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DIPLOMARBEIT

Development of a defined medium for the heterotrophic cultivation of *Metallosphaera sedula*

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unter der Supervision von

Associate Prof. Dipl.-Ing. Dr.nat.techn. Oliver Spadiut
und Projektass. Dipl.-Ing. Viktor Laurin Sedlmayr, BSc

durch

Maximilian Luger

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Abstract

Metallosphaera sedula is an obligate aerobe, thermoacidophilic Crenarchaeon within the order Sulfolobales. As a model organism for CO_2 fixation of the genus *Metallosphaera* it utilizes the 3-Hydroxypropionate/4-Hydroxybutyrate cycle for autotrophic growth.

The organism is best known for its lithoautotrophic mode of growth and a process called bioleaching which describes the extraction of heavy metals from ores. The CO_2 fixation via the 3-HP/4-HB cycle is the feature of this organism with the most scientific attention in the past because of its incredible potential the pathway itself, but above that the enzymes within have, especially when being heterologously expressed in specific other organisms.

In addition to that, *M. sedula* also has the ability to grow heterotrophically on complex organic molecules, but as a downside is not able to metabolize any kinds of sugar, a metabolic feature that is common within the genus *Metallosphaera*. Nevertheless, there are some limitations that complex media come with such as contaminations with components of the biological matrix.

In this study, the complex media component tryptone could be replaced with a mixture of amino acids. Starting from a complex carbon source, the first defined cultivation medium for the heterotrophic cultivation of *M. sedula* could be developed. This medium consists of 16 amino acids as carbon and nitrogen sources. After analysis of substrate uptake rates the number of substrates in the medium could be reduced to 5 resulting in the final 5AA medium. Additionally, the lipid pattern of *M. sedula* grown on complex media was compared to the one of *Sulfolobus acidocaldarius*.

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Introduction

1 *Metallosphaera sedula*

Metallosphaera sedula is an obligate aerobe, thermoacidophilic Crenarchaeon within the order Sulfolobales. Discovered in 1989 in solfataric fields in Italy, it was the first species of the genus *Metallosphaera* and is to this day by far the most studied one.[1]

The organism is best known for its lithoautotrophic mode of growth and a process called bioleaching which describes the biotic extraction of heavy metals from ores by conversion into soluble salts. Nevertheless, the CO_2 fixation via the 3-Hydroxypropionate/4-Hydroxybutyrate (3-HP/4-HB) cycle is probably the feature with the most scientific attention in the past due to the incredible potential of the pathway itself, but especially of the involved enzymes when being heterologously expressed for diverse biotechnological applications.[2,3]

Additionally, *M. sedula* is also able to grow heterotrophically on complex organic molecules, but is not able to metabolize any kinds of sugar, a metabolic feature that is common within the genus *Metallosphaera*.[4]

2 Autotrophy

2.1 Autotrophic CO_2 fixation

The autotrophic lifestyle has a very broad spectrum of ways in which it can occur. The most important one for oxygenic life on earth is photoautotrophy. This process is used by plants and primary producers in general to produce oxygen using light and carbon dioxide as energy and carbon sources.[5] The other type of autotrophy is the chemoautotrophic lifestyle which is used by many microorganisms and among others also archaea of the order Sulfolobales. Chemo(litho)autotrophic organisms use energy from the transfer of electrons from a donor to an acceptor in order to convert an inorganic carbon source (in organisms within the order Sulfolobales CO_2) into organic compounds.[6] In doing so, there are several different pathways through which this can happen. A brief overview of the cycles that are known to exist in archaea, their active CO_2 compound and the key enzymes is shown in Table 1.[7]

Table 1: Ways of CO_2 fixation in archaea

Pathway	Alternative name	Active CO_2 compound (enzyme dependent)	Key enzyme(s)	Reference
Reductive acetyl-CoA pathway	Wood-Ljungdahl pathway	CO_2/HCO_2^-	Acetyl-CoA synthase – CO dehydrogenase	8
Dicarboxylate/ 4-Hydroxybutyrate cycle	---	HCO_3^-	4-Hydroxybutyryl-CoA dehydratase	9
3-Hydroxypropionate/ 4-Hydroxybutyrate cycle	---	CO_2/HCO_3^-	Acetyl-CoA/ Propionyl-CoA carboxylase	2,10

While the reductive acetyl-CoA pathway and the dicarboxylate/4-Hydroxybutyrate cycle only occur in strictly anaerobic species, the 3-HP/4-HB cycle is the CO_2 fixation pathway of autotrophic species within the order Sulfolobales and is one source of the physiological versatility of *M. sedula*.

2.2 3-HP/4-HB cycle

First described in 2007, the cycle consists of 16 reactions catalyzed by 13 enzymes. When the cycle was discovered in *M. sedula*, those reactions were thought to be catalyzed by the same number of enzymes. For the most of those enzymes, no specific gene could be assigned to them, but over time, more and more candidate genes were ruled out leading to the present state of 10 enzymes catalyzing one reaction and the following 3 multifunctional enzymes:[11]

- 3-hydroxypropionyl-CoA dehydratase/crotonyl-CoA hydratase[12]
- Acetyl-CoA/Propionyl-CoA carboxylase[10]
- Malonyl-CoA/Succinyl-CoA reductase[13]

The reactions of the cycle can be divided into two parts. In the first half, succinyl-CoA is formed from acetyl-CoA via the first eponymous intermediate, 3-hydroxypropionate, incorporating 2 CO_2 molecules in the form of carbonate ions.[14,15] In the second part of the cycle, the resulting succinyl-CoA is then converted to 2 molecules of acetyl-CoA via the second name-giving intermediate 4-hydroxybutyrate.[16]

The overall balance of the cycle leading to the formation of one molecule acetyl-CoA is as follows:

$$2\,HCO_3^- + 4\,NADPH/H^+ + 4\,ATP \rightarrow Acetyl\text{-}CoA + 4\,NADP^+ + 4\,ADP + 4\,P_i$$

The cycle with intermediates and the enzymes catalyzing the reactions are shown in Figure 1.

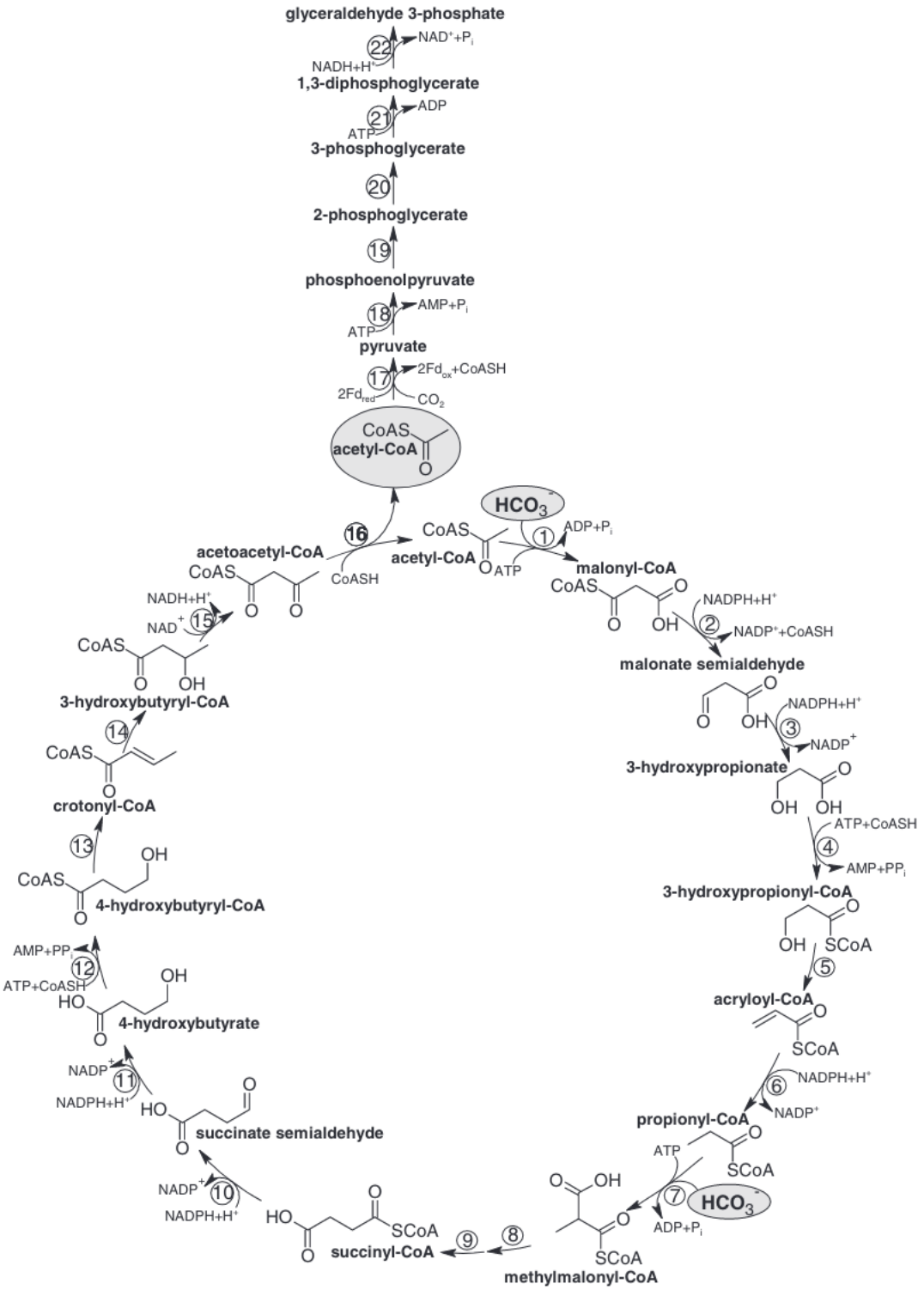

Figure 1: Reactions and catalyzing enzymes of the 3-HP/4-HB cycle. Enzymes include: 1. Acetyl-CoA carboxylase; 2. Malonyl-CoA Reductase; 3. Malonate semialdehyde reductase; 4. 3-Hydroxypropionyl-CoA synthetase; 5. 3-Hydroxypropionyl-CoA dehydratase; 6. Acryloyl-CoA reductase; 7. Propionyl-CoA Carboxylase; 8. Methylmalonyl-CoA Dehydratase; 9. Methylmalonyl-CoA mutase; 10. Succinyl-CoA reductase; 11. Succinate semialdehyde reductase; 12. 4-Hydroxybutyryl-CoA synthetase; 13. 4-Hydroxybutyryl-CoA dehydratase; 14. Crotonyl-CoA hydratase; 15. 3-Hydroxybutyryl-CoA dehydrogenase; 16. Acetoacetyl-CoA β-ketothiolase[2]

Note: Reactions catalyzed by enzymes 1/7, 2/10 and 5/14 are catalyzed by one promiscuous enzyme each [10,12,13]

So, as can be seen in Figure 1, one of two molecules of acetyl-CoA can be further metabolized via the incorporation of another CO_2 molecule leading to conversion to pyruvate catalyzed by a pyruvate synthase. The other acetyl-CoA stays in the cycle to serve as a substrate for another turn producing two further molecules of acetyl-CoA.[17]

