a 5 L bioreactor) (Chen et al., 2016; Coussement et al., 2017; Xu et al., 2018). Among the archaea, genetically engineered *H. mediterranei* has shown the most promise in its commercialization for lycopene production. Although it does not naturally accumulate the bacterioruberin precursor lycopene, it has been shown to grow on a large variety of carbon sources, has a higher specific growth rate than any know member of the Halobacteriaceae, and under optimized conditions, produces large amounts of bacterioruberin (Chen et al., 2015; Oren and Hallsworth, 2014). High lycopene accumulation in *H. mediterranei* was achieved through metabolic engineering, specifically by disrupting bacterioruberin and PHBV biosynthesis as well as heterologous expression of phytoene-synthase (CrtB) and desaturase (CrtI) from the carotenogenic haloarchaea *Haloarcula hispanica*. The engineered strain, producing lycopene at 119 mg g^{-1} CDM, was further adapted for industrial production by reintroduction of a functional *pyrF* gene, allowing for the omission of uracil from the medium and consequently reducing cultivation costs (Zuo et al., 2018). Although the production conditions of the engineered lycopene producing *H. mediterranei* strain were not yet optimized that the strains grow at the same specific growth rate or to the same OD value as the *E. coli* strains, the extraction of lycopene from halophiles is much simpler and thus cheaper than from *E. coli*. Halophilic archaea, therefore, remain an interesting addition to the collection of industrialized lycopene sources and, based on the analysed publications, archaea-based lycopene production has been assessed to be at B-TRL 3 (Pfeifer et al., 2020).

9.4.2. ß-carotene

The lycopene-derived ß-carotene is a retinal precursor and as such, one of the most important provitamin A supplies in the human diet (Weber and Grune, 2012). It's role as a vitamin A source as well as its colouration, anticancer and antioxidant properties have made ß-carotene the most important industrially produced carotenoid (Barreiro and Barredo, 2018). Although the share of biologically sourced ß-carotene is increasing, industrially the majority of ß-carotene is produced through chemical synthesis. Natural ß-carotene is either extracted from plants such as carrots (99 mg g^{-1} wet weight), kale (54 mg g^{-1} wet weight) or red paprika (32 mg g^{-1} wet weight) or produced through microbial biosynthesis. The highest industrially

utilized ß-carotene producers include the eukaryotic algae *D. salina* (100 mg g^{-1} CDM) and *B. trispora* (29 mg g^{-1} CDM), but additional organisms with industrial potential have been described (Barreiro and Barredo, 2018; Wang et al., 2019). In archaea, ß-carotene is vital as a precursor of retinal, one of the two building blocks of bRs (Dummer et al., 2011). Trace amounts of ß-carotene were detected in *Haloferax alexandrines, H. salinarum*, and *Halobrum* sp. TBZ126, but targeted ß-carotene production in archaea has not been reported (Asker and Ohta, 2002; El-Sayed et al., 2002; Hamidi et al., 2014). Research into bR production has shown that haloarchaea can produce large quantities of ß-carotene as an intermediate in rhodopsin biosynthesis, which could hold potential for metabolic engineering. Archaeal ß-carotene production has been determined to be at B-TRL 1 (Pfeifer et al., 2020).

9.4.3. Canthaxanthin

Canthaxanthin, an oxygenated ß-carotene derivative, is a high-value secondary metabolite that is commercially available. It is used in applications in cosmetics, chemical-, medical and the food industry, where it is used as a feed additive (E 161g) for enhancing the red colour of egg yolk, salmon and rainbow trout meat, as well as shrimp and other crustaceans (Esatbeyoglu and Rimbach, 2017; Inc, 2018). The vast majority of available canthaxanthin is produced through chemical synthesis, while natural canthaxanthin produced through microbial fermentation of algae, bacteria and filamentous fungi is gaining in popularity (Barreiro and Barredo, 2018). The highest microbial productions to date of 206 mg g^{-1} CDM was achieved using the fungus *Aspergillus carbonarius* in batch (Gharibzahedi et al., 2013; Krupa et al., 2010). The only investigation into archaeal canthaxanthin production achieved a yield of 0.7 mg g^{-1} CDM, utilizing *H. alexandrinus*. Although this yield is lower than that achieved with other organisms, the non-sterile growth conditions and the ease of canthaxanthin extraction from this halophilic organism compared to the extraction from fungi or bacteria, could compensate the inferior yield in potential industrialization of *H. alexandrines* (Asker and Ohta, 2002). Therefore, the current publications on canthaxanthin production by *H. alexandrinus* allow for the technology to be at B-TRL 2 (Pfeifer et al., 2020).

9.4.4. Bacterioruberin

Bacterioruberin is a bright red, lycopene-based C_{50} carotenoid produced by most members of the Haloferacaceae as well as some bacteria, such as *Rubrobacter radiotoleransis* (Rodrigo-Baños et al., 2015; Saito et al., 1994). It has been shown to protect the cells from gamma-ray radiation, oxidative stress and UV-radiation, and to be involved in DNA repair (Dundas and Larsen, 1963; Shahmohammadi et al., 1998, 1997; Singh and Gabani, 2011). A recent study compared the antioxidant capacities of various carotenoids using a Trolox-Assay (Trolox Equivalent Antioxidant Capacity), and found that bacterioruberin had the highest antioxidant capacity, with capacity 2.8 fold higher than that of ß-carotene (Mandelli et al., 2012). In this context, a recent study showed that the addition a bacterioruberin-rich extract to freezing and thawing sperm significantly improved the viability and mobility of sperm by integrating into the sperm membrane (Zalazar et al., 2019). Industrially, bacterioruberin has found applications as a skin-protective ingredient in personal care products and as an effective antioxidant, providing a natural alternative to synthetic antioxidants such as butylhydroxytoluol (BHT). In its application as a colouring agent in the food industries, a few milligrams are enough to uniformly stain 100 kg of product (personal correspondence with Halotek). These properties make bacterioruberin extremely interesting for commercial applications.

Bacterioruberin has been identified as the major carotenoid in *Haloferax volcanii*, *Haloarcula japonica*, *H. salinarum*, *Halorubrum sodomense*, *H. mediterranei* and *Haloarcula vallismortis* (Jehlička et al., 2013; Mandelli et al., 2012; Naziri et al., 2014; Ronnekleiv, 1995; Yatsunami et al., 2014), and various approaches have been attempted to increase the yield of bacterioruberin. Interestingly, it was observed that the production of bacterioruberin in

these archaea increases when the organisms are subjected to osmotic stress. It is therefore believed that bacterioruberin has a membrane stabilizing function (Asker et al., 2002; Bidle et al., 2007; D'Souza et al., 1997; Fang et al., 2010; Montero-Lobato et al., 2018; Naziri et al., 2014). For example, the yield of bacterioruberin from *H. volanii* increased 1.7-fold under low salt conditions (Bidle et al., 2007). However, the increased production under osmotic stress brought the problem of decreased cell growth with it. Addressing this, a 2-step production of cultivation of *H. mediterranei* was tested in a 20 L jar-fermenter. For this cultivation, biomass was first produced under optimal growth conditions, after which the cells were transferred to a hypoosmotic medium optimized for bacterioruberin production. This increased the production 6.4-fold from 0.095 A_{494nm} mL^{-1} broth to 0.604 A_{494nm} mL^{-1} broth (Fang et al., 2010). Although increasing yield, this process also increased the number of cultivation steps, thus increasing the amount of work needed for the production. A later study showed that both the bacterioruberin yield from *H. mediterranei* and its biomass concentrations could be increased in a single step cultivation by cultivating at lower salt concentrations with optimized conditions (Chen et al., 2015). *H. mediterranei* produced 125 mg L^{-1} carotenoids at a salt concentration of 230 g L^{-1} with a maximal cell density of $7.7\ 10^9$ cells mL^{-1}, whereas a maximum cell density of $9.2 \cdot 10^9$ cells mL^{-1} was reached at a salt concentration of 144 g L^{-1}, resulting in a carotenoid production of 555 mg L^{-1} containing 52.4% (291 mg L^{-1}) bacterioruberin. This increase in productivity, corresponding to a 4.4-fold increase in yield and a 20% increase in biomass, is the highest recorded production by a wild-type strain (Chen et al., 2015). While studying the role of the *H. volcanii* LonB protease in membrane composition control, it was observed that *lonB* conditional mutants were hyperpigmented. These hyperpigmented mutants (HVLON3) showed a 14-fold increase in bacterioruberin production (220 mg g^{-1} CDM) compared to the wild type (13.8 mg g^{-1} CDM); the highest yield ever observed in haloarchaea (Cerletti et al., 2014; Zalazar et al., 2019).

As with other haloarchaeal productions, the extraction of bacterioruberin through cell lysis can be achieved by washing the cells with deionized water. This gives the halophiles an advantage over the more elaborate extraction from *R. radiotoleransis.* Although productions have only been published on laboratory scale, bacterioruberin is commercially available as "Halorubin" produced by Halotek in Germany. Bacterioruberin production by archaeal cell factories has therefore been determined to be at B-TRL 3C (Pfeifer et al., 2020).

10. New frontiers in metabolic engineering of archaea

10.1. Acetate

Acetate and acetic acid are used as platform chemicals for the production for compounds such as vinyl acetate, acetic anhydride or esters (Vidra and Németh, 2018) used in products such as include glues, paints, synthetic fibres, bottles or film (David et al., 2020; Erickson et al., 2012; Vidra and Németh, 2018). Acetate can also serve as a substrate for many microorganisms, which may convert it to a product of choice in a two-stage process. An example for this would be the acetate based synthesis of ethanol (Nissen and Basen, 2019). The biosynthesis of acetate by anaerobes usually involves acetyl-coenzyme A (acetyl-CoA) as an intermediate, which in turn is a precursor in many microbial pathways ending in products of commercial interest, such as short-chain fatty acids, lipids, isoprenes or PHA (Krivoruchko et al., 2015). The annual global market for acetic acid in 2015 was about ~13 million tons (Vidra and Németh, 2018), of which about 90% are produced chemically. Biologically, acetate can be produced under oxic conditions from ethanol by acetic acid bacteria, and anaerobically by acetogens either though the fermentation of sugars or from H_2 and CO_2. The aerobic production of acetate from ethanol is utilized in the production of vinegar, where, *S. cerevisiae* first converts sugars to ethanol, which is subsequently oxidized by aerobic acetic acid bacteria. On a laboratory scale, titres, productivities and yields of up to 200 g L^{-1}, 1.8 g L^{-1} h^{-1} and 0.96 g g^{-1} have been achieved, respectively (Vidra and Nemeth,

2018). In terms of yield, anaerobic (homo)acetogens are preferred biocatalysts, since theoretically, they convert one glucose molecule to three molecules of acetate. They do so by re-channelling the electrons from sugar oxidation to the Wood-Ljungdahl pathway to reduce the (produced) CO_2 to a third molecule of acetate (Müller, 2019). Moreover, they can be employed to utilize syngas for the production of acetate.