The 16 reactions in this cycle are catalyzed by 13 different enzymes while 3 of them catalyze more than one reaction. Within the cycle, different kinetic parameters have been monitored and in a very detailed study by Loder et al. (2016)[18], a kinetic model was developed for all 16 reactions with side reactions including the production of biomass from acetyl-CoA and succinate. The key messages of this study were the development of a kinetic model for the whole cycle and the analysis of the applicability of the cycle in metabolic engineering. Regarding reaction kinetics, Flow Control Coefficients (FCCs) were determined for all enzymes, including two promiscuous ones, acetyl-CoA/propionyl-CoA carboxylase and malonyl-CoA/succinyl-CoA reductase. Both enzymes showed high impact values for the predicted production of biomass from acetyl-CoA as well as succinate. This explains the large proportion of those enzymes compared to the whole enzyme mass. In contrast to that, enzymes like succinic semialdehyde reductase or 4-hydroxybutyryl-CoA synthetase were shown to have a highly positive effect on the production of biomass from acetyl-CoA, but a rather negative influence on the biomass produced from succinate. This is the case because of the catalyzed reactions leading to the consumption of succinyl-CoA and the production of acetyl-CoA. There are some enzymes with FCCs supporting the vice versa case, but according to the model, those enzymes make up only a very small portion of the total enzyme mass. Furthermore, the model predicted that the maximum biomass production is achieved when only acetyl-CoA/propionyl-CoA carboxylase and malonyl-CoA/succinyl-CoA reductase make up almost 70% of the total enzyme mass. This result is also consistent with the ones found in other, similar studies.[10,19] Especially the former of the two enzymes also plays an important role in the applicability of the 3-HP/4-HB cycle in metabolic engineering. With 3-HP not occurring in high amounts naturally, but being a very important chemical as a precursor for the production of 1,3-propanediol or biopolymers, its production in microbials got a lot of attention in the past.[20] For example, taking the heterotrophic production of 3-HP in the anaerobic and hyperthermophilic archaeon *Pyrococcus furiosus* which is the most promising species to produce this chemical. Enzymes of the 3-HP/4-HB cycle are heterologously expressed in *P. furiosus* making it able to fixate CO_2 and through that producing 3-HP with 2 carbons of the produced chemical coming from a sugar source and

one carbon coming from the fixated CO_2. The key enzyme in this whole process is acetyl-CoA/propionyl-CoA carboxylase making up over 90% of the whole enzyme mass involved in this cycle which makes it a perfect regulator for acetyl-CoA usage within the cycle (note: the higher the activity of enzyme, the more acetyl-CoA is used for the cycle and the less of it is used for biomass production). This and the fact that the ATP/CO_2 ratio is 2.6-fold lower in the engineered cycle producing 3-HP compared to the native cycle makes it a very valuable application concerning production of an important chemical and CO_2 fixation.[18,21,22]

2.3 Lithoautotrophy

Another very important property of *M. sedula* is its ability to bioleach, a process which includes the biological metal extraction from ores. In order to carry out this, *M. sedula* uses its lithoautotrophic lifestyle by using CO_2 as carbon source to build up cell material and by deriving energy from inorganic matter (from Greek: 'feed on stones').

For bioleaching, there are some basic requirements that must be met. First, the process must take place in a water-rich environment in order to dissolve soluble salts. Second, the ores must contain substances that can be oxidized by microorganisms. These are often iron or sulfur compounds. When using bioleaching as a biotechnological application under controlled conditions, those compounds can also be added if the ores do not contain enough oxidizable components.[23] Last but not least, the necessary growth substrates for the bioleaching microorganisms must of course be present.

Bioleaching proceeds in several steps. As an example, the leaching steps of pyrite (FeS_2) and chalcopyrite ($FeCuS_2$), the main ores used in application of *M. sedula*, are as follows: First, both the iron and sulfur components of the ores must be oxidized. Since *M. sedula* is capable of both, this can be done by the organism itself instead of the conventionally applied chemical oxidation by the addition of Fe(III) ions. Then, the resulting products must be distinguished from each other. Pyrite produces thiosulfate ($S_2O_3^{2-}$) and chalcopyrite produces elemental sulfur in oxidizing conditions like low pH value used in the cultivation of thermoacidophilic archaea. Subsequently, the resulting Fe(II) ions are oxidized by the organism to Fe(III) ions and the oxidized sulfur compound formed in each case is further oxidized to the soluble sulfate salt (note: elemental sulfur is inert and can only be oxidized biologically to sulfuric acid).[24–27]

Using *M. sedula* for bioleaching, cultivation conditions are advantageous, leading to increased leaching rates. Among these are both the low pH (2-3) during cultivation, which leads to higher

oxidation rates (depending on the organism there is a certain point after which at lower pH values the leaching rates rapidly drop) and the high temperature (75°C), which generally accelerates the processes of bioleaching.[28,29] Nevertheless, there are ways to further increase the leaching rates of *M. sedula*.[30]

Since thermoacidophilic organisms in particular have great potential in terms of bioleaching due to their extreme growth conditions, several metal- and acid-resistant strains have been developed in the past. In the case of *M. sedula*, for example, this was achieved by McCarthy et al. using adaptive laboratory evolution or directed evolution. They isolated a strain (*M. sedula* SARC-M1) that could grow at pH 0.9, an acidity level at which the native strain no longer exhibits growth. However, even at pH 1.2 (a level at which the native strain still grows), the newly isolated strain achieved higher leaching rates (25% higher) of copper from enargite (Cu_3AsS_4).[31] The metals copper and arsenic contained in enargite are also good examples of another way to increase leaching rates in archaea. In this specific case, it is a matter of inducing resistance of the cells to high concentrations of heavy metals. Copper and arsenic are a special case, as it has been shown in the past that resistance to these metals can also occur together in one strain. To date, a total of four so-called cross-resistant strains have been isolated (ARS50-1/2 and ARS120-1/2), with all four developed starting from the copper-resistant strain CuR1 via adaptive laboratory evolution.[31–34]

3 Heterotrophy

3.1 Heterotrophic growth within the order Sulfolobales

Compared to autotrophy, the heterotrophic metabolism of *M. sedula* is by far not as well studied. In the past, mainly protein-based complex carbon sources were used for heterotrophic cultivation. These include tryptone, peptone, or yeast extract, among others. With the help of media containing these carbon sources, many studies have already been carried out, most of which using growth kinetics and cell densities of heterotrophic growth as a comparison to autotrophic or mixotrophic (autotrophic + organic carbon source) cultures.[21,34,35]

Species from the order Sulfolobales and especially from the genus *Sulfolobus* (obligate aerobes and largely chemoorganoheterotrophic organisms) are known for their diverse carbohydrate metabolism. Carbohydrates that can be metabolized by *Sulfolobus* spp. include various hexoses (glucose, mannose) as well as pentoses (arabinose), although the latter can only be used by *S. solfataricus* and *S. acidocaldarius*. In addition to these, di- and polysaccharides as well as alcohols, sugar and amino acids can also be metabolized as carbon sources for heterotrophic growth.[36–43]

Despite the diversity of metabolic substrates, the way they are metabolized varies in archaea compared to bacteria and eukaryotes. In a large study in 2014, Bräsen et al. were able to show in great detail how the pathways differ between the domains. Roughly speaking, the differences are that diverse modifications in central carbohydrate metabolism arose in archaea and thus, classical pathways such as the Embden-Meyerhof-Parnas (EMP) pathway and the Entner-Doudoroff (ED) pathway are not found in archaea. In addition, the pentose phosphate pathway is rarely, if ever, found in archaea, and the degradation of pentoses generally differs greatly between the domains.[44]

However, it is known that *M. sedula* has an incomplete citric acid cycle (TCA cycle) because the enzyme alpha-ketoglutarate dehydrogenase is missing. On the one hand, this is used to produce important metabolites because the unreacted and thus accumulating alpha-ketoglutarate is used to produce the carbon skeleton for the amino acids glutamine, proline and arginine. On the other hand, due to the incompleteness of the TCA cycle, the classical regeneration of reduction equivalents (NADH, $FADH_2$) is missing. Instead, M. sedula uses an an alpha-ketoglutarate:ferredoxin-oxidoreductase catalyzing the reversible conversion of alpha-

ketoglutarate to succinyl-CoA replacing the irreversible reaction catalyzed by the missing alpha-ketoglutarate dehydrogenase. This is contrasted with some other archaea from the order Sulfolobales such as *S. acidocaldarius*, an organism that has a complete TCA cycle and can also introduce pentoses into it as alpha-ketoglutarate via the aldolase independent Weimberg pathway. Furthermore, it possesses a gluconate dehydratase making the metabolization of glucose possible by entering the ED pathway[45–49]

Since *M. sedula* lacks the enzymatic repertoire for the aforementioned pathways, the organism is unable to metabolize sugars besides mannose to some extent. In general, within the genus *Metallosphaera*, only *M. cuprina* is known to possess a complete TCA cycle and thus to be able to metabolize diverse sugars as well.[4]

The inability to metabolize sugars also raises the question of how one could potentially get to a defined medium. A very large part of the media known so far for species of this order consist of sugars or their derivatives as carbon sources. Since this is somewhat difficult in the case of *M. sedula*, one must ask why it is necessary to cultivate with defined media at all and why one cannot simply use the established complex media.

3.2 Purpose and necessity of a defined medium

Complex, largely protein-based substrates have been widely used in heterotrophic and mixotrophic cultivation of *M. sedula* and the growth kinetics of the organism on tryptone are already well known. However, despite the good growth, there are some aspects of such media that can be a hindrance in their application. In general, complex media can be described as media whose exact composition is not known, since they are substrates of biological origin. Concerning this study, we are talking about tryptone, an enzymatic cleavage product of casein, which consists mainly of peptides and partly of free amino acids. In addition to this, however, tryptone also contains various unwanted components resulting mainly from the biological matrix but also from the manufacturing process. This in turn causes certain batch-to-batch variations, which also makes reproducibility difficult.[50]

Another property of tryptone is that it contains traces of uracil. Auxotrophy to uracil has been used as a selection marker in the past in relation to genetic manipulation. In this case, the complete uracil had to be removed from the complex C source first, a very laborious process.

With chemically defined media, one would be able to avoid this problem since no unknown or unwanted component would be found here.

Defined media have the advantages over complex ones of being less contaminated and also having less to no variation. However, a very important factor, especially for large-scale applications, is also the economic feasibility of the process. On a small scale, especially defined media with many components are still very expensive compared to complex ones. The larger the scale, the less expensive defined media become, with complex media remaining constant in their cost per liter.[51]

4 Lipids in extremophilic archaea

Species within the phylum *Crenarchaeota* have a very characteristic membrane lipid composition consisting of a core structure of isoprenoids which can be composed of diterpenoids or tetraterpenoids. The connection is made via one or two glycerols, to each of whose sn-2 and sn-3 positions these chains are attached via ether bonds. Connected to the sn-1 position of the glycerol, polar head groups modify those lipids on an additional level and mainly consist of inositolphosphate or (poly)-hexoses (Hex). The basic core structure consists of isoprenoid chains with a length of 20 carbon atoms (C_{20}) in sn-2 and sn-3 position, building a diterpenoide with a C_{40} core structure called archaeol (diether lipid, DEL). Condensation of two archaeol molecules gives rise to a tetraterpenoide with a C_{80} core structure which is known as caldarchaeol (tetraether lipid, TEL).[52–54]

Further important membrane components are glycerol dialkyl calditol/nonitol tetraether (GDNT) lipids which represent the major class in species within the order Sulfolobales making up around 80% of all core lipids. As this class of lipids is responsible for the acid resistance of these thermoacidophilic archaea, in almost all species, it is indispensable. However, in 1999, strain TA-2 was obtained from an acidic hot spring in Japan. It represents a GDNT deletion strain and therefore lacks those lipids. After phylogenetic analysis it was discovered that TA-2 was closely related to *M. sedula* and *M. prunae*.[55]

The core lipids and especially the DEL/TEL ratio vary depending on the species and more importantly they vary upon growth conditions. In contrast to that, the polar head groups of the lipids are an important and unique taxonomic marker. These head groups are either phospholipids or glycosides, but can also be combined to phosphoglycolipids. As a taxonomic marker, it was shown in the past, that there is a correlation between polar lipid composition and the 16S rRNA.[56–58]

However, not only the lipid compositions of archaea is unique, but also the possible applications of archaeal lipids and archaeal liposomes (archaeosomes). One example is the usage as drug or gene delivery system. Archaeosomes have many advantages as delivery systems such as stability to enzymatic hydrolysis and they are also self-adjuvant meaning that the lipid itself accelerates and prolongs the immune response.[59]

Therefore, archaeosomes can be used as a promising delivery system.

Paper Draft

Development of a defined medium for the heterotrophic cultivation of *Metallosphaera sedula*

Maximilian Luger[1], Viktor Sedlmayr[1], Julian Quehenberger[1], Oliver Spadiut[1*]

[1]*Institute of Chemical Engineering, Vienna University of Technology*
**Correspondence; E-mail:* oliver.spadiut@tuwien.ac.at

Abstract

Metallosphaera sedula, a thermoacidophilic Crenarchaeon, is a model organism for autotrophic CO_2 fixation using its 3-Hydroxypropionate/4-Hydroxybutyrate cycle. Heterotrophic growth has not been the focus of many studies in the past. Since its first isolation in 1989, complex organic media based on tryptone or yeast extract were used for the heterotrophic cultivation of this organism. Complex media have several drawbacks, including batch-to batch variation and a high number of different contaminations, limiting its applicability in genetic manipulation of uracil auxotrophic strains.

In this study, these limitations were addressed and a defined medium was developed for heterotrophic growth. Starting from Casamino Acids, we were able to imitate amino acid composition and achieve cell growth in a medium containing 16 amino acids. After analysis of substrate uptake, the number of different amino acids could be reduced to five while maintaining a comparable cell growth to complex organic media. In this final 5AA medium, glutamic acid, proline and cysteine were identified as essential amino acids while valine and phenylalanine had a growth promoting effect.

Keywords: *Metallosphaera sedula*, defined cultivation medium, FS medium, 5AA medium, Amino Acids

1 Introduction

Metallosphaera sedula was isolated for the first time from a sulfur-rich, volcanic field in Italy by Huber et al.[1] in 1989. With optimal growth conditions at 75°C and a pH value of 2.0-2.5 it belongs to the group of thermoacidophilic archaea.

Dealing with cultivation of archaea, the order Sulfolobales is a highly studied group of organisms and within this order, there are some model organisms for different modes of cell growth. For heterotrophic growth, the genus *Sulfolobus* is a frequently used in cultivation studies and many of its species are well characterised.[60,61] Regarding autotrophic growth on the other hand, the genus *Metallosphaera* is one of the best known and most interesting species. When comparing *Metallosphaera* spp. with other genera and species within the order Sulfolobales, they stand out due to their diversity of growth modes.[62]

Probably the most studied *Metallosphaera* species is *M. sedula* which has made a name for itself through its CO_2 fixation pathway (3-hydroxypropionate/4-hydroxybutyrate cycle), the enzymes of which have already been deciphered in many studies and are very well understood.[10,12,13,17–19,63–67] Enzymes from this cycle have been used in metabolically engineered strains of other organisms for the production of valuable chemicals like 3-Hydroxypropionate.[68–72] In addition, its application in biomining has been widely investigated and enhancement of bioleaching activity has been the objective of many studies. This is mostly accomplished by enhancing acid or heavy metal resistance, through amplification of cell growth or by regulation of the redox environment. The exact mechanism of those heavy metal resistances is not yet well known, but in general, there are seven different postulated ways of how acidophilic organisms are able to tolerate high heavy metal concentrations of which the most common regulation is via membrane transport.[73] Several different *M. sedula* strains have been developed carrying one of those or even cross-resistances. Most of these adapted strains were isolated by adaptive laboratory evolution (ALE) by continuously increasing heavy metal concentration or acidity. The first isolated acid resistant strain (SARC-M1) is able to grow at a pH value of 0.9 while the wild-type is only able to survive down to pH 1. Regarding metal resistance, five major strains were isolated in the near past. CuR1 is the first highly copper resistant strain (containing 20 distinct mutations) isolated and can grow in media containing up to 200 mM copper compared to the parental strain only surviving up to 75 mM, but even then, with a long lag phase. The other heavy metal to which resistant strains have already been

isolated is arsenic. A consortium of 4 strains, namely ARS50-1/2 and ARS120-1/2, with enhanced resistance to 75 mM arsenic and a total of 12 and 13 mutations, respectively, were developed (Note: arsenic-resistant strains were not adapted from the DSM 5348 strain, but from CuR1, resulting in a cross-resistance to arsenic and copper in those strains). The major advantage of all these adapted strains is the wider range of application in bioleaching, as they are able to perform either at low pH, conditions conducive to bioleaching, or at high heavy metal concentrations. This leads to more efficient bioleaching being carried out by these strains.[26,30–32,34,73–77]

When speaking about the heterotrophic lifestyle of certain thermoacidophilic archaea within the order Sulfolobales, it is common practice to use diverse complex organic protein-based substrates in addition to whatever C-source(s) is (are) suitable for the particular organism. Products like N-Z-amines, yeast extract, tryptone/peptone or casein hydrolysates are typically used to enhance growth, especially within the initial phase.[78,35,79–81] Several organisms within the order Sulfolobales are able to metabolize a great variety of sugar variants, including mono-, di-, but also polysaccharides. In the past, metabolization of sugars, amino acids and several other substrates influencing growth of thermoacidophilic archaea has been extensively studied and for some of species withing the order Sulfolobales, defined cultivation media have already been developed.[78,79] One of the major distinctions between organisms of the genera *Sulfolobus* and *Metallosphaera* is the difference in the metabolization of sugars. While only specific *Metallosphaera* spp. are able to use a very small number of sugars, *Sulfolobus* spp. are able to metabolize almost all kinds of sugars with little to no exceptions.[4,82,83]

Application of *M. sedula* in bioleaching and its autotrophic lifestyle have been the main focus of research in this field, while the heterotrophic lifestyle is the least extensive studied field regarding this organism. At least two attempts of developing a defined cultivation medium can be found in the literature, both of which were unsuccessful. It has been shown that this organism is not able to metabolize any kinds of sugar and until now, in every study containing heterotrophically grown *M. sedula* cells, complex protein-based C-sources were used. Most of those studies dealt with a comparison of heterotrophic, autotrophic and mixotrophic growth conditions.[11,26,84,85]

A defined cultivation medium should enable easy genetic manipulation using the versatile toolboxes found in the literature. These often foresee the utilization of uracil auxotrophies as a selection marker, a task that has been proven to be error prone and laborious with complex,

organic C-sources due to contamination with components of the biological matrix. To overcome this problem, McCarthy et al. made efforts to cultivate *M. sedula* on free amino acids as C-source and with that, develop a defined medium. This was also the first attempt to develop a defined medium for heterotrophic cultivation of this organism since Huber et al. in 1989.[4,84,86] In this study, the steps towards the development of a defined medium for reproducible heterotrophic and mixotrophic cultivations are presented and a characterization of *M. sedula* regarding substrate uptake is conducted.

2 Materials and Methods

2.1 Growth conditions

M. sedula DSM 5348 was cultivated aerobically at 75°C at an initial pH value of 2.2. In order to avoid evaporation and create a reflux condensing environment, long neck shake flasks (100 mL) were used in a shaking oil bath with 50 mL initial culture medium to provide enough gas space for an adequate oxygen supply. For the growth experiments in this study, cells from precultures were used as inoculum. In order to prevent a carryover of media components, the cell broth of the preculture was centrifuged (5.000 g, 5 min) and resuspended in 1 mL of the culture medium. Initial OD_{600} after inoculation ranged between 0.005 and 0.01 in all experiments.

2.2 Media preparation

The basal salt medium was prepared according to Brock et al.[60] (Table 2), with only the original iron source being replaced by Fe(III)-citrate, and used in all experiments within this study. As complex C-sources, tryptone (Carl Roth, Germany) and Casamino Acids (Thermo Fisher Scientific, USA) were used at a concentration of 1 g/L.

The amino acid composition of the FS medium is shown in Table 4. The proportion of amino acids in the FS as well as the simplified media further used is based on Casamino Acids (CAAs). To allow direct comparability and reproducibility, the total concentration of AAs in the defined media were normalized to 1 g/L. Additionally, Wolin's vitamin solution (Table 3) was added to defined media.[87]

Table 2: Composition of brock basal medium and complex C-sources used

Brock medium basal salts and trace elements				
$(NH_4)_2SO_4$	1.30	g/L	9.84	mM
KH_2PO_4	0.28	g/L	2.06	mM
$MgSO_4$*7 H_2O	0.25	g/L	1.01	mM
$CaCl_2$*2 H_2O	0.07	g/L	0.48	mM
Fe(III)-citrate*H_2O	19.5	mg/L	72.14	µM
$Na_2B_4O_7$*10 H_2O	4.50	mg/L	11.80	µM
$MnCl_2$*4 H_2O	1.80	mg/L	9.10	µM
$ZnSO_4$*7 H_2O	0.22	mg/L	0.765	µM
$CuCl_2$* 2 H_2O	0.05	mg/L	0.293	µM
Na_2MoO_4*2 H_2O	0.03	mg/L	0.124	µM
$VOSO_4$*2 H_2O	0.03	mg/L	0.151	µM
$CoCl_2$*6 H_2O	0.01	mg/L	0.036	µM

Table 3: Wolin's vitamin solution used with all media expect for the ones supplemented with tryptone

Vitamin	Final concentration [mg/L] 100x Stock
Biotin	2
Folic acid	2
Pyridoxine*HCl	10
Riboflavin	10
Thiamine	5
Nicotinic acid	5
Pantothenic acid (Ca salt)	5
Vitamin B12	0.1
4-Aminobenzoic acid	5
Lipoic acid	5

Table 4: Amino acid composition of the FS medium (1 g/L)

Amino Acid	Concentration [mg/L]	Molarity [mmol/L]
L-Glutamic acid	203	1.57
L-Proline	142	1.46
L-Lysine	80	0.62
L-Aspartic acid	74	0.64
L-Cysteine	72	0.70
L-Methionine	61	0.47
L-Leucine	57	0.50
L-Valine	57	0.58
L-Serine	52	0.60
L-Threonine	39	0.39
L-Arginine	34	0.22
L-Isoleucine	34	0.30
L-Alanine	32	0.45
L-Phenylalanine	25	0.17
Glycine	19	0.33
L-Histidine	19	0.14

2.3 Analytical methods

Cell densities were determined by measuring the optical density photometrically at 600 nm with an ONDA V-10 Plus spectrophotometer (GIORGIO BORMAC, Italy). Each sample taken for further analysis was centrifuged (21913 g, 4°C, 10 min) with a SIGMA 3-18KS universal refrigerated table top centrifuge (Sigma Laborzentrifugen GmbH, Germany). The supernatant was stored at -20°C until measurement.