Acetate is a common product among the anaerobic archaea. When for example, H_2 is produced at high yields (4 mole H_2 per mole C6 sugar) in Thermococcales, acetate is produced at a rate of 0.5 acetate per H_2. Nonetheless, it has not been proposed in the literature to utilize or extract the remaining acetate from the bioreactor, or to supply it in a second stage bioprocess. Furthermore, it has been shown, that some methanogens possess the ability to reverse the methanogenesis pathway and perform "trace CH_4 oxidation" to produce acetate. This reversal has been shown to be facilitated by the mcr found in anaerobic methanotrophic archaea. Furthermore, the mcr of ANME-1 (anaerobic CH_4-oxidizing archaea-1) was shown to be up to 25 times more efficient than that found in *M. marburgensis*, with an estimated specific enzyme activity of up to 280 nmol min^{-1} mg Mcr $protein^{-1}$ (Hinrichs et al., 1999; Timmers et al., 2017). Through heterologous expression of the ANME-1, Mcr in *M. acetivorans*, an acetate producing strain that can be cultivated anaerobically on CH_4 was created (Soo et al., 2016). This mutant was shown to consume 15% of CH_4 at a production rate of 86 µmol L^{-1} h^{-1} acetate; three times the efficiency of ANME-1. Although further improvements are necessary, these findings show the possibility of converting CH_4 into acetate (Soo et al., 2016). The study should primarily be seen as proof-of-concept for engineered CH_4 oxidation to a product, rather than a proof-of concept for CH_4 conversion to acetate. Nonetheless, in a future process, the produced acetate could be used as a precursor in industrial synthesis of commercial chemicals such as butanol, or as a feed for microbial factories. The current state of research has allowed us to place the archaeal production of acetate in B-TRL 2 (Table 8) (Pfeifer et al., 2020).

10.2. Ethanol and other alcohols

Biologically produced ethanol and other alcohols are of prime interest as alternative fuels in a low carbon economy. By production volume, ethanol is the most important biotechnological product, with an annual global production of about 100 billion litres (Jansen et al., 2017). Currently, the main production process is the fermentation of cane sugar or hydrolyzed corn starch by *S. cerevisiae* (which is, of course, also utilized in the production of wine and beer), at >90% theoretical yield, titres of 210 g L^{-1} and productivities of 2-3 g L^{-1} h^{-1} (Jansen et al., 2017). Numerous demonstration plants for the multi-step production of ethanol from lignocellulose, in which the last step is the fermentation of soluble sugars to ethanol have been built (Rosales-Calderon and Arantes, 2019).

Anaerobic bacteria, especially those belonging to the class *Clostridia* harbour some advantages over yeasts in the production of bio-alcohols. One particular advantage being the different substrate spectra. Some *Clostridia* spp. are natively able to utilize polysaccharides such as crystalline cellulose (Olson et al., 2015) with other, acetogenic species able to utilize syngas for the production of ethanol. While the starting point of ethanol formation from sugars in *Zymomonas mobilis* or in *S. cerevisiae* is acetaldehyde, anaerobic bacteria decarboxylate pyruvate to acetyl-CoA, which is subsequently reduced in two steps via acetaldehyde to ethanol (Pei et al., 2010). Among the thermophilic bacteria, engineered strains of *Geobacillus thermoglucosidasius* and *Thermoanaerobacterium saccharolyticum* are reported to produce 15.9 L^{-1} and 61 g L^{-1} from glucose and xylose, respectively; with yields close to the theoretical maximum (2 mol ethanol per mol glucose) and productivities of around 2.1 g L^{-1} h^{-1} (Olson et al., 2015). The latter two examples actually demonstrate the potential of unusual microorganisms, generally not seen as industrial workhorses; not unlike most archaea.

Butanol can be utilized as a paint additive or plasticizer (Rosales-Calderon and Arantes, 2019), and, like isobutanol, its properties as a fuel are superior to those of ethanol. It is a native product in some solventogenic species such as *Clostridium acetobutylicum or Clostridium beijerinckii*. With engineered strains of *C. acetobutylicum*, achieving concentrations of up to 20 g L^{-1} and productivities of up to 0.38 g L^{-1} h^{-1} were possible (Li et al., 2020). Isobutanol is utilized as platform chemical for e.g., the production of coatings, paints and pharmaceuticals (Rosales-Calderon and Arantes, 2019). It is not natively produced in microorganisms. However, a recombinant strain of *E. coli* has been shown to convert 36 g L^{-1} glucose to 22 g L^{-1} isobutanol in 112 h, with a yield of 0.35 g g^{-1} (Atsumi et al., 2008).

Archaea are not known to natively produce ethanol or other alcohols as major fermentation end-products (Basen et al., 2014; Keller et al., 2017; Machielsen et al., 2006). However, the insertion of bacterial bifunctional aldehyde/alcohol dehydrogenase *adhE* genes from different thermophilic ethanol producers into the chromosome of the hyperthermophilic archaeon *P. furiosus* led to the production of up to 0.2 g L^{-1} (4.1 mmol L^{-1}) ethanol (Basen et al., 2014; Keller et al., 2017). In contrast, a strain overproducing a primary NADPH-dependent alcohol dehydrogenase A only produced 1.2 g L^{-1} ethanol from maltose or cellobiose during growth, at a productivity of 0.02 g L^{-1} h^{-1} (Basen et al., 2014) .

The *P. furiosus* strain A, lacking aldehyde dehydrogenase described above, produced ethanol from acetate by direct reduction via the enzymes aldehyde:ferredoxin oxidoreductase (AOR) and alcohol dehydrogenase (ADH), and not from acetyl-CoA, termed the AOR-ADH pathway (Basen et al., 2014). An advantage of the AOR-Adh pathway is the broad substrate specificity of both AOR and Adh, enabling the production of a variety of alcohols. Consequently, cell suspensions of the Adh overexpressing *P. furiosus* strain A (strain ADHA, MW608) reduced different aliphatic-, branched-chain- and aromatic-organic acids to their corresponding alcohols (Basen et al., 2014) at concentrations of 20 to 40 mmol L^{-1} (van den Ban et al., 1999). The insertion of the CO dehydrogenase operon of *T. onnurineus* further enabled it to reduce organic acids with electrons derived from CO (and H_2). When providing 105 mmol L^{-1} of isobutyrate and CO in the headspace, strain A/CODH achieved a concentration of 70 mmol L^{-1} isobutanol at a rate of 1 mmol L^{-1} h^{-1} (Basen et al., 2014). New evidence is increasingly revealing that the AOR-Adh pathway is more widespread among the archaea than previously assumed (Nissen and Basen, 2019). Novel homologues may lead to an improvement of rates, yields and concentrations in archaeal acid reduction in the future.

Lastly, butanol production from maltose has been reported for engineered strains of *P. furiosus* (MW164 and MW196) with a product concentration of 1.0 mmol L^{-1} (Keller et al., 2015). This was achieved by heterologous expression of a “hybrid” butanol fermentation pathway found in various thermophilic bacteria. While rates, yields and concentrations are not yet competitive with other means of production, there are only a few other examples of butanol production by thermophilic microorganisms (Bhandiwad et al., 2014; Tian et al., 2019), and this proof-of-concept study is an example of what is possible with genetic engineering, and how it could help shape the future of Archaea Biotechnology.

While archaea have not yet been engineered primary producers of alcohols, particularly the broad substrate range of the AOR-Adh pathway for acid reduction opens up new possibilities to produce different alcohols. Moreover, the high cultivation temperatures of archaeal thermophiles, facilitates alcohol removal from culture supernatant (Zeldes et al., 2015). Based on the presented data, the production of alcohols in archaea is currently at B-TRL 3 with a proof-of-concept based on genetically engineered strains and successful improvement of strains addressing bottlenecks (Table 8) (Pfeifer et al., 2020).

10.3. Lactic acid

Lactic acid has wide range of industrial applications ranging from cosmetics and food to pharmaceuticals, where it is used among others as an exfoliant, preservative and an electrolyte in intravenous solutions, respectively. Furthermore, optically pure lactic acid is the precursor of poly(lactic acid), a bio-degradable plastic (McAnulty et al., 2017). The demand for lactic acid was around 490,000 t in 2017 (Miller et al., 2019) with an annual increase of 5-8% (Abdel-Rahman et al., 2013). Biologically, lactate may be produced from different sources such as glycerol, (biomass-derived) oligosaccharides, complex polysaccharides starch or lignocellulose, or by CO_2 reduction (Abdel-Rahman et al., 2013). The key enzymatic step is the reduction of pyruvate (e.g., from glycolysis) to lactate by lactate dehydrogenase (Ldh). Key organisms in the fermentation of sugars to lactate are lactic acid bacteria and fungi of the genus *Rhizopus*, but the ability to produce lactate has been transferred by cloning *ldh* genes into recombinant industrial workhorses such as *E. coli*, *Corynebacterium glutamicum* or *S. cerevisiae*. Titres achieved from batch fermentation of lignocellulosic material or agricultural waste using strains of *Lactobacillus* sp. or *Bacillus* sp. are >90 g L^{-1}, with conversion efficiencies of up to 0.98 g g^{-1} and productivities of 5.4 g L^{-1} h^{-1} (Abdel-Rahman et al., 2013).

Although lactic acid is not a common product of fermentation by archaea, the hyperthermophile *P. furiosus*, a well-studied member of the *Thermococcales* ,also referred to as the "*E. coli* of hyperthermophiles" (Kengen, 2017) was genetically engineered to produce lactate as major catabolic product from sugars. This was achieved by heterologously expressing a *ldh* gene from the extreme thermophilic bacterium *Caldicellulosiruptor bescii* (Basen et al., 2012). When this new strain was grown in a 15 L bioreactor, 3 mmol L^{-1} lactate was produced from cellobiose at 0.1 mmol L^{-1} h^{-1}. While it is currently unlikely that *P. furiosus* will be an industrially-relevant lactic acid producer, given the performance of other microbial systems, the study was a first proof-of-concept for heterologous production in *P. furiosus*, and one of the first genetic engineering approaches in any archaeon. Moreover, fermentation at high temperatures by a recombinant hyperthermophile demonstrated here harbors advantages to fermentations at lower temperatures, especially with regard to the conversion of recalcitrant lignocellulose (Kataeva et al., 2013).