Amino acids in the supernatant were analyzed using HPLC which was carried out using an Ultimate 3000 (Thermo Fisher Scientific, USA) system. For analyte separation, a reversed-phase column column (Agilent ZORBAX Eclipse AAA; 150x3 mm, 3.5 µm) equipped with the corresponding precolumn (Agilent Eclipse AAA; 12.5x3 mm, 5 µm) in a column oven heated to 40°C were applied. For the gradient in this method (Table 5), eluent A (40 mM NaH_2PO_4*H_2O, pH 7.8) and eluent B (MeOH/ACN/MQ, 45/45/10, v/v/v) at a flowrate of 1.2 mL/min were used. Injection volume was set to 10 µL. The gradient applied can be found in Table 5.

Table 5: Gradient of HPLC method analyzing amino acids

t (min)	-1.0	0.0	2.5	20.0	20.5	23.0	23.5	26.0
% A	100	100	100	51.5	0	0	100	100
% B	0	0	0	48.5	100	100	0	0

For detection, an in-line derivatization step was performed. For primary amino acids, ortho-phthaldialdehyde containing 1% 3-mercaptopropionic acid and for secondary amino acids, 9-fluormethylencarbonylchloride was used as a derivatization agent. Norvaline (primary amino acids) and sarcosine (secondary amino acids) served as internal standards and were each added with a final concentration of 1.25 mM to all samples. Analytes were detected by a fluorescent detector run at 340/450 nm (excitation/emission) for primary and 266/305 nm for secondary amino acids.

2.4 Calculation of dry cell weight and specific reaction rates

With the obtained data of cell growth and substrate concentrations over the course of the cultivation, the following values and rates could be calculated:

- Dry cell weight, **DCW [g/L]**, was calculated by multiplication of the measured OD_{600} values with a factor of 0.171, which was derived from an OD_{600}:DCW correlation curve of *M. sedula* cells grown on Brock basal + 1 g/L tryptone
- Specific growth rate, **μ [h^{-1}]**, between sampling points was calculated by dividing the difference in DCW by the mean DCW and the elapsed time between those sampling points
- Specific substrate uptake rate, **q_s [$g_s/g_x/h$]**, between sampling points was calculated by dividing the difference in substrate concentration by the mean DCW and elapsed time between those sampling points

3 Results and Discussion

3.1 Cultivation with complex media

3.1.1 Selection of a suitable complex C-source for further experiments

The state of the art C source for *M. sedula* is tryptone, with which high cell densities can be achieved. However, tryptone also shows a high level of contamination with salts and the biological matrix of the milk protein casein, from which it is produced by enzymatic cleavage. In addition, it consists largely of oligopeptides, which also makes analysis more difficult. For this reason, it was decided to search for an alternative C source with the motivation of facilitating analysis while still achieving suitable cell growth.

As alternatives, two different acidic hydrolysis products of casein were tested with Casamino Acids (CAAs) and casein hydrolysate. These consist almost exclusively of free amino acids as a result of the manufacturing process. After evaluating the growth kinetics, experiments were continued using CAAs due to higher cell densities.[88–94]

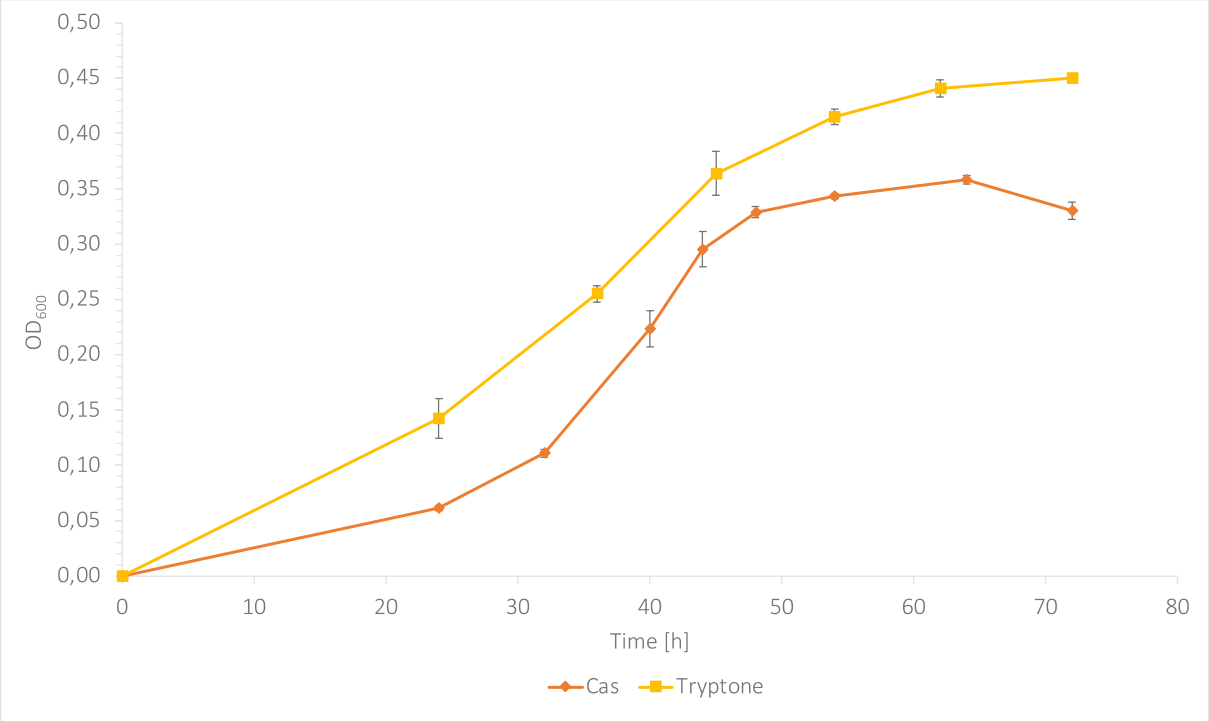

Figure 2: Comparison of growth kinetics of heterotrophically grown M. sedula cells using Brock basal medium and tryptone (orange) and CAAs supplemented with Wolin's vitamin solution (blue) as C-source. Both complex C-sources were used at a concentration of 1 g/L

Within the process of deciding which alternative complex C-source to use for the experiments, not only contamination and analyzability have been considered as important properties, but especially suitable cell growth was crucial. Several complex C-sources have been tested for cultivation of *M. sedula*, but there was no further knowledge about growth kinetics when comparing different C-sources. A comparison of growth kinetics of heterotrophically grown *M. sedula* cells using tryptone and CAAs as C-sources has been performed (Figure 2). It can be concluded, that CAAs enable cellular growth similar to the one observed when using tryptone. The composition of CAAs used in this study was determined (Table 6). For that purpose, the same batch of CAAs was analyzed in triplicates. (Note: Tryptophane is depleted during the acid hydrolysis treatment.)

Table 6: Amino acid pattern of CAAs under cultivation conditions (75°C, pH 2.2)

Amino acid	Concentration in CAAs [mg/g]	SD [mg/g]
Glu	154,3	2,3
Pro	94,4	3,2
Lys	65,7	4,6
Asp	51,5	1,0
Leu	43,9	0,3
Val	40,1	2,2
Ser	38,8	1,1
Cys	29,0	4,0
Thr	28,6	0,4
Arg	26,7	1,4
Ile	26,5	0,8
Phe	26,5	5,1
Ala	24,3	0,3
Met	23,3	3,0
His	18,0	0,5
Gly	14,6	0,5

When comparing the amino acid composition to tryptone, the amino acid pattern does not differ greatly from the CAAs. Nevertheless, the amino acids in tryptone are mainly bound to longer peptide chains which cannot be analyzed easily, but especially cannot be purchased commercially. Furthermore, peptides as a C-source in a defined medium would increase costs immeasurably resulting in no longer feasibility.[78,95]

3.2 Cultivation with defined media

3.2.1 The FS medium and a comparison to CAAs in liquid culture

With the knowledge gained from the performed experiment, the development of the first defined medium for the heterotrophic cultivation of *M. sedula* was possible. This newly developed FS medium contains 16 of the 20 proteogenic amino acids.

The FS medium was created as an imitation of the amino acid pattern of the CAAs. A comparison of the growth kinetics of *M. sedula* cells grown in FS medium as well as in Brock basal supplemented with 1 g/L CAAs is shown (Figure 3). The initial growth phase is comparable between both media whereas after 30 h of cultivation time, cells grown in FS medium started to grow faster than the ones cultivated on CAAs. After around 50 h, both cultures reached their stationary phase, but the FS cultures ended up growing to higher maximum cell densities ($OD_{600,max}$ = 0.454) than the CAAs cultures ($OD_{600,max}$ = 0.378). Interestingly, cell densities of the CAAs cultures decreased rapidly after reaching the stationary phase and ended up at an OD_{600} value of 0.231 after 120 h cultivation time. On the other hand, cells grown in FS medium could maintain the stationary phase until the end of cultivation after 120 h with a final OD_{600} value of 0.422. As for specific maximal growth rates, cultures in both media were comparable with values of μ_{max} = 0.081 h^{-1} for the FS cultures and μ_{max} = 0.076 h^{-1} for CAAs cultures.