In another approach, the acetate producing strain of *M. acetivorans* expressing ANME-1 *mcr* mentioned above was further engineered to convert the produced acetate to lactic acid. By expressing a modified version of the butanol production pathway from *Clostridium acetobutylicum,* in which the butyryl-CoA dehydrogenase was replaced by a trans-enoyl-CoA reductase (ter) from *Treponema denticola,* 1 mmol L^{-1} of optically pure L-lactate was produced in 28 mL culture tubes from methane (McAnulty et al., 2017). Collectively, the archaea-based lactate production has been shown to be at B-TRL 2 (Pfeifer et al., 2020).

10.4. 3-Hydroxypropionate

3-hydroxypropionate (3-HP) has received increased attention ever since it had been identified by the US Department of Energy (DoE) in 2004 as one of the TOP 12 building blocks with the potential to displace petroleum-based technologies on the path to sustainability (Choi et al., 2015). 3-HP is a platform chemical used for the synthesis of a variety of high value chemicals such as bioplastics, 1,3-propanediol, acrylic acid or acrylamide (Matsakas et al., 2018). In bacteria, 3-HP is mainly produced from glycerol and glucose, using different pathways and organisms. To date, high concentrations and conversion efficiencies have only been achieved with engineered bacterial strains (Matsakas et al., 2018). One of the highest concentrations to date was reached with a genetically modified strain of the enterobacterium *Klebsiella pneumoniae.* This strain was engineered to overproduce a native aldehyde dehydrogenase, while competing pathways producing

acetate and lactate were removed, allowing for a production of 0.93 mol L^{-1} 3-HP from glycerol when cultivated in a 5 L bioreactor (Li et al., 2016). Another engineered strain of the Gram positive bacterium *C. glutamicum*, converted glucose to 0.69 mol L^{-1} 3-HP at a productivity of 9.7 mmol L^{-1} h^{-1} in a fed-batch mode (Chen et al., 2017).

In archaea, 3-hydroxypropionate production was achieved from sugars, H_2 and CO_2 via the malonyl-CoA pathway. 3-HP is an important intermediate in the 3-hydroxypropionate/4-hydroxybutyrate cycle for CO_2 fixation in the thermophilic acidophilic archaeon *Metallosphaera sedula* (Berg et al., 2007). The heterologous expression of *M. sedula* genes encoding the first 3 enzymes of the cycle in *P. furiosus*, allowed for to the production of 0.6 mmol L^{-1} 3-HP from maltose and CO_2 (Keller et al., 2013). Furthermore, cell-free extracts of the SP1 expressing *P. furiosus* strain catalysed 3-HP production from acetyl-CoA and CO_2 with electrons from H_2 only, showing that H_2 may serve as additional substrate for whole cell biocatalysts. *In vivo*, a threefold improvement of 3-HP production was achieved by deleting acetyl-CoA synthase (ACSIα or ACSIIα) (Thorgersen et al., 2014). To further improve 3-HP production, the SP1 *P. furiosus* strain was modified to additionally express carbonic anhydrase and biotin protein ligase (BPL) from *M. sedula* (Hawkins et al., 2015). When cultivated in a 3 L bioreactor, a 3-HP concentration of 276 mg L^{-1} and a productivity of 11 mg L^{-1} h^{-1} was achieved by the corresponding strain MW78 (Table 8). The proof-of-concept by genetic engineering and the optimization in reactor studies justify B-TRL level 3 (Hawkins et al., 2015; Lian et al., 2016; Pfeifer et al., 2020).

10.5. Acetoin and 2,3-butanediol

Acetoin is a flavouring agent widely used in the food industry because of its buttery taste, which contributes an essential sensory component to fermented milk products like kefir. It has also found application in cigarettes, cosmetics, detergents, as an insect attractant in biological pest controls and as a precursor for the synthesis of heterocyclic compounds such as 2,3,5,6-tetramethylpyrazine (TTMP). Moreover, acetoin acts as intermediate product in the production of diacetyl, which is an even stronger flavouring compound; amongst others, it provides Czech beer with its characteristic taste. The majority of the market demand is met through chemical synthesis from fossil resources, but the demand for natural alternatives has driven research into biotechnological production (Xiao and Lu, 2014). A *S. cerevisiae* mutant has recently been reported to produce 100 g L^{-1} acetoin with a yield of 0.44 g g^{-1} glucose (Bae et al., 2016). In archaea, it has been observed that wild type strains of the hyperthermophilic archaeon *P. furiosus* (MW608) produced 4 mmol L^{-1} acetoin at 70 to 78°C (Supplementary table 30) as a side product of sugar oxidation, and has been improved to 6 mmol L^{-1} through metabolic engineering (Nguyen et al., 2016). Deletion of the acetolactate synthase (*als)* gene showed that acetoin is produced by decarboxylation of acetolactate, a metabolite synthesized to circumvent the bottleneck of pyruvate oxidation to acetyl-CoA by pyruvate:ferredoxin oxidoreductase (POR) (Nguyen et al., 2016).

Acetoin can further be reduced to 2,3-butanediol (2,3-BD) using a secondary alcohol dehydrogenase (*adh*). The US DoE has recently classified 2,3-BD as a platform chemical with enormous potential applications. 2,3-BD can be used as a precursor for the synthesis of other high value compounds and has been used as a drop-in fuel additive (Yang and Zhang, 2019). As with acetoin, the vast majority of the global demand is met through chemical synthesis, but it can be produced by various organisms from a wide range of substrates. One of the highest productions of 140 g L^{-1} was achieved with *Enterobacter aerogenes* grown on sugarcane molasses. Archaeal production is limited to an engineered *T. onnurineus*, which was shown to produce 3.3 mmol L^{-1} 2,3-BD from pyruvate at yield of 0.1 mol mol^{-1} (G. B. Lee et al., 2019). This yield was achieved through homologous expression of an *adh* from *Thermoanaerobacter guayamacensis*, and the inhibition of a competing acetate utilizing pathway by deletion of the gene encoding acetyl-CoA synthase IIIα (ACSα). ASCα, which

produces acetate from acetyl-CoA. Furthermore, the supply of extra reductant in the form of CO to the natively CODH producing *T. onnurineus* increased specific 2,3-butanediol production (Table 8). The production of acetoin and 2,3-BD is currently at level B-TRL 2 with genetically engineered strains (Supplementary table 19).

10.6. Compatible solutes

A common strategy among microorganisms to cope with high salt concentrations in the environment is to produce small, soluble compounds that are osmotically active. These osmolytes or compatible solutes are not primary products of catabolism, but nonetheless are produced in high concentrations. Because of their osmotic activity, these compatible solutes including trehalose, glycine, betaine, or ectoine are of biotechnological interest. Halophilic microorganisms are a prime choice for the production of these compounds, since they are naturally adapted to thrive in high-salt environments. Bacterial ectoine is an aspartate-derived, high-value compatible solute (9 to $17 \cdot 10^3$ € kg^{-1}) that is used as skin protectant, and that may be used as an additive in wastewater treatment (Czech et al., 2018). It is commercially produced by both native and engineered strains of *Halomonas* sp. (Czech et al., 2018), with an estimated annual production of $1.5 \cdot 10^4$ tons. Ectoine is harvested through a process coined “bacterial milking”, where the cells are cyclically exposed to hyper- and hypoosmotic conditions (Sauer and Galinski, 1998). So far, only twelve archaeal species were found to encode the ectoine/hydroxyectoine gene cluster, among them are some methanogens and the marine chemolithotrophic thaumarchaeon *Nitrosopumilus maritimus*. The latter was found to natively produce 0.32 µmol ectoine mg^{-1} protein and 0.19 µmol mg^{-1} hydroxyectoine protein (Widderich et al., 2016). The production of ectoine by *N. maritimus* is currently only an interesting observation, and it has neither been tried nor suggested to utilize this slow-growing chemolithotroph for an industrial ectoine production process.

The non-reducing sugar trehalose holds potential for a variety of applications ranging from food preservatives to its use as a cryoprotectant for the conservation of vaccines at room temperature (Schiraldi et al., 2002). It is currently commercially produced from maltooligodextrins or starch using two bacterial enzymes, homologues of which were also found in the Sulfolobales (Nakada et al., 1996). An alternative approach may be the production of trehalose from maltose by trehalose synthase, an enzyme which can be purified from the archaeon *Picrophilus torridus* (Cai et al., 2018). Although studies towards whole cell production are lacking, some archaeal phyla, including the hyperthermophile *Thermoproteus tenax* (Zaparty et al., 2013) and members of the Sulfolobales (Martins et al., 1997) produce trehalose. The latter have been suggested as production platforms for trehalose, since they are genetically accessible and grow up to high cell densities (117 g L^{-1}) in bioreactors (Table 8) (Krahe et al., 1996; Quehenberger et al., 2017). Based on the analysed publications, the industrial production of compatible solutes by archaea is at B-TRL 1 (Supplementary table 20).

11. Biocatalysts

Archaeal enzymes have become vital components of industrial processes and research, and will play an even bigger role in the future. The established practice for archaeal enzymes harbouring biotechnological potential is to clone the biocatalyst of interest into mesophiles such as *E. coli* or fungal hosts for characterizations and large-scale productions (Littlechild, 2015, 2011; Martinez-Espinosa, 2020). This allows for quick scale-up in established production systems and, in the case of thermophilic enzymes, for an easy downstream processing through heat-shock precipitation (Restaino et al., 2018). The extent of archaeal enzymes produced through heterologous expression in bacterial or fungal cell-factories, their applications and potentials are outside of this review and addressed in detail elsewhere (Cabrera and Blamey, 2018; Littlechild, 2015); instead, this review will focus on enzymes produced by archaeal cell factories.

Heterologous expression or homologous overexpression of recombinant archaeal enzymes in archaea are mostly limited to functional studies. With a few exceptions, such as hydrogenase production in *P. furiosus,* biotechnological approaches towards the utilization of methanogenic, anaerobic organoheterotrophic archaea have focused on chemicals and biofuel production (Chandrayan et al., 2015, 2012; Wu et al., 2018). Halophiles, on the other hand, have been studied as hosts for protein production. Their halotolerant proteins are of great commercial interest; however, the overexpression in hosts like *E. coli* is often problematic, since low cytoplasmic slat concentrations can affect protein folding and because metallo-cofactors may sometimes not be properly inserted into the apoproteins (Haque et al., 2020; Martinez-Espinosa, 2020). Therefore, desirable proteins have successfully been overproduced homologously or heterologously in other halophiles. Due to the limitation of genetic systems in most halophiles, homologous expressions have been limited to *H. volcanii* and *Halobacterium* sp. NRC-1 (Haque et al., 2020; Martinez-Espinosa, 2020). Compared with other halophilic archaea, *H. volcanii* is better suited for heterologous pathway engineering due to its faster growth, readily available genetic toolkit and stable genome (Allers, 2010; Haque et al., 2020; Leigh et al., 2011). With few exceptions, the overexpression in halophilic archaea has been focused on studying the biochemical properties of proteins and identifying industrially relevant proteins from uncultured or low yield strains (Martinez-Espinosa, 2020). For example, a β-galactosidase gene from the cold-adapted *Halorubrum lacusprofundi* was cloned into *Halobacterium* sp. NRC-1, achieving a 20-fold overexpression. The heterologously expressed β-galactosidase was further shown stable and active in 10 to 20% ethanol, isoamyl alcohol, methanol or n-butanol and thus could be of interest for industrial applications (Karan et al., 2013). The function of a novel alkaline serine protease (halolysin), identified in the genome of an uncultured halophile, was studied by heterologous expression in *H. volcanii*. Furthermore, *H. volcanii* was used as an expression system for a mammalian olfactory receptor, successfully integrating the protein into its lipid membrane. This showed that *H. volcanii* could not only successfully express mammalian proteins, but it was proposed to be potentially utilized for the production of nanovesicle-based hybrid biosensors (Lobasso et al., 2015).

In efforts to further develop the biotechnological potential of *H. volcanii*, a few studies have achieved improvement in enzyme production by adapting cultivation conditions. For example, esterase and lipase production in the halophiles *Natronococcus* sp. TC6, *H. marismortui* and *Halobacterium* sp. NRC-1 were improved by 6.3-, 1.6- and 2.8-fold respectively, by shifting cultivation from submerged fermentation to solid-state fermentation (Martin et al., 2015). The production of alcohol dehydrogenase in an engineered strain of the *H. volcanii* was optimized in a 1 L continuously stirred-tank bioreactor to produce 16.8 mg g CDM^{-1} enzymes from 6.5 g L^{-1} biomass (Strillinger et al., 2016)

Although production of industrially relevant biocatalysts in archaea has improved in recent years, there are still challenges to overcome in respect to production and purification of large quantities of biocatalysts utilizing archaea. The available publications have shown the archaeal production of biocatalysts to be at B-TRL 3 (Supplementary Table 21).

12. Extracellular polymeric substance

The acronym EPS was historically used as an abbreviation for “exopolysaccharides”, which today, as we know now, are only one component of the complex “Extracellular Polymeric Substances” (EPS) containing among others enzymes, lipids, nucleic acids, polysaccharides and structural proteins (Costa et al., 2018). Naturally, these EPS are produced by bacteria, microalgae, yeast, fungi and protists, where they are vital for the formation of cell clusters and biofilms (Flemming, 2016). EPS components from various organisms have found technological applications and can largely be produced from industrial waste. For example, the bacterium *Pseudomonas curdlan* (6 g L^{-1}) shows promise as a vaccine adjuvant and

encapsulation of nucleic acids for transport into cells; the FDA approved gellan produced by the bacterium *Sphingomonas paucimobilis* (36 g L^{-1}) is used as a thickener and stabilizer in the food industry and as an agar alternative in solid media cultivations; xanthan produced by the bacterium *Xanthomonas campestris* (15 g L^{-1}) has been widely used in oil refineries to enhance oil recovery and as a stabilizer and emulsifier in the food industry; and the fungal EPS components ß-glucan, schizophyllan and scleroglucan are promising antitumor compounds (Jindal and Singh Khattar, 2018; McGuffey et al., 2018; Tiwari et al., 2020; Wang et al., 2020). Apart from the biotechnological interest in EPS components, bacterial EPS have also been studied in detail in efforts to understand biofilm formation of pathogens such as *P. aeruginosa*, the yeast *Saccharomyces aerus* and the bacterium *K. pneumoniae* involved in hospital related infections (Vasdev et al., 2018).

EPS is also widespread among the archaea, where its role in both the planktonic lifestyle and formation of biofilm communities has been studied (Table 9) (Orell et al., 2017; van Wolferen et al., 2018). While bacterial EPS components are widely used industrially, research into archaeal EPS production has mainly focused on the biological function of EPS and there have not been any attempts to improve EPS yields (Antón et al., 1988; Hamidi et al., 2019; Lü et al., 2017; Nicolaus et al., 1999, 1993; Paramonov et al., 1998; Parolis et al., 1996, 1999; Rinker and Kelly, 2000; Sowers and Gunsalus, 1988; Squillaci et al., 2016; R. Zhang et al., 2019). Mechanistically, EPS production in haloarchaea typically goes in parallel with PHA accumulation; the shift towards one or the other target product can be accomplished by changing environmental conditions. It has been shown that moderate salinity favours EPS formation by *H. mediterranei*, while increased salt levels favour direction of the carbon source towards PHA biosynthesis (Cui et al., 2017). To date, the highest production of total EPS in archaea was 3 g L^{-1} by *H. mediterranei* in a 25 L pH-stat fermenter, which is 10 times higher than any other recorded archaeal EPS production, but the study did not include the targeted production of specific EPS components (Antón et al., 1988; Parolis et al., 1996). A recent study has found that large percentage of species in the orders Halobacteriales (21%), Haloferacales (31%) and Natrialbales (.9%) have the ability to produce fructans, a group of fructose-based carbohydrates, as part of their EPS, but yield of fructans were not discussed (Kırtel et al., 2019). Although there are currently no industrial or medical applications of archaeal EPS, there might be potential for applications in the future, as it has been shown that the EPS of *Haloterrigena turmenica* produced at 206 mg L^{-1} has a higher moisture-retention ability than hyaluronic acid (Squillaci et al., 2016). Furthermore, archaeal biofilms have become important in bioremediation and bioleaching consortia. Based on the analysis of EPS related publications, we conclude that based on advancements achieved with *H. mediterranei*, EPS production in archaea is currently at B-TRL 2 (Supplementary table 22).

13. Amino acid production in methanogens

Amino acid production by archaea was not the scope of any study until the discovery that amino acids are actively excreted by *Methanothermococcus okinawensis* up to 0.7 mmol L^{-1}. (Taubner et al., 2019). Subsequent quantitative comparative studies using three other methanogens revealed that the amino acid production patterns are characteristic for each methanogen as well as temperature and substrate concentration dependent (Taubner et al. manuscript submitted for publication). This is a novel field of Archaea Biotechnology research and only at B-TRL 1 (Supplementary table 23).

14. Trimethylaminuria and atherosclerosis Prevention

Trimethylamine (TMA) is a bacterial metabolite, produced by gut microbiota from substrates such as choline and phosphatidylcholine found in cheese, eggs, fish, red meat, and some vegetables. Once absorbed into the blood stream, it is oxidized to plasma trimethylamine oxide (pTMAO) in the liver by the flavin–containing monooxygenase 3 (FMO3) and excreted through the renal system. In one out of 40,000 people, a mutation of

the FMO3 or a disfunction in its transcriptional regulation can lead to the build-up of TMA in the blood stream also known as Trimethylaminuria. Affected people excreted the TMA through various bodily fluids, and thus tend to give off a strong, rotten fish-like odour. In individuals with renal deficiencies, the pTMAO build-up has been linked to atherosclerosis, hence, the formation of atheromatous plaque. It has been proposed that a possible treatment for these diseases could be periodic inoculations of TMA utilizing natural gut microbes (Brugère et al., 2014; Hania et al., 2017).

The first metagenomic studies of human faeces had revealed the existence of an unknown archaeon in the human gut (Mihajlovski et al., 2010, 2008). Since then, the isolation and purification of the strain *Methanomassiliicoccus luminyensis* B10 has led to the establishment of the archaeal order Methanomassiliicoccales (Dridi et al., 2012; Iino et al., 2013). *M. luminyesis* B10 has been shown to utilize TMA as a substrate for methanogenesis and has therefore been proposed to be used as pharmabiotics/archaebiotics to treat trimethylamine-related disorders in humans, such as Trimethylaminurea (fish-odour syndrome) (Borrel et al., 2013b, 2013a; Brugère et al., 2014, 2014; Gorlas et al., 2012; Tottey et al., 2015). To our knowledge, these studies are still in the fundamental research and ideation stage of the B-TRL scale.

15. Archaea in bioleaching

In an era, where Rare Earth Elements (REE) have become essential components in a vast array of modern technologies spanning almost all industries, the mining of these elements is accompanied by water scarcity and environmental degradation. It has therefore become evident that new sustainable processes are needed in this industry. Bioleaching is a biohydrometallurgical technology, in which microorganisms are utilized to aid in the extraction of REE from sulphide- and pyrite-rich ores (Fathollahzadeh et al., 2019). As an example, bioleaching to extract copper has been employed since 1958, and today 20% of the world's copper is mined through bioleaching. Other REE which are extracted using bioleaching include gold, uranium, cadmium, nickel and zinc (Fathollahzadeh et al., 2019). In recent decades, the application of bioleaching was expanded from its application in bioprocessing metal ore, to the leaching of REE from industrial, electronic, and incinerated municipal waste streams. While most biohydrometallurgic applications involve members of the bacterial genera *Acidithiobacillus* and *Leptospirillum* (Atashgahi et al., 2018), studies have highlighted the potential for archaea in these applications. *Acidianus brierleyi* (Konishi et al., 1999), *Sulfulobus metallicus* (Howard and Crundwell, 1999) and *Metallosphaera sedula* (Auernik and Kelly, 2010; Blazevic et al., 2019; Han and Kelly, 1998) were examined regarding their bioleaching properties.

A. brierleyi is a pleomorphic chemolithoautotrophic archaeon, which can oxidize sulphur and iron at temperatures ranging from 45 to 70°C with a pH optimum of 2 (Brierley and Brierley, 1973). It has been cultivated in a 1 L stirred batch bioreactor at 500 rpm, which was air-sparged continuously at a flow rate of 1 L min^{-1}. The growth yield and the specific growth rate were investigated, but bioleaching rates were not reported. However, chalcopyrite ($CuFeS_2$) bioleaching with *A. brierleyi* can be optimally performed at an inoculum size of 1014 cells m^{-3} and an initial mineral-liquid loading ratio of 5 and 10 kg m^{-3} (Konishi et al., 1999).