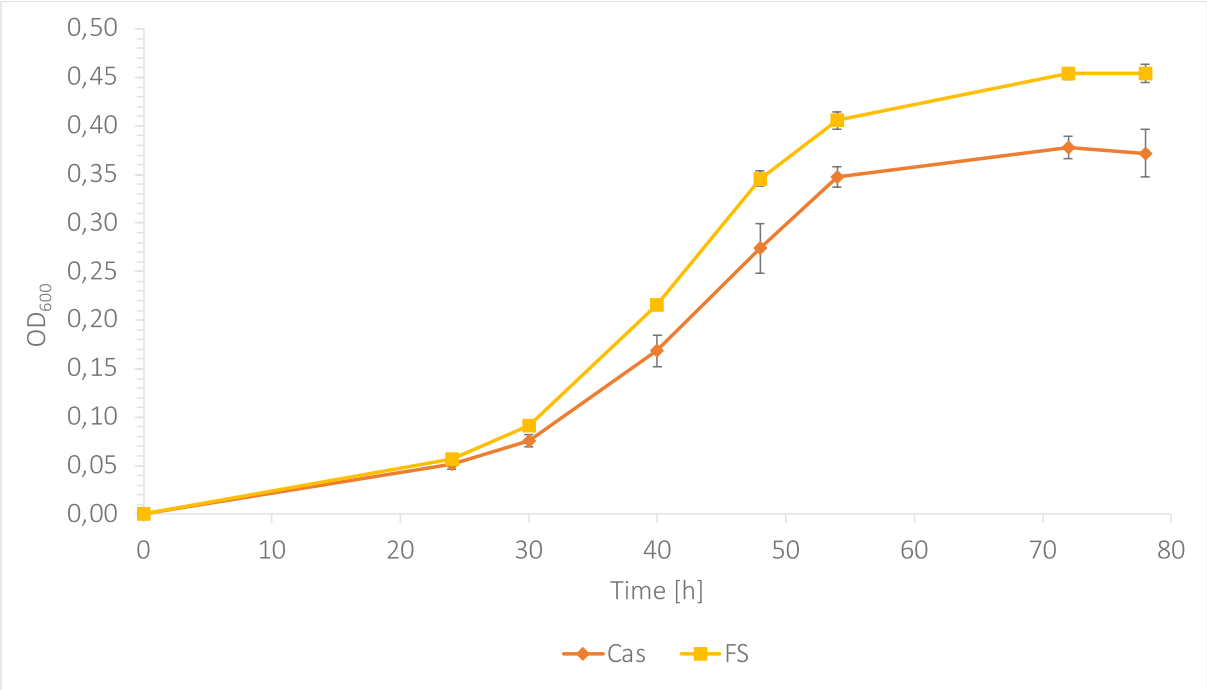

Figure 3: Comparison of growth kinetics of heterotrophically grown M. sedula cells using the newly developed FS medium (orange) and CAAs (blue) as C-source. Both media were supplemented with Wolin's vitamin solution, CAAs were used at 1 g/L.

Figure 4 shows the specific substrate uptake rates for each of the 16 amino acids present in both the CAAs and the FS medium.

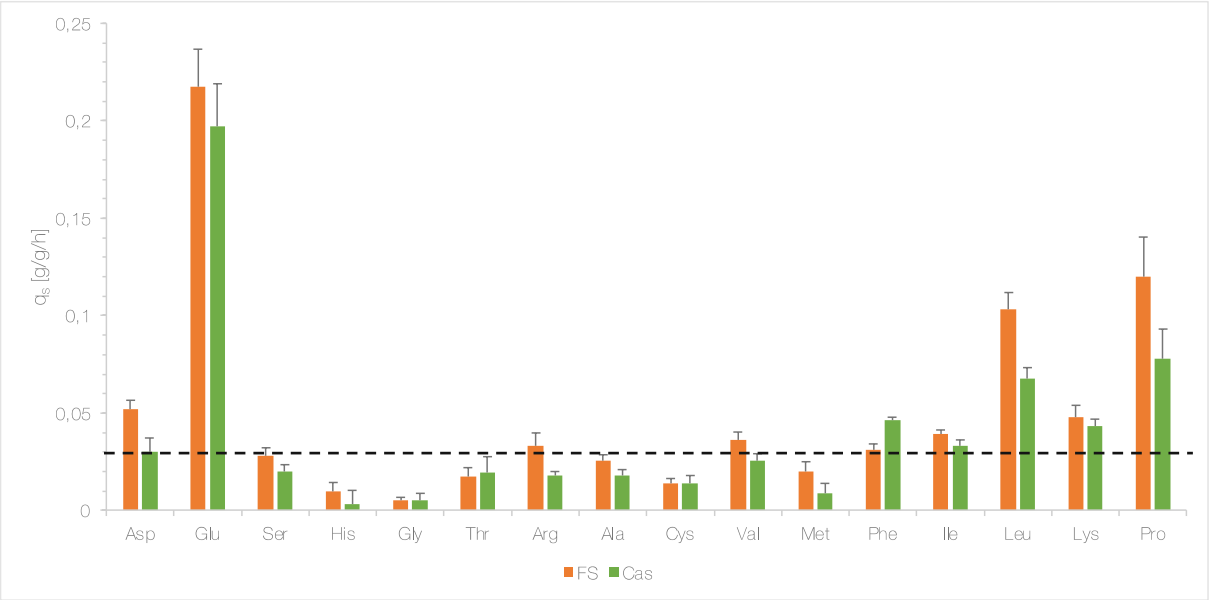

Figure 4: Specific substrate uptake rates for the 16 amino acids contained in both the CAAs and the FS medium. The average uptake rates between start of exponential phase (24h) until either reaching stationary phase (72h) or substrate depletion were calculated. The black, dashed line marks the threshold of q_s above which the amino acids were taken to get to the PS medium.

What immediately stands out is the consistency of the average substrate uptake rates in the exponential phase with the growth kinetics with the only exception being phenylalanine which is taken up more by cells grown on CAAs than by those grown in the FS medium. As can be seen, glutamate was by far taken up at the highest rate followed by proline. Glutamate acts as a central metabolite in many biosynthesis pathways and is used a sole C-source in the cultivation of other species within the order Sulfolobales.[78] In addition, glutamate is an immediate substrate for the TCA cycle, since it can be converted directly to alpha-ketoglutarate, catalyzed by a glutamate dehydrogenase and an aspartate aminotransferase, and thus participates in the formation of an intermediate of the TCA cycle.[96,97]

The second highest specific uptake rate was $q_{S,Proline}$. Proline has been described in several studies to act as a thermal stabilizer in proteins. The higher uptake rate in the initial growth phase could be due to the increased production of heat shock proteins in order to keep the cell alive at low cell densities. In general, proline is an important structural component increasing rigidity and thermal stability of proteins due to its less rotatable amide bond. This is also consistent with the higher uptake rate of proline in the initial growth phase and is an indication of proline being taken up as a structural component and not that much as an energy source.[98–100] There are several studies dealing with the occurrence of amino acids in thermophilic

proteins and what stands out is the very frequently mentioned ratio (Glu+Lys)/(Gln+His) which is typically higher in (hyper)thermophilic proteins while also salt-bridges between glutamate and lysine play an important role in thermophilic proteins.[101,102] When looking at the uptake rates of *M. sedula* regarding the amino acids, it can be seen that the rates of glutamate and lysine are higher than the one of histidine which supports that finding. Glutamine is not present in the medium due to transamination to turn into glutamate. In conclusion, the high uptake rate of glutamic acid and lysine suggests that, like proline, they serve both as energy sources and as structural elements in proteins. In addition, glutamate has a highly hydrophobic character in its protonated form supporting its uptake as a structural amino acid to form the favorable hydrophobic protein core.[102,103]

After characterizing the heterotrophic growth of *M. sedula* cells on CAAs and the FS medium, the defined medium still contained 16 different C-sources. In order to generate a more simple medium, the next objective was to reduce the number of amino acids.

3.2.2 Screening for possible ways to reduce the number of substrates of the FS medium

The first step towards a simplification of the FS medium was to look at the specific uptake rates of each of the amino acids during the cultivation. A threshold of 0.03 g/g/h was set (black, dashed line in Figure 4) and reduced the composition to 10 amino acids. However, using this medium, no growth was observable. After several screening experiments (data not shown), we deciphered glutamic acid, proline and cysteine as essential amino acids to obtain observable cell growth. After reduction of components, the PS (partly simplified) medium which contained amino acids with a q_s value greater than 0.03 g/g/h supplied with cysteine was tested. Growth kinetics of *M. sedula* cultivated in this medium are shown in Figure 5.

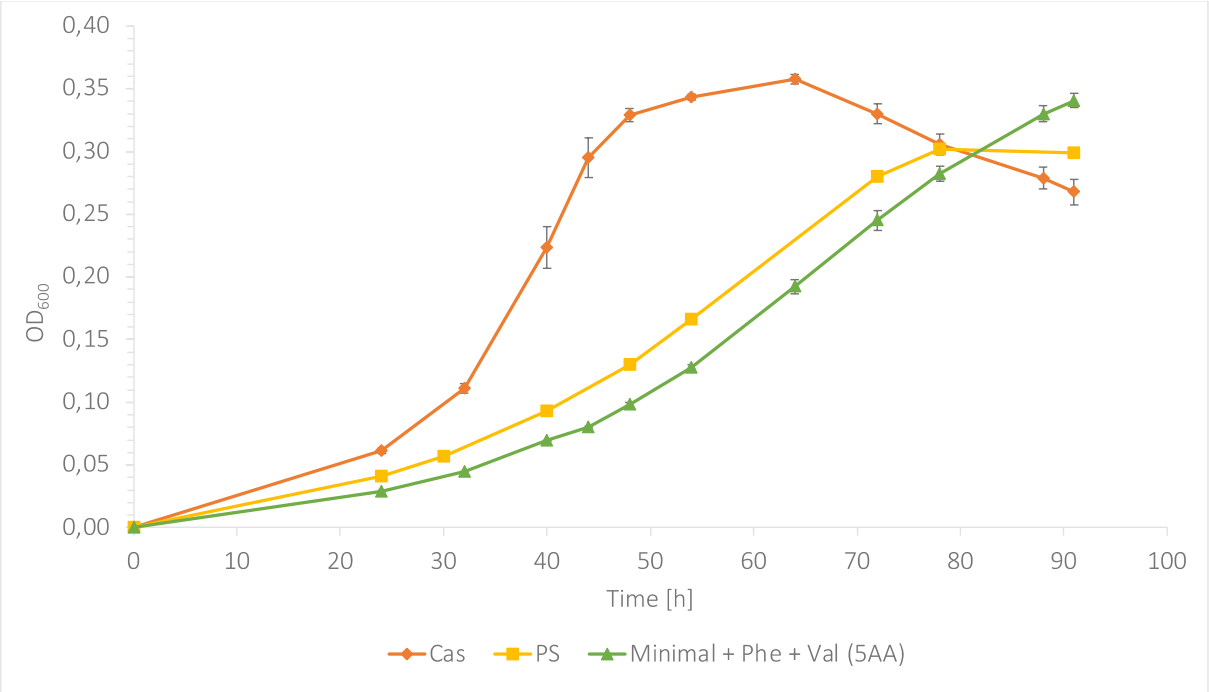

Figure 5: Comparison of growth kinetics of M. sedula cells grown in CAAs containing medium (orange), PS medium (yellow) and 5AA medium (green).

Since cell growth in a medium containing only the three essential amino acids (Glu, Pro, Cys) as C-sources was not satisfactory, amino acids were further tested for their growth-promoting properties. For this purpose, amino acids from the PS medium were added both individually and in groups to determine which amino acids improved growth. The first amino acid to be identified was phenylalanine, and based on this, in a further experiment, valine was also identified as growth-promoting, with phenylalanine showing these effects mainly in the later phases of cultivation and valine in the initial phase (Figure 6).

These experiments led to the final 5AA medium (Table 7). Growth kinetics were comparable to the ones of cells grown in PS medium, but were inferior to the complex medium. There still are some growth promoting components missing, especially in the initial growth phase compared to cells grown CAAs containing media, but final cell densities are comparable (Figure 5).