S. metallicus is a an aerobic, chemolithoautotrophic archaeon, growing on sulfidic ores like pyrite, sphalerite, chalcopyrite and on elemental sulphur at 50 to 75°C and a pH of 1.0 to 4.5 (Huber and Stetter, 1991). Repetitive batch experiments in bioreactors were performed using *S. metallicus*. The 2 L bioreactors were operated at 68±1°C and an initial pH of 1.1 and with continuous aeration and agitation. The aims of the study were to optimize the starting concentrations of ferric iron and to compare the bioleaching rates to the leaching rates of the abiotic control. The initial $CuFeS_2$ bioleaching rate of *S. metallicus* was 2.3 mg L^{-1} h^{-1}. The

maximum rate of leaching of $CuFeS_2$ was 24.6 mg L^{-1} h^{-1} at an initial concentration of 50 mmol L^{-1} ferric ions, obtaining $1.65 \cdot 10^9$ cells mL^{-1} (Howard and Crundwell, 1999).

M. sedula is an extremely thermoacidophile growing optimally at 70 to 75°C and pH of 2 (Huber et al., 1989), performing the dissimilatory oxidation of iron and sulphur. In $CuFeS_2$ bioleaching, ferric iron precipitation is reduced during bioleaching and the specific bioleaching rates are negatively impacted in the presence of H_2. The specific bioleaching activity for H_2-supplemented cultures was $5.1 \cdot 10^{-10}$ mg Fe $cell^{-1}$ compared to $2.8 \cdot 10^{-9}$ mg Fe $cell^{-1}$ for the control. However, the cell densities and faster growth rates were detected in the H_2-supplemented cultures, which indicated that H_2 served as an alternate energy source for *M. sedula*. Therefore, supplementing *M. sedula* bioleaching cultures with H_2 might offer a method to grow the organism faster to higher biomass concentrations and reducing undesirable ferric iron compound precipitation (Auernik and Kelly, 2010). When *M. sedula* was used for iron pyrite (FeS_2) bioleaching in batch experiments, the leaching rates were $6.7 \cdot 10^{-7}$ mg Fe^{3+} $cell^{-1}$ h^{-1} at 79°C compared to $4.3 \cdot 10^{-7}$ mg Fe^{3+} $cell^{-1}$ h^{-1} at 73°C. Furthermore, the specific bioleaching rate of *M. sedula* was 5-fold and 3-fold higher at 81°C or 79°C than at 73°C, respectively (Han and Kelly, 1998). *M. sedula* was recently used for bioleaching of scheelite ($CaWO_4$). It was shown that the total soluble tungsten is significantly higher in batch cultures containing *M. sedula* grown on $CaWO_4$ than the abiotic control. The maximum bioleaching rate was 76 ng tungsten L^{-1} h^{-1} (Blazevic et al., 2019). These studies on different archaea and ores are still in the fundamental research and ideation stage of the B-TRL scale.

16. Concluding Remarks

Currently, the vast majority of microbial cell factories utilized for the production of value-added and high-value compounds on an industrial scale are bacterial, fungal or algae based, but as cultivation and genetic systems of archaea improve, they are becoming ever more relevant. Some of the main advantages archaeal cell factories, are the ability to culture many strains under non-sterile conditions and to utilize cheap feedstocks often toxic to bacteria, thus drastically reducing cultivation costs. Currently, the only commercially available products of archaeal cell factories are bR, squalene, bacteriorhodopsin and diether-/tetraether-lipids, all of which are produced by Halotek utilizing haloarchaea. Other products such as PHA and CH_4, which have already been developed to a higher B-TRLs, are promising technologies for the emerging sustainability trend in industrial productions, and in the case of CH_4 driven by Krajete GmbH, Electrochaea GmbH, and Micropyrus GmbH. The growing demand for sustainable and biological products could also be an opportunity to fund and develop other archaeal products, such as carotenoids and H_2, which are currently largely produced from petrochemicals. As the number of isolated strains, the understanding of the cultured stains, as well as the development of genetic systems increases, so will the biotechnological potential of the archaea.

Author declarations

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Figure 1: B-TRLs of archaeal cell factories. This figure is a depiction of the *cell factory* producing the various products analysed through this review and shows their currect B-TRLs. This image has been designed using resources from Freepik.com.

Figure 2: A brief overview of established TRLs. The TRLs established by the NASA include risk and cost assessments at the various Technology Readiness Levels. As Technologies progress along the scale, the number of Technologies decreases and the costs and risks associated with advancing the remaining technologies rises.

Table 1: B-TRLs of Archaea Biotechnology

Basic and Applied Research	**1**	**Fundamental Research/ Ideation**	Analytically or experimentally identifying a Fundamental Concept. Identification of suitable archaea or components thereof
	2 / 2C	**Proof of Principle**	Development of experimental designs Basic principles observed on plate/flask scale experiments Systematically screening suitable archaea or components thereof
	3 / 3C	**Concept demonstration**	Identification and optimization of scale-up parameters and cultivation systems (fed-batch/ closed batch/ continuous culture) Identification of possible downstream processes **<10L**
	4 / 4C	**Proof of Concept**	Transferring optimized parameters to a bioreactor level Optimization of up/downstream processes **10<100L**
Technology Development	**5 / 5C**	**Technology demonstration**	Discontinuous production of target compound in bioreactor Process development (including upstream/downstream processing) **10<100** L
	6 / 6C	**Technology validation**	Continuous and reproducible operation/extraction under production conditions
System Optimization	**7 / 7C**	**Pilot -Scale**	Demonstration of the technology in actual environment continuously producing relevant product quantity and quality
	8 / 8C	**Pre-commercialization**	Analysing true costs, technology certification and quality control
Commercialization	**9 / C**	**Commercialization**	Technology(-system) has shown to function continuously and economically Financing and construction of commercial production

Table 2: Archaeal CH_4 production*

Cultivation	Gassing rate / vvm	Genus	Species	Strain	CH_4 offgas / Vol.-%	MER / mmol L^{-1} h^{-1}	Scale / L	Reference
Fed-batch	2.00	*Methanothermobacter*	*marburgensis*	DSM 2133	13.50	476.50	2.3 (CV)	(Abdel Azim et al., 2017)
	2.00	*Methanobacterium*	sp.	KN-15	NS	464.00	1 (WV)	(Nishimura et al., 1991)
	0.60	*Methanothermobacter*	*thermoautotrophicus*	DSM 3590	NS	114.00	10 (WV)	(Gerhard et al., 1993)
	1.00	*Methanobacterium*	*thermaggregans*	DSM 3266	NS	107.00	1.67 (WV)	(Mauerhofer et al., 2018)
	0.02	*Methanosarcina*	*barkeri*	DSM 800	NS	5.54	1.3 (WV)	(Weimer and

								Zeikus, 1978)
	5.00	*Methanobacterium*	sp.	KN-15	15.49	1,280.00	1 (WV)	(Nishimura et al., 1992)
	2.01	*Methanothermobacter*	*marburgensis*	DSM 2133	61.00	953.42	4.99 (WV)	(Seifert et al., 2014)
	5.00	*Methanobacterium*	sp.	KN-15	9.60	930.00	1 (WV)	(Nishimura et al., 1992)
	NS	*Methanothermobacter*	*marburgensis*	DSM 2133	96.00	535.38	1.03 (WV)	(Peillex et al., 1989)
	5.00	*Methanobacterium*	sp.	KN-15	3.88	450.00	1 (WV)	(Nishimura et al., 1992)
Continuous	1.38	*Methanothermobacter*	*thermoautotrophicus*	DSM 1053	26.00	426.12	0.03 (WV)	(Jee et al., 1987)
	0.41	*Methanothermobacter*	*marburgensis*	DSM 2133	NS	165.00	0.8 (WV)	(Rittmann et al., 2012)
	NS	*Methanocaldococcus*	*jannaschii*	DSM 2661	NS	130.00	1.5 (WV)	(Tsao et al., 1994)
	0.04	*Methanothermobacter*	*thermoautotrophicus*	DSM 3590	84.65	18.46	3.5 (WV)	(Martin et al., 2013)
	0.01	*Methanothermobacter*	*marburgensis*	DSM 2133	96.60	7.96	25 (WV)	(Hoffarth et al., 2019)

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NS = Not Supplied; MER = Methane Evolution Rate; WV = Working Volume; CV = Container Volume

Table 3: Archaeal H_2 production*

	Cultivation	Substrate	Genus	Species	Strain	$Y_{(H2/S)}$ / mol mol^{-1}	HER / mmol L^{-1} h^{-1}	Scale / L	Reference
WT	Closed batch	Peptone	*Thermococcus*	*litoralis*		NS	6.75	0.02 (WV)	(Bálint et al., 2005)
		Formate	*Thermococcus*	*onnurineus*	NA1	1.00	3.83	0.02 (WV)	(Bae et al., 2012)
		Keratin	*Pyrococcus*	*furiosus*		NS	3.25	0.02 (WV)	(Bálint et al., 2005)
		Starch	*Thermococcus*	*onnurineus*	NA1	3.13	2.66	0.02 (WV)	(Bae et al., 2012)
		Maltose, tryptone	*Pyrococcus*	*furiosus*	DSM3638	NS	2.30	0.05 (WV)	(Hensley et al., 2016)
		Yeast extract, tryptone, sea water components	*Pyrococcus*	*furiosus*		NS	2.00	0.05 (WV)	(Malik et al., 1989)
		Cellulose	*Pyrococcus*	*furiosus*		NS	1.80	0.05 (WV)	(Oslowski et al., 2011)
		Cellobiose	*Pyrococcus*	*furiosus*		NS	1.60	0.05 (WV)	(Oslowski et al., 2011)
		Maltose	*Pyrococcus*	*furiosus*		NS	1.60	0.05 (WV)	(Hensley et al., 2016)
		CO	*Thermococcus*	*onnurineus*	NA1	0.98	1.55	0.02 (WV)	(Bae et al., 2012)
		Maltose, acetate	*Pyrococcus*	*furiosus*	DSM3638	NS	1.40	0.05 (WV)	(Hensley et al., 2016)
		Tryptone	*Pyrococcus*	*furiosus*	DSM3638	NS	1.10	0.05 (WV)	(Hensley et al., 2016)
		Casein hydrolysate	*Pyrococcus*	*furiosus*		NS	0.50	0.05 (WV)	(Oslowski et al., 2011)
		Yeast extract	*Pyrococcus*	*furiosus*		NS	0.40	0.05 (WV)	(Oslowski et al., 2011)
		Keratin	*Thermococcus*	*litoralis*		NS	0.07	2.5 (WV)	(Bálint et al., 2005)
		Formate	*Thermococcus*	*onnurineus*		1.25	NS	0.08 (WV)	(Lee et al., 2012)
		Pyruvate	*Pyrococcus*	*furiosus*		1.20	NS	0.001 (WV)	(Schäfer and Schönheit, 1991)
		Yeast extract, formate	*Thermococcus*	*onnurineus*		1.42	NS	0.08 (WV)	(Lee et al., 2012)
	Batch	Formate	*Thermococcus*	*onnurineus*	NA1	NS	235.70	15 (WV)	(Bae et al., 2015)
		Cellulose	*Desulfurococcus*	*amylolyticus*	DSM 16532	NS	6.50	1.5 (WV)	(Reischl et al., 2018)
		Fructose	*Desulfurococcus*	*amylolyticus*	DSM 16532	NS	1.60	1.5 (WV)	(Reischl et al., 2018)
		Peptone	*Thermococcus*	*litoralis*		NS	0.21	2.5 (WV)	(Bálint et al., 2005)
		Yeast extract,	*Pyrococcus*	*furiosus*		NS	0.02	NS	(Fiala and

		peptone							Stetter, 1986)
		Pyruvate	*Pyrococcus*	*furiosus*		0.80	NS	0.4 (WV)	(Schäfer and Schönheit, 1991)
	Fed-batch	Formate	*Thermococcus*	*onnurineus*	NA1	NS	2,820.00	1 (WV)	(Lim et al., 2012)
		CO	*Thermococcus*	*onnurineus*	NA1	NS	32.90	2 (WV)	(Kim et al., 2013)
	Continuous	Cellobiose	*Pyrococcus*	*furiosus*		3.80	6.24	1 (WV)	(Chou et al., 2007)
		Pyruvate	*Thermococcus*	*kodakarensis*		1.09	3.88	7 (WV)	(Kanai et al., 2005)
		Starch	*Thermococcus*	*kodakarensis*		3.33	3.16	7 (WV)	(Kanai et al., 2005)
GMO	Closed batch	Maltose	*Pyrococcus*	*furiosus*	COM1	NS	48.75	0.251 (CV)	(Chandrayan et al., 2012)
		Maltose, formate	*Pyrococcus*	*furiosus*		NS	3.50	NS	(Lipscomb et al., 2014)
		CO	*Thermococcus*	*onnurineus*	NA1 (MC01)	1.07	32.90	0.05 (WV)	(Kim et al., 2013)
		Chitin	*Thermococcus*	*kodakarensis*	KU216 (KOD1ΔpyrF)	[illegible]3	0.31	0.015 (WV)	(Aslam et al., 2017)
	Batch	Formate	*Thermococcus*	*onnurineus*	FO	N[illegible]	118.50	1.5 (WV)	(S. H. Lee et al., 2019)
	Fed-batch	CO	*Thermococcus*	*onnurineus*	NA1 (1567)	NS	361.00	3 (WV)	(Kim et al., 2017)
	Continuous	Pyruvate	Thermococcus	kodakarensis	MAH1	NS	27.10	0.5 (WV)	(Kanai et al., 2015)

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NS = Not Supplied; HER = H_2 Evolution Rate; WV = Working Volume; CV = Container Volume

Table 4: Archaeal PHA production*

Cultivation	Substrate	Genus	Species	Stain	PHA	PHA CD M^{-1} / %	Q / g L^{-1} h^{-1}	$Y_{PHA/s}$ / g g^{-1}	Scale / L	Reference
Batch	Starch	*Halogeometricum*	*borinquense*	E3	PHBHV	74.19	NS	NS	0.5 (WV)	(Salgaonkar et al., 2019)
	Glucose	*Halogeometricum*	*borinquense*	E3	PHBHV	73.51	0.0252	NS	NS	(Salgaonkar and Bragança, 2015)
	Hydrolysed Whey extract	*Haloferax*	*mediterranei*	DSM1412	PHBHV	73.00	0.0900	0.29	42 (CV)	(Koller et al., 2008)
	Raw stillage	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	1.00	0.1700	0.35	0.1 (WV)	(Bhattacharyya et al., 2014)
	Vinasse - pretreated 25%	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	70.00	0.2100	0.87	0.1 (WV)	(Bhattacharyya et al., 2012)
	Raw stillage + recovered salts	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	69.00	0.1700	0.34	0.1 (WV)	(Bhattacharyya et al., 2014)
	Glucose	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	66.67	NS	NS	0.1 (WV)	(Ghosh et al., 2019)
	Hydrolysed Microalga (seaweed) Ulva sp. (25%)	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	58.10	0.0350	NS	0.1 (WV)	(Ghosh et al., 2019)
	Sucrose	*Haloarcula*	sp.	IRU1	PHB	55.74	NS	NS	0.1 (WV)	(Taran, 2011b)
	Fructose	*Haloarcula*	sp.	IRU1	PHB	55.13	NS	NS	0.1 (WV)	(Taran, 2011b)
	Corn starch Nutrient Broth	*Natrinema*	*palladium*	1KYS1	PHBV	53.14	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Sodium acetate	*Halopiger*	*aswanensis*	DSM13151	PHBHV	53.00	0.0045	0.54	6 (WV)	(Hezayen et al., 2000)
	Sugarcane bagasse 25%	*Halogeometricum*	*borinquense*	E3	PHBHV	50.00	0.0095	0.448	NS	(Salgaonkar and Bragança, 2017)
	Glucose and Valerate	*Haloferax*	*mediterranei*	ES1	PHBHV	50.00	NS	NS	50 (WV)	(Han et al., 2015)
	Synthetic seaweed medium	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	48.15	0.0300	-	0.1 (WV)	(Ghosh et al., 2019)

	Whey Nutrient Broth	*Natrinema*	*palladium*	1TK1	PHBV	47.69	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Petrochemical wastewater	*Haloarcula*	sp.	IRU1	PHB	46.60	NS	NS	0.1 (WV)	(Taran, 2011a)
	Sugarcane bagasse 50%	*Halogeometricum*	*borinquense*	E3	PHBHV	46.00	0.0113	0.253	NS	(Salgaonkar and Bragança, 2017)
	Glucose and Galactose	*Haloferax*	*mediterranei*	ATCC 33500	PHBHV	46.00	0.0550	0.66	0.1 (WV)	(Pais et al., 2016)
	Cassava waste (CW)	*Halogeometricum*	*borinquense*	E3	PHBHV	44.70	NS	NS	0.5 (WV)	(Salgaonkar et al., 2019)
	Olive Mill Wastewater (OMW)	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	43.00	NS	NS	0.1 (WV)	(Alsafadi and Al-Mashaqbeh, 2017)
	Butyric acid	*Halobiforma*	*haloterrestris*	DSM1307 8	PHB	40.00	NS	NS	NS	(Hezayen et al., 2002)
	Palmitic acid	*Haloarcula*	sp.	IRU1	PHB	39.47	NS	NS	0.1 (WV)	(Taran, 2011b)
	C4:0/C4:0 mix (56:44%) + Tween80	*Haloferax*	*mediterranei*	DSM 1413	PHBHV	37.40	0.0050	0.2	0.1 (WV)	(Ferre-Guell and Winterburn, 2019)
	Xylose	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	33.40	NS	NS	0.1 (WV)	(Ghosh et al., 2019)
	Crude Oil	*Haloarcula*	sp.	IRU1	PHB	31.30	NS	NS	0.1 (WV)	(Taran, 2011a)
	Tomato Nutrient Broth	*Natrinema*	*palladium*	1TK1	PHBV	3[illegible]7	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Melon Nutrient Broth	*Natrinema*	*palladium*	2KYS1	PHBV	26.30	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Volatile fatty acids (VFA) C4:0	*Haloferax*	*mediterranei*	DSM 1411	PHBV	18.70	NS	NS	0.1 (WV)	(Ferre-Guell and Winterburn, 2018)
	Volatile fatty acids (VFA) C5:0	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	16.00	NS	NS	0.1 (WV)	(Ferre-Guell and Winterburn, 2018)
	Apple Nutrient Broth	*Natrinema*	*palladium*	1TK1	PHBV	15.25	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Casamino Acids	*Halogranum*	*amylolyticum*	TNN58	PHBHV	8.30	NS	NS	3 (WV)	(Zhao et al., 2015)
	Glycerol	*Halogranum*	*amylolyticum*	TNN58	PHBHV	7.00	NS	NS	3 (WV)	(Zhao et al., 2015)
	Butyrate	*Halogranum*	*amylolyticum*	TNN58	PHBHV	4.40	NS	NS	3 (WV)	(Zhao et al., 2015)
	Sucrose Nutrient Broth	*Natrinema*	*palladium*	3TL4	PHBV	4.11	NS	NS	0.1 (WV)	(Danis et al., 2015)
	Carrot waste	*Haloterrigena*	*hispanica*	DSM 18328	PHB	0.13	NS	NS	1.2 (WV)	(Di Donato et al., 2011)

Table 4: Archaeal PHA production (continued)*

Cultivation	Substrate	Genus	Species	Stain	PHA	PHA CDM^{-1} / %	Q / g L^{-1} h^{-1}	$Y_{PHA/S}$ / g g^{-1}	Scale / L	Reference
Fed-Batch	Hydrolysed Whey extract	*Haloferax*	*mediterranei*	DSM1411	PHBHV4HB	87.50	0.1400	0.2	10 (CV)	(Koller et al., 2007a).
	GLP + Meat and Bone Meal (MBM)	*Haloferax*	*mediterranei*		PHBHV	75.00	0.0390	0.18	42 (CV)	(Koller et al., 2005)
	Crude glycerol phase (CGP)	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	74.7	0.1200	0.19	10 (CV)	(Hermann-Krauss et al., 2013)
	Glycerol	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	72.8	0.1200	0.37	10 (CV)	(Hermann-Krauss et al., 2013)
	Hydrolysed Whey extract + Recycling of Cell Debris (SF)	*Haloferax*	*mediterranei*	DSM1411	PHBHV	70.00	NS	NS	7 (WV)	(Koller, 2015a)