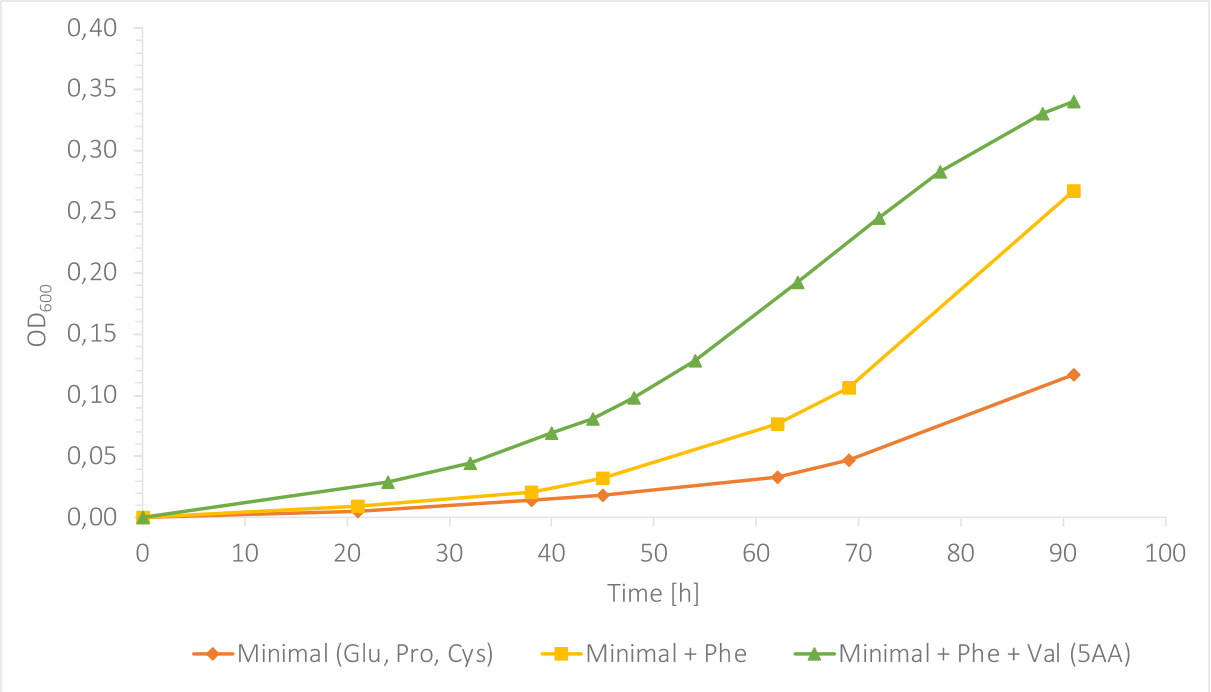

Figure 6: Evaluation of growth-promoting effect of amino acids when being added to a medium containing the essential amino acids (Glu, Pro, Cys)

Table 7: Amino acid composition of the 5AA medium (1 g/L)

Amino Acid	Concentration [mg/L]	Molarity [mmol/L]
L-Glutamic acid	406	3,15
L-Proline	285	2,94
L-Cysteine	145	1,41
L-Valine	114	1,15
L-Phenylalanine	49	0,33

4 Conclusion

While cultivation of *M. sedula* in complex media yields high cell densities and is easy to handle when considering cell growth as the only crucial parameter, it is very hard to apply any genetic work to an organism when not being able to do proper selection. With the development of a defined medium, the gate towards metabolic engineering of *M. sedula* and its application in heterotrophic and mixotrophic cultivation has been opened.

The amino acid composition of Casamino Acids was determined and we could specify crucial amino acids. With the collected data, the first fully defined medium for heterotrophic cultivation of *M. sedula* was developed, a novelty in *Metallosphaera* literature. The FS medium contains 16 of the 20 proteinogenic amino acids and does not contain any further carbon source. Growth kinetics as well as substrate uptake rates of all amino acids were consistent and also comparable with Casamino Acids and the FS medium.

With further experiments, the number of substrates in the medium could be reduced to get to the final "5AA" medium with 4 of the 5 amino acids (glutamate, proline, cysteine, phenylalanine) in the medium being crucial for growth and the 5th (valine) having a growth promoting effect when added.

Additional experiments within this thesis

1 Screening experiments for obtaining the 5AA medium

As the data obtained from this experiment were not shown in the paper draft, the 2 steps of getting from the PS to the final 5 AA medium are shown in this chapter. The initial objective was to reduce the number of substrates to as little as possible. Therefore, the decision of trying to do so in a stepwise approach was made. In the end, the approach contained two major steps.

1.1 First step of simplifying the PS medium

Starting from the PS medium (containing 9 amino acids, selected via the criteria of a specific substrate uptake rate higher than 0.03 g/g/h in cultivations using the FS medium), the first objective was whether the cysteine could be replaced by the second sulfur containing amino acid methionine.

The concentration of free cysteine varied relatively strongly during cultivation in FS medium, especially in the initial growth phase and after reaching stationary phase. A possible reason for this could be that in the analytical method, cysteine is analyzed as a cystine dimer, with the prerequisite for derivatization with ortho-phthalic dialdehyde (OPA) in this case being that the monomer is present. As an additional bias, the fluorescence signal of the cystine derivative is very weak, which may be due to a competitive reaction of OPA with the sulfhydryl group of cysteine, which competes with the thiol group of 3-mercaptopropionic acid.[104,105]

However, the results (shown in Figure 7) demonstrate that cysteine could not be replaced with methionine because there was no growth at all when exchanging those two amino acids. So, cysteine was determined as essential for the defined medium.

Subsequently, amino acids contained in the PS medium were classified as 2 major metabolic groups:

- Glutamate, Proline, Cysteine (essential amino acids for cultivation of *M. sedula*)
- Phenylalanine, Valine, Leucine, Isoleucine, Alanine, Aspartate and Arginine (potential growth promoting amino acids)

As the “minimal” medium, Brock basal supplemented with the 3 amino acids which were already known to be indispensable (glutamate, proline, cysteine) and Wolin’s vitamins was used. In addition to that, all the other amino acids mentioned were added in different combinations to get an idea which of them support cell growth and to point out the ones that do not. Already in this step, the main objective was the reduction of media components and when looking at the growth kinetics, a certain tendency towards the media with a larger number of amino acids could be seen. Nevertheless, one important thing could be observed. Comparing the growth kinetics of the experiments containing phenylalanine with those that do not, with the rest being equal, it was enhanced by only phenylalanine in each of them.

Therefore, the “minimal” medium was expanded by phenylalanine and the screening was continued with a new basis.

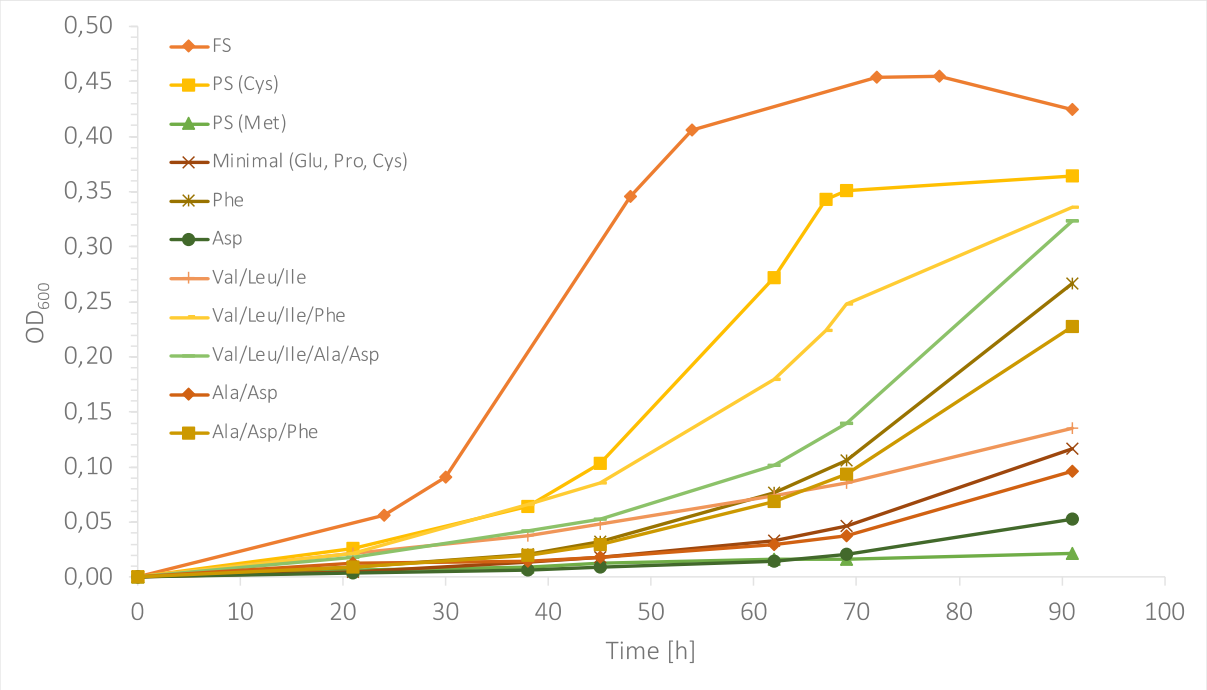

Figure 7: Second step of screening for necessity of certain amino acids with phenylalanine promoting growth as a result

1.2 Second and final step of simplifying the PS medium

After gaining knowledge about phenylalanine being a growth promoting factor, the second experiment towards the final medium was performed.

For that purpose, the new "minimal" medium (containing Glu, Pro, Cys, Phe) was supplemented with the metabolic group of BCAAs, but this time, the group was split and each of the amino acids assigned to it was supplemented on its own in 3 separate experiments. Additionally, all 3 combinations of pairs of those amino acids were tested.

Due to the PS medium also containing arginine which has not been assigned to any of those metabolic categories, its effect on growth was also monitored. It can be seen that in none of the experiments containing arginine, the growth was significantly better, so it was concluded that its growth promoting effect is neglectable.

Of the group of each of the BCAAs, the different effects on the growth can be seen clearly. Isoleucine had big growth inhibiting effect when being supplemented to the minimal medium by itself. In contrast to that, when being jointly added with valine, the growth was better than with the minimal medium. Therefore, it was concluded that valine had to have a very good growth promoting effect on the cells and this also led to the final 5AA medium due to valine having a strong growth promoting effect when being added alone. In contrast to that, when combining valine with leucine the growth was also inhibited (Figure 8).