	50%									
	Glucose	*Haloferax*	*mediterranei*	DSM1411	PHBHV	70.00	0.2100	0.23	7 (WV)	(Koller, 2015b)
	Crude glycerol phase (CGP) + y-butyrolactone	*Haloferax*	*mediterranei*	DSM 1411	PHBHV4HB	66.5	0.1000	0.16	10 (CV)	(Hermann-Krauss et al., 2013)
	Hydrolysed Whey extract	*Haloferax*	*mediterranei*	DSM1411	PHBHV	66.00	NS	NS	220 (WV)	(Koller, 2015a)
	Raw stillage	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	63.00	0.1400	0.27	14 (WV)	(Bhattacharyya et al., 2015)
	C4:0/C4:0 mix (56:44%) + Tween80	*Haloferax*	*mediterranei*	DSM 1423	PHBHV	58.90	0.0102	0.18	1.2 (WV)	(Ferre-Guell and Winterburn, 2019)
	Extruded rice bran (ERB) and extruded cornstarch (ECS) 1/8 (g/g)	*Haloferax*	*mediterranei*	ATCC 33500	PHBHV	56.60	NS	NS	2.75 (WV)	(Huang et al., 2006)
	Corn starch	*Haloferax*	*mediterranei*	ATCC 33500	PHBHV	51.00	NS	NS	6 (CV)	(Chen et al., 2006)
	Volatile fatty acids (VFA) C4:0:C5:0 (29:71)	*Haloferax*	*mediterranei*	DSM 1411	PHBHV	25.00	NS	NS	0.2 (WV)	(Ferre-Guell and Winterburn, 2018)
Continuous	Starch	*Haloferax*	*mediterranei*	Q4	PHBHV	60.00	NS	0.324	1.5 (WV)	(Lillo and Rodriguez-Valera, 1990)

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NS = Not Supplied; CDM = Cell Dry Mass; WV = Working Volume; CV = Container Volume

Table 5: Applications of S-layer proteins

S-layer protein construct	Task	Application	Reference
S-layer protein fragments (ghosts)	*Sulfolobus acidocaldarius* as bionanomask for nanostructure formation	Nanostructuring of surfaces Nanocluster formation Molecular lithography	(Douglas et al., 1992, 1986; Winningham et al., 2001, 1998)
S-layer ultrafiltration membrane (SUM)	Defined surface and molecular sieving properties Controlled immobilization of functional molecules (enzymes, monoclonal antibodies, etc.)	Ultrafiltration membrane Amperometric and optical bioanalytical sensors Dipstick-style immunoassay	(Breitwieser et al., 1996; Neubauer et al., 1996, 1993)
S-layer microparticle (SMP)	Immobilization matrix (Protein A, antibodies, etc.)	Escort-particles in affinity cross-flow filtration for isolation and purification of antibodies Functional affinity microparticles for e.g., ELISA Extracorporeal Blood Purification	(Küpcü et al., 1996; Neubauer et al., 1994; Weber et al., 2001)
S-layer self-assembly product (SAP)	Immobilization matrix (antigens, haptens, etc.)	Intrinsic adjuvant property for weakly immunogenic antigens and haptens, mucosal vaccines	(Jahn-Schmid et al., 1996a, 1996b; Smith et al., 1993)
S-layer-coated liposomes and emulsomes	Drug targeting and delivery systems	Curcumin delivery vehicle	(Ucisik et al., 2013)
Self-assembled S-layer proteins on solid supports	Matrices for controlled biomineralization (Au, Ag, Pt, ZnO, CdS, CdSe nanoparticles) Templates to form regularly arranged nanoparticles	Biosensor, memory element, non-linear optics, templates for biomineralization	(Györvary et al., 2004; Patel et al., 2010; Pollmann et al., 2006; Velásquez and Dussan, 2009)
S-layer supported functional lipid membranes	Incorporation of membrane-active peptides (gramicidin, alamethicin, valinomycin, anti-microbial peptide) and membrane proteins (□-hemolysin, ryanodine receptor, voltage-dependent anion-selective channel)	Biosensor, High-throughput device for lead compounds	(Gufler et al., 2004; Schrems et al., 2013; Schuster and Sleytr, 2002)

Table 6: Archaeal bacteriorhodopsin production*

Cultivation	Complex C- and N-source	Genus	Species	Stain	CDM / g L^{-1}	Bacteriorhodopsin / mg L^{-1}	Q / mg L^{-1} h^{-1}	Scale / L	Reference

Batch	Casamino acids / meat extract / corn steep powder	*Halobacterium*	*salinarum*	PTCC 1685	29.4	234.6	2.4	4 (WV)	(Ghasemi et al., 2008)
					11.5	183.2	2.0	0.05 (WV)	(Ghasemi et al., 2008)
Repeated batch	Tryptone	*Halobacterium*	*salinarum*		4.8	201.8	0.961	0.8 (WV)	(Shiu et al., 2015)
Fed-batch / cell cycle	Yeast extract	*Halobacterium*	*salinarum*	R1	30.3	282.0	1.2	1.5 (WV)	(Lee et al., 1998)
Fed-batch and adsorption	Peptone / yeast extract	*Halobacterium*	*salinarum*	KSK-03307	45.0	1,750.0	0.6	3 (WV)	(Kalenov et al., 2016)

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); CDM = Cell Dry Mass; WV = Working Volume

Table 7: Archaeal carotenoid production*

Cultivation	WT/GMO	Genus	Species	Stain	Carotenoids / mg g^{-1} CDM	ß-carotene /mg g^{-1} CDM*	Canthaxanthin / mg g^{-1} CDM	Bacteriorubberin /mg g^{-1} CDM*	Lycopene / mg g^{-1} CDM*	Scale / L	Reference
Batch	WT	*Haloarcula*	*hispanica*	ATTC 33960	1.35	NS	NS	NS	NS	NS	(Calo et al., 1995)
		Haloarcula	*japonica*	TR-1	0.34	NS	NS	0.23	NS	0.4 (WV)	(Yatsunami et al., 2014)
		Halobacterium	*halobium*	M8	7.63	NS	NS	NS	NS	0.1 (WV)	(Abbes et al., 2013)
		Halobacterium	*salinarum*	NRC-1	2.40	NS	NS	NS	NS	NS	(Calo et al., 1995)
		Halobacterium	*salinarum*	ATCC 33171	0.05	NS	NS	0.03	NS	0.4 (WV)	(Mandelli et al., 2012)
		Halobacterium	*salinarum*	Oyon Moussa-16	12.2 nmol / 10^{10} cells	2.8 nmol / 10^{10} cells	NS	3.9 nmol / 10^{10} cells	NS	0.5 (WV)	(El-Sayed et al., 2002)
		Halobrum	sp.	TBZ126	16.34 mg L^{-1}	0.2 mg L^{-1}	NS	16 mg L^{-1}	0.1 mg L^{-1}	0.09 (WV)	(Hamidi et al., 2014)
		Halococcus	*morrhuae*	ATCC 17082	0.09	NS	NS	0.06	NS	0.4 (WV)	(Mandelli et al., 2012)
		Haloferax	*alexandrinus*	JCM	6.45	0.19	0.70	3.82	NS	0.5 (WV)	(Asker and Ohta, 2002)
		Haloferax	*mediterranei*	ATCC 33500	0.70	NS	NS	NS	NS	NS	(Calo et al., 1995)
		Haloferax	*mediterranei*	ATCC 33500	NS	NS	NS	0.604 A494nm mL^{-1}	NS	15 (WV)	(Fang et al., 2010)
		Haloferax	*mediterranei*	R4	3.74 mg L^{-1}	NS	NS	NS	NS	0.1 (WV)	(Montero-Lobato et al., 2018)
		Halorubrum	sp.	SH1	25 mg L^{-1}	NS	NS	NS	NS	0.5 (WV)	(Vega et al., 2016)
		Natrialba	sp.	M6	9.80	NS	NS	NS	NS	0 (WV)	(Hegazy et al., 2020)
	GMO	*Haloferax*	*mediterranei*	50B6I6ΔphaEC	>119	NS	NS	NS	119.00	0.05 (WV)	(Zuo et al., 2018)
		Haloferax	*volcanii*	H26	NS	NS	NS	13.80	NS	NS	(Cerletti et al., 2014)
		Haloferax	*volcanii*	HVLON3	183 (OD600=1.7) mg L^{-1}	NS	NS	220.00	NS	NS	(Cerletti et al.,

											2014)
Fed-batch	WT	*Haloferax*	*mediterranei*	ATCC 33500	556 mg L^{-1}	NS	NS	291.344 mg L^{-1}	NS	NS	(Chen et al., 2015)

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NS = Not Supplied; CDM = Cell Dry Mass; WV = Working Volume; * = unit unless other is given

Table 8: Other archaeal products*

Product	Cultivation		Genus	Species	Strain	Substrate	Yield / mol mol^{-1}	Productivity / mmol L^{-1} h^{-1}	Titer / mmol L^{-1}	Scale / L	Reference
1-butanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.21	25	0.01	Basen et al., 2014
	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.4	40	0.01	Basen et al., 2014
1-decanol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.13	NS	0.13	0.05	van den Ban et al., 1999
1-hexanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.05	6.0	0.01	Basen et al., 2014
1-propanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.23	28	0.01	Basen et al., 2014
2,3-butanediol	Batch	GMO	*Thermococcus*	*onnurineus*	BP002	Pyruvate	0.1	NS	3.3	NS	Lee et al., 2019
3-hydroxy-propionic acid	Batch	GMO	*Pyrococcus*	*furiosus*	MW110	Maltose	NS	0.017	0.2	0.05	Thorgersen et al. 2014
	Batch	GMO	*Pyrococcus*	*furiosus*	RKM12	Maltose, yeast extract	NS	NS	4.1	1	Lian et al. 2016
3-phenyl-1-propanol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.69	NS	0.69	0.05	van den Ban et al., 1999
4-phenyl-1-butanol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.39	NS	0.39	0.05	van den Ban et al., 1999
5-phenyl-1-pentanol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.42	NS	0.42	0.05	van den Ban et al., 1999
6-phenyl-1-hexanol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.25	NS	0.25	0.05	van den Ban et al., 1999
Acetate	Batch	GMO	*Methanosarcina*	*acetivorans*		CH_4	0.36	0.09	0.005	0.1	Soo et al. 2016
Acetoin	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	NS	6.0	0.05	Nguyen et al., 2016
Benzyl alcohol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.27	NS	0.27	0.05	van den Ban et al., 1999
CH4	Batch	GMO	*Methanosarcina*	*acetivorans*	C2A (pDL203)	NS	0.6	0.0006	0.06	0.01	Lessner et al. 2010
Cinnamyl alcohol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.67	NS	0.67	0.05	van den Ban et al., 1999
Crotyl alcohol	Batch	WT	*Pyrococcus*	*furiosus*	DSM3638	Starch	0.17	NS	0.17	0.05	van den Ban et al., 1999
Ectoine	Batch	WT	*Nitrosopumilus*	*maritimus*	SCM1	NS	NS	NS	NA	15	Widderich et al. 2016
Ethanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Cellobiose, maltose	0.7	0.45	21.4	0.01	Basen et al., 2014
	Batch	GMO	*Pyrococcus*	*furiosus*	MW325	Maltose	1.2	0.047	4.2	0.05	Keller et al. 2017
Geraniol	Batch	GMO	*Methanococcus*	*maripaludis*	S0027	Formate	NS	NA	0.003	0.005	Lyu et al. 2016
Isobutanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.25	30	0.01	Basen et al., 2014
Lactate	Batch	GMO	*Pyrococcus*	*furiosus*	LAC	Cellobiose	NS	*0.10*	3.0	15	Basen et al., 2012

L-lactate	Batch	GMO	*Methanosarcina*	*acetivorans*	C2A (pES1-MATbiohol-B4)	CH_4	0.1	0.01	1.06	0.005	McAnulty et al. 2017
Phenylethanol	Batch	GMO	*Pyrococcus*	*furiosus*	MW608 (ADHA)	Maltose	NS	0.125	15	0.01	Basen et al., 2014
Phytoene	Batch	GMO	*Thermococcus*	*kodakarensis*		Pyruvate, maltodextrin	NS	NA	0.005	0.01	Fuke et al., 2018

*Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NS = Not Supplied

Table 9: Archaeal EPS production

Cultivation	Genus	Species	Stain	EPS /mg L^{-1}	EPS yield / mg g^{-1} CDM	Carbohydrates / % of EPS	Proteins / % of EPS	Sulfates / % of EPS	nucleic acids / % of EPS	Uronic acid / % EPS	EPS composition	Scale / L	Reference
Batch	*Haloferax*	*mediterranei*	R4	3,000.00	NS	58.25	7.50	6.80	NS	2.25	NS	20.5 (WV)	(Antón et al., 1988)
	Halorubrum	sp.	TBZ112	480.00	NS	70.0	0.8	NS	2.5	8.3	Man, GlcN, GalA, Ara, GlcA, Xyl, Gal, Glc, Rib and Rha	NS	(Hamidi et al., 2019)
	Haloarcula	sp.	T5	370.00	NS	NS	NS	2.00	NS	NS	Man : Gal : GlcA = 2 : 1 : 3	0.01 (WV)	(Nicolaus et al., 1999)
	Haloterrigena	*turkmenica*	DSM 5511	206.80	41.60	91.00	1.40	2.80	NS	NS	Glc : GlcNH2 : GlcA : Gal : $GalNH_2$ 1 : 0.65 : 0.24 : 0.22 : 0.02	0.5 (WV)	(Squillaci et al., 2016)
	Haloarcula	sp.	T6	45.00	NS	NS	NS	2.00	NS	NS	Man : Gal : Glc = 1 : 0.2 : 0.2	0.01 (WV)	(Nicolaus et al., 1999)
	Haloarcula	*hispanica*	ATCC33960	30.00	NS	51.00	NS	26.00	NS	NS	Man : Gal : Glc = 1 : 0.77 : 0.02	1 (WV)	(Lü et al., 2017)
Continuous	*Thermococcus*	*litoralis*	5473	120.00	2.00	NS	NS	2.00	NS	NS	NS	1 (WV)	(Rinker and Kelly, 2000)
NS	*Halobacterium*	*volcanii*	1539	300.00	NS	NS	NS	0.60	NS	NS	Man. Hexuronic acids	NS	(Severina et al., 1990)

Adapted from Pfeifer *et al.* (Pfeifer et al., 2020); NP = Not Supplied; CDM = Cell Dry Mass; WV = Working Volume

Highlights

- Review of the current state and future potential of Archaea in Biotechnology
- Introduction of microbial cell factory-specific Bio-Technology Readiness Levels
- Archaeal PHA and CH_4 productions at B-TRL 5 show potential for commercialization
- Archaeal lipids, squalene and bacterioruberin are commercially available
- Some archaeal bioproducts could replace fossil-based compounds and polymers

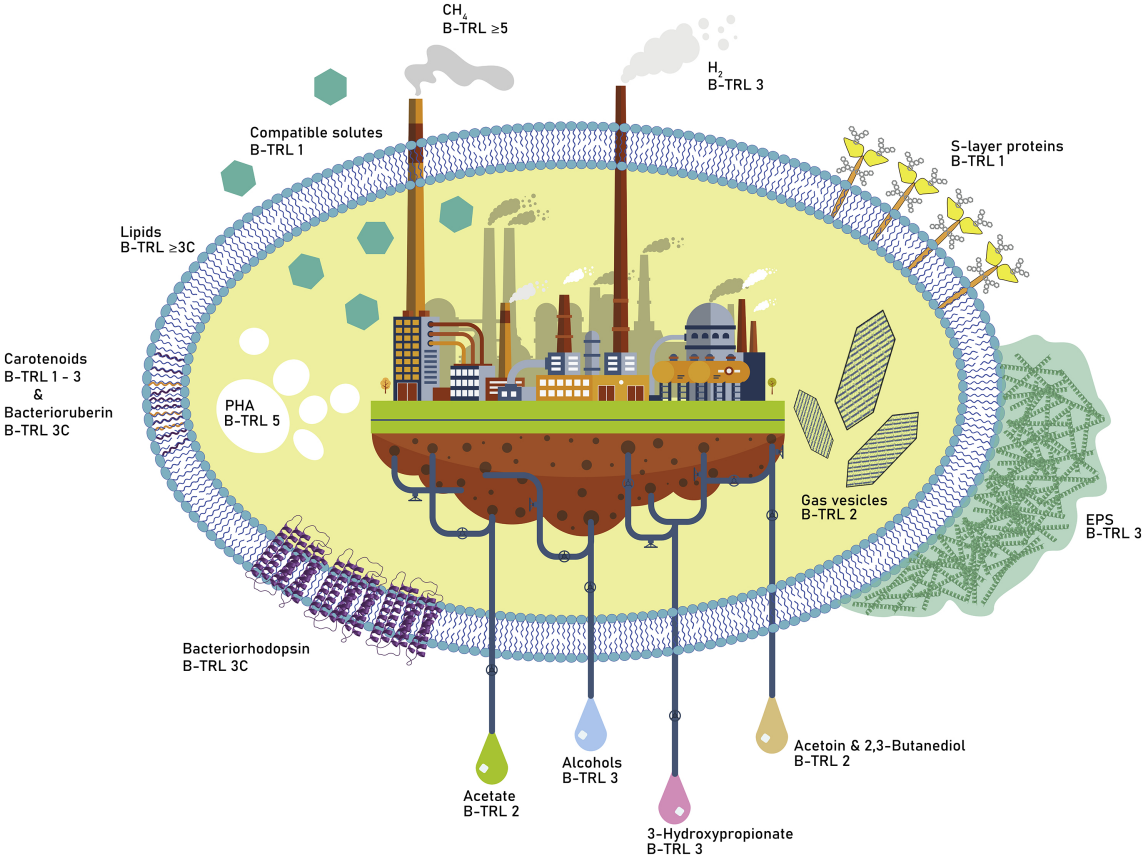
CH_4
B-TRL ≥5
H_2
B-TRL 3
Compatible solutes
B-TRL 1
S-layer proteins
B-TRL 1
Lipids
B-TRL ≥3C
Carotenoids
B-TRL 1 - 3
&
Bacterioruberin
B-TRL 3C
PHA
B-TRL 5
Gas vesicles
B-TRL 2
EPS
B-TRL 3
Bacteriorhodopsin
B-TRL 3C
Acetate
B-TRL 2
Alcohols
B-TRL 3
3-Hydroxypropionate
B-TRL 3
Acetoin & 2,3-Butanediol
B-TRL 2

Figure 1

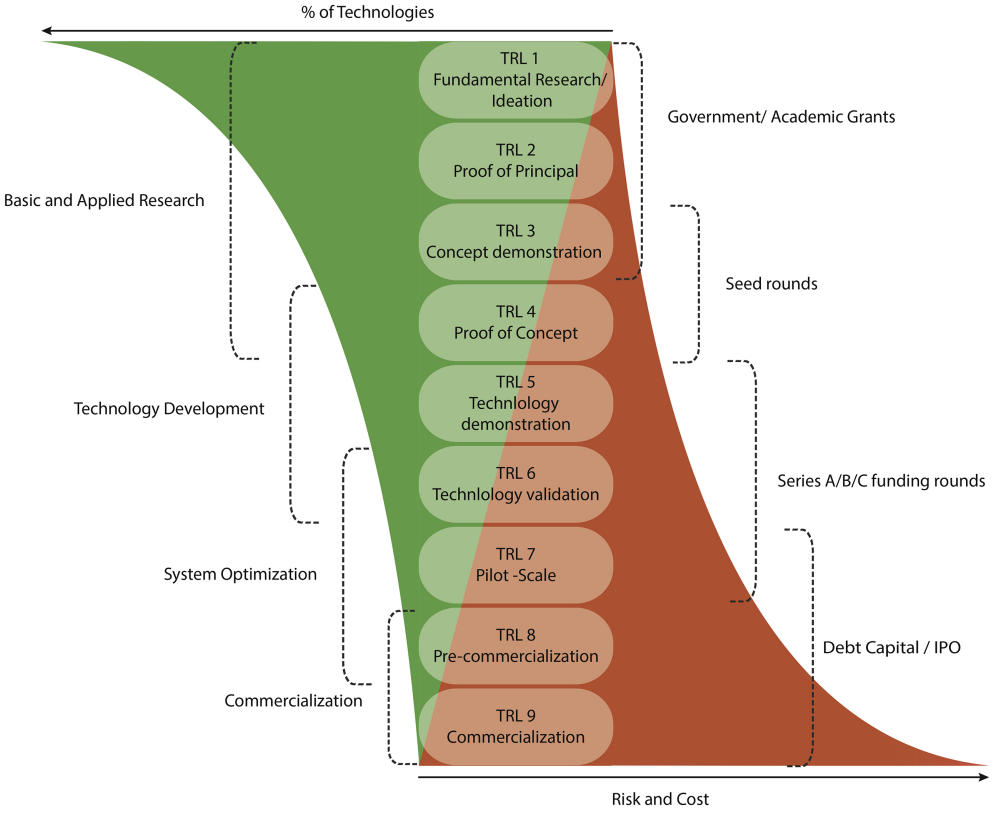
% of Technologies
TRL 1
Fundamental Research/
Ideation
TRL 2
Proof of Principal
TRL 3
Concept demonstration
TRL 4
Proof of Concept
TRL 5
Technlology
demonstration
TRL 6
Technlology validation
TRL 7
Pilot -Scale
TRL 8
Pre-commercialization
TRL 9
Commercialization
Government/ Academic Grants
Seed rounds
Series A/B/C funding rounds
Debt Capital / IPO
Basic and Applied Research
Technology Development
System Optimization
Commercialization
Risk and Cost

Figure 2