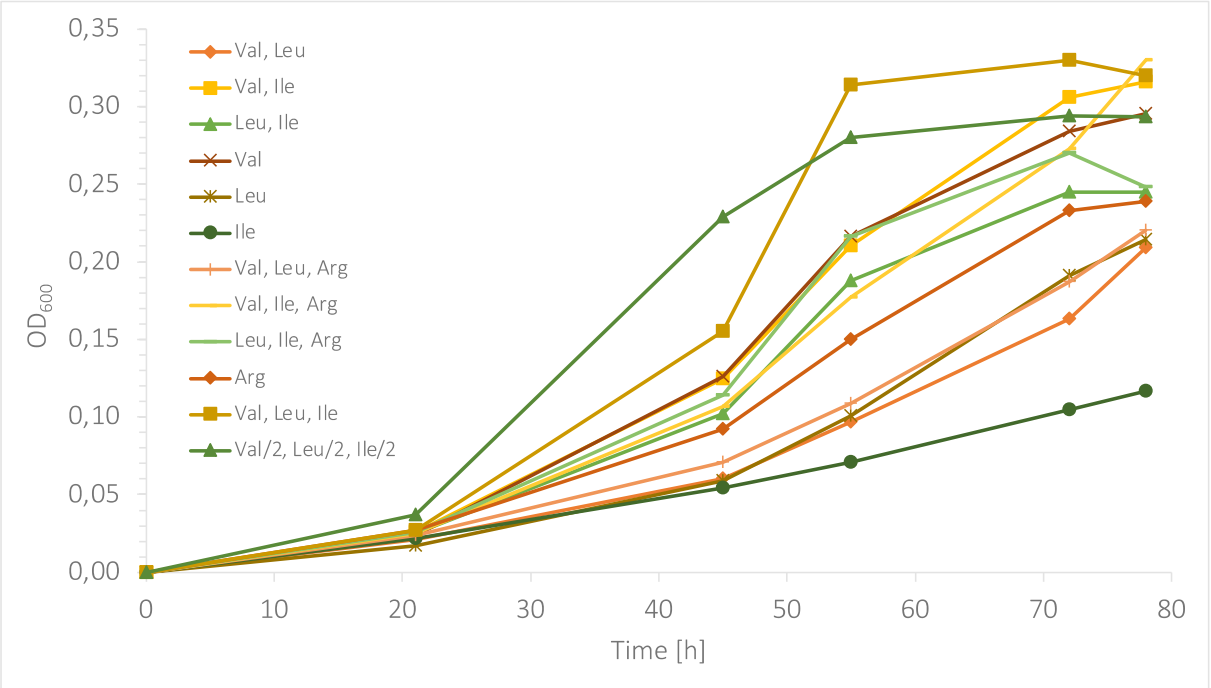

Figure 8: Second and final step of screening for necessity of certain amino acids with valine promoting growth as a result

2 Analysis of lipid pattern

Another experiment conducted within this study was the analysis of the lipid pattern of *M. sedula* cells grown on Brock basal medium supplemented with 1 g/L tryptone.

2.1 Lipid extraction

First, lipids were extracted using a slightly modified Folch extraction according to Folch et al. (1957).[106] After centrifugation of 50 mL cell broth, the cell pellet was resuspended in 3 mL of 155 mM ammonium acetate. The resuspended cells were then sonicated (Branson 450 digital sonifier, Branson Ultrasonics, USA) on ice 3 times for 30 seconds with 30 seconds of cooling time in between and afterwards diluted with 155 mM ammonium acetate to reach an OD_{600} value of 0.5. The cell suspension was then mixed with a 2:1 (v/v) mixture of chloroform/methanol with a ratio of 1:4.95 (v/v) in a glass vial with all steps the rest of the way being carried out in glassware to avoid extraction of plastic components. After shaking for several hours or overnight at 30 rpm and centrifugation (1000 g, 4°C, 2 min.), the upper aqueous phase was removed and the lipid containing lower organic phase was collected through the protein and nucleic acid containing interphase. In order to dry the lipids, the solvent was evaporated using a rotavapor. The dried lipids were then stored at -20°C until measurement.

2.2 Lipid analysis

All measurements were performed using a Shimadzu MALDI 7090 tandem time-of-flight (TOF) mass spectrometer (Shimadzu-Kratos Analytical, Manchester, UK). The instrument has a frequency-tripled 2 kHz Nd-YAG laser (λ = 355 nm) operated at a repetition rate of 200 Hz and desorbed/ionized analyte molecules are extracted with an accelerating voltage of ± 20 kV. All MALDI mass spectra are acquired in the positive- and negative-ion reflectron TOF mode, respectively (average of 10.000 spectra per final mass spectrum, 1 spectrum corresponds to the average of 5 individual laser shots). 2,4,6-Trihydroxyacetophenone was used as MALDI-matrix for all experiments (roughly 15 mg/1 ml methanol). Matrix and analyte solution were mixed 1:1 prior to sample preparation on standard stainless steel targets (roughly 0.8 µl per

spot, dried droplet technique). For positive- and negative-ion mass calibration the commercial Shimadzu TOF mix (Laser Biolabs, Valbonne, France) based on known peptides masses prepared on α-cyano-4-hydroxy cinnamic acid (CHCA) matrix was used.[52,107,108]

2.3 Results of lipid analysis and comparison to Sulfolobus acidocaldarius

As an example, the structure of a GDGT lipid with an inositolphosphate head group is shown in Figure 9. As can be seen in this structure, the high number of carbon atoms between the glycerols also enables cyclic structures to build within the isoprenoid chains. The average cyclisation number varies not only between organisms, but also between different growth rates and growth phases.[52]

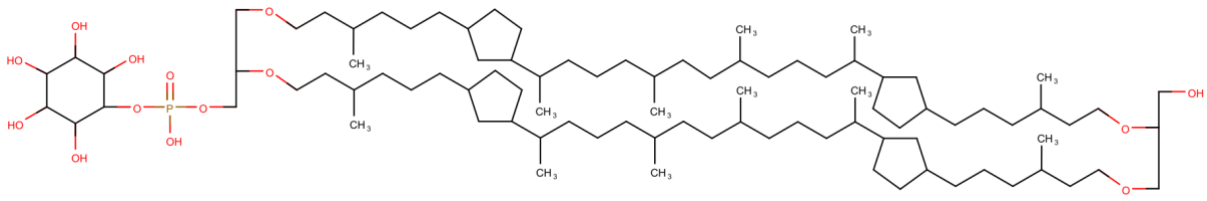

Figure 9: Example of the structure of an IP-GDGT lipid

Now, coming to the lipids present in the membrane of *M. sedula*, the results of the MALDI-TOF MS analysis can be seen in Figure 10 and Figure 11 (positive and negative ion mode). The most abundant and most important lipids contained are shown in Table 8.

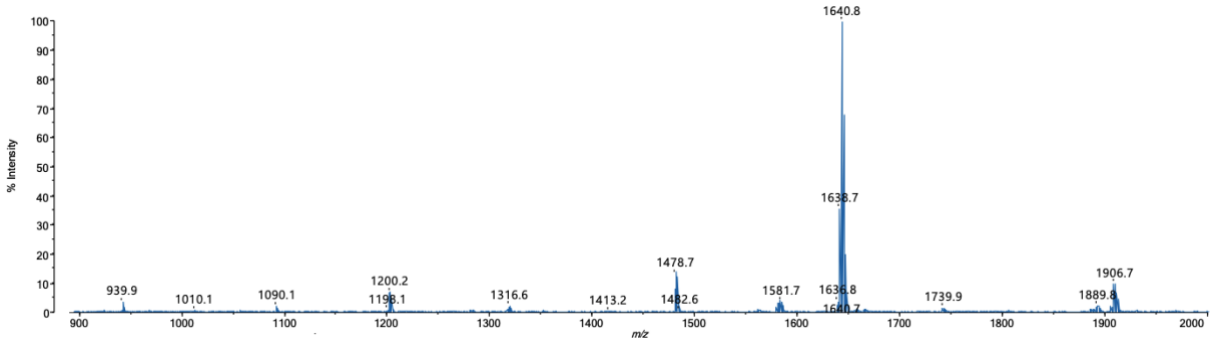

Figure 10: Mass spectrum of M. sedula lipids. MALDI-TOF analysis in positive ion mode spiked with NaCl was conducted.

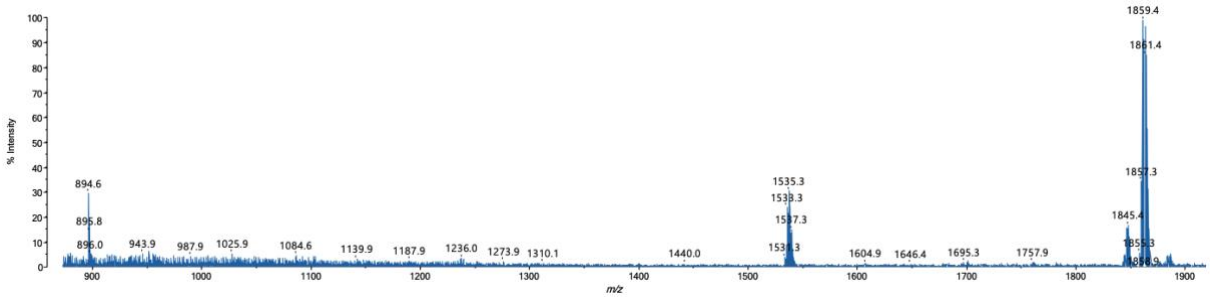

Figure 11: Mass spectrum of M. sedula lipids. MALDI-TOF analysis in negative ion mode was conducted. Phosphate-free lipids cannot be detected here.

Table 8: Peak results of MALDI-TOF analysis in positive and negative ion mode with lipid classes, peak types (ionization forms) and m/z values

Ionisation mode	Lipid class detected	Peak type	m/z
+	IP-DGD	$[M+2Na-H]^+$	939.9
+			1200.2
+	GDGT	$[M+Na]^+$	1316.6
+	Hex-GDGT	$[M+Na]^+$	1478.7
+	IP-GDGT	$[M+2Na-H]^+$	1581.7
+	Hex2-GDGT	$[M+Na]^+$	1640.8
+	Hex2-GDGT-IP	$[M+2Na-H]^+$	1906.7
-	IP-DGD	$[M-H]^-$	894.6
-	IP-GDGT	$[M-H]^-$	1535.3
-	Hex2-GDGT-IP	$[M-H]^-$	1859.4

Furthermore the lipid pattern was compared to the one of *S. acidocaldarius* analysed by Quehenberger et al. (2020).[52]

When looking at mass spectra generated by analyzing the lipids of heterotrophically grown *S.acidocaldarius* cells, the lipid pattern looks very similar which could be expected since the species both belong to the genus Sulfolobales. However, when comparing the ratios between the distinct lipid classes, there are indeed some differences, two of which kind of stand out.

First, the content of IP-DGD (m/z = 939.9 (pos.), 894.6 (neg.)) is lower in *M. sedula* than in *S. acidocaldarius*. When comparing both positive ion mode (with NaCl) spectra, IP-DGD was second highest in *S. acidocaldarius*, but barely detectable in *M. sedula*. In general, the DEL:TEL ratio seems to be lower in *M. sedula*.

The second thing that stood out was when analyzing in the positive ion mode, a certain m/z ration of 1200.8 appears with a non-neglectable signal strength which could not be assigned to any of those lipids classes and also does not appear in the spectra generated by the lipids of *S. acidocaldarius*. It may be an adduct of a lipid within one of the lipid classes or also cleavage product of it, but this is still unknown and has to be further investigated.

3 Analysis of trace elements (TEs) in heterotrophic cultivations of *M. sedula*

3.1 Evaluation of composition of tryptone via ICP-MS

The first approach of this work was to find a defined medium as close as possible to the composition of a complex medium, knowing that cell growth is good in those media. However, since not only C-sources are present for cultivation, but also various salts and trace elements are needed, it was necessary to find out this particular composition of a complex medium to identify potential missing components in a defined medium or to adapt certain concentrations. Therefore, an ICP-MS analysis of tryptone in powder form was performed and the mass percentages of the individual elements in tryptone were compared to those in the Brock basal medium (Table 9).

Table 9: Ratio of w% of mineral and trace elements present in tryptone compared to Brock basal

Ratio of w% in tryptone compared to Brock basal	
P	136
S	22
Mg	8
Ca	15
K	195
Na	58304
Fe	2
Zn	1248
V	156
Mn	2

What immediately stands out is the high concentration of sodium (Na) in tryptone (3.2%, 58304-fold higher than in Brock basal) which probably has its roots in the high NaCl content in tryptone. However, since *M. sedula* is able to grow on tryptone and is not inhibited by the high salt concentration, the focus in the next step was shifted to TEs and to analyze whether growth is better when in addition to tryptone, no further TEs are added (Figure 12). With this experiment, the objective was to determine the effect of Brock basal TEs on cell growth in complex media and to be able to make an initial assessment of the concentration ranges in which TEs should be used in defined media.

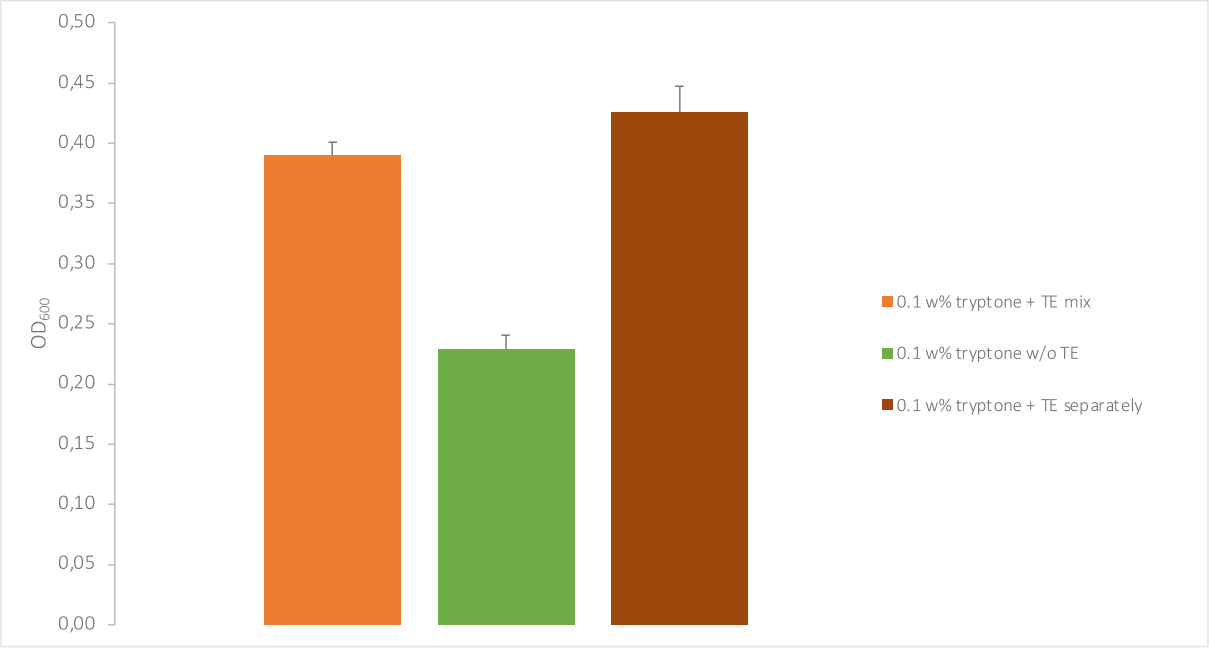

Figure 12: Comparison of OD_{600} values (measured at reaching stationary phase after 72 h) of M. sedula cells grown in different media compositions regarding TEs: Brock basal + 1 g/L tryptone (blue), Brock w/o TEs + 1 g/L tryptone (orange) and Brock (+ TEs just not from stock solution, but put in separately) + 1 g/L tryptone

As can be seen, *M. sedula* cells grew to lower maximum cell densities without additional TEs, a result that shows that TEs in Brock basal are essential for the cells. Since cell growth was evident even without supplementation of TEs, it must be assumed that tryptone alone is sufficient as a source of TEs. This in turn led to the conclusion that tryptone is obviously so complex that a complete compositional breakdown seems impossible. Therefore, the less complex Casamino Acids were used for the next experiment.

3.2 A Design of Experiment to evaluate suitable TE concentrations using CAAs

The following step was a DoE dealing with the evaluation of TE concentrations suitable for the cultivation. The DoE was performed by cultivations in 6-well plates and designed using the software “MODDE” and was designed as a fractional factorial design with 3 center points. As input variables, all TEs contained in the Brock basal medium with Nickel in addition were used. The output variable was the OD_{600} value. As it was expected that the TEs were not inhibiting growth, their concentrations were only varied at levels at or higher than the one in the Brock basal medium. The 3 concentration levels were chosen to be Brock basal (level 1), 5 times the concentration as in Brock basal (level 2) and the 20-fold concentration as in Brock basal (level 3).

So, as a basic prerequisite, the normal distribution after a negative logarithmic transformation of the variables shown on the left hand side of Figure 13 was acceptable.

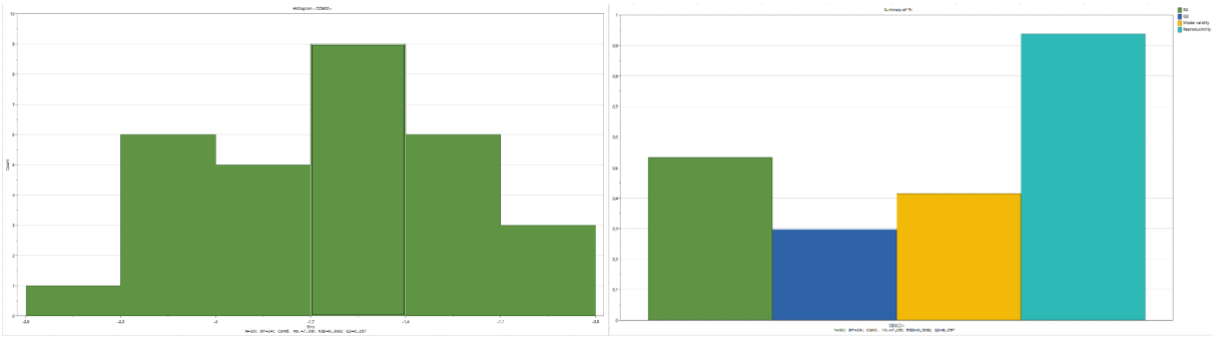

Figure 13: Histogram of response distribution after negative logarithmic transformation (left). Statistical parameters of the model; R2 (green), Q2 (blue), validity of the model (yellow) and reproducibility of the model (turquoise) (right)

The statistical parameters of the model are shown on the right hand side of Figure 13. The R^2 value (coefficient of determination) had a value of 0.532 which is a moderate value according to several publications dealing with PLS (partial least square) analysis, but since this value was mentioned for the first time, the ranges have been adapted several times.[109–111] That means that 53.2% of the variability can be explained by the model. The Q^2 value which describes the predictive of the model had a value of 0.297. In the past, there was a threshold for this value set at 0 above which the model can be considered as predictive relevant.[112,113]

Nevertheless, some sources state that R^2 and Q^2 should be as similar as possible to represent a good model. Here, commonly a threshold of $\Delta 0.3$ is set.[114] As this model had $\Delta(R^2,Q^2)$ of 0.235, it can still be accepted. The model validity and reproducibility showed values of >0.25 and >0.5, thresholds over which, according to Dennison et al.[115], both represent a good fitting model.

As the next part of the results, the significance of terms was considered in order to point out which variables have a strong effect on the outcome of the experiment. So, significant are those terms which have a value of the output variable at a large distance from 0, but also have a level of uncertainty which does not cross the 0-line. On the other hand, insignificant are those terms having a value of the output variable close to 0 and additionally those which have a level of uncertainty crossing the 0-line.[116]

Now looking at the results of this particular model on the left hand side of Figure 14, it can be seen that the linear terms of copper (Cu) and vanadium (V) with the latter not to a similar extent, were significant. Within the model, also quadratic interactions were found to be significant and included, in this case for iron (Fe) and copper. Interestingly, the linear term of iron had almost no effect on the output of this model, but the quadratic term affected the

output pretty much while both had a positive effect. On the other side, the linear term of copper had a positive while the quadratic term had a huge negative effect.

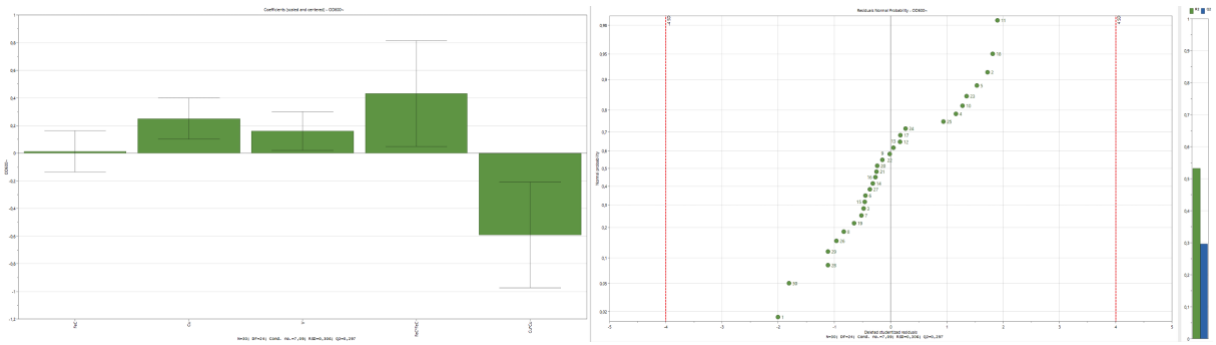

Figure 14: Coefficient plot with linear and quadratic terms of the significant variables which in this case are iron (Fe), copper (Cu) and vanadium (V). (left) Residuals normal probability plot showing no outliers in the experiment and a normal distribution of the residuals due to the points following a straight line. (right)

To make sure there were no outliers in the model, one can have a look at the residuals normal probability plot (right hand side of Figure 14). As there are no values outside the 4 standard deviations, all values were considered as valid for being considered for the model.

Concluding, the DoE shows an effect of iron, copper and vanadium on the growth of *M. sedula* cells, but since cell densities were relatively low during the whole experiment ($OD_{600,max}$ = 0.154), the experiment should probably be repeated to gain more information about the effect of TEs on the cell growth, especially due to the center points not being real center points concerning the output (Figure 15).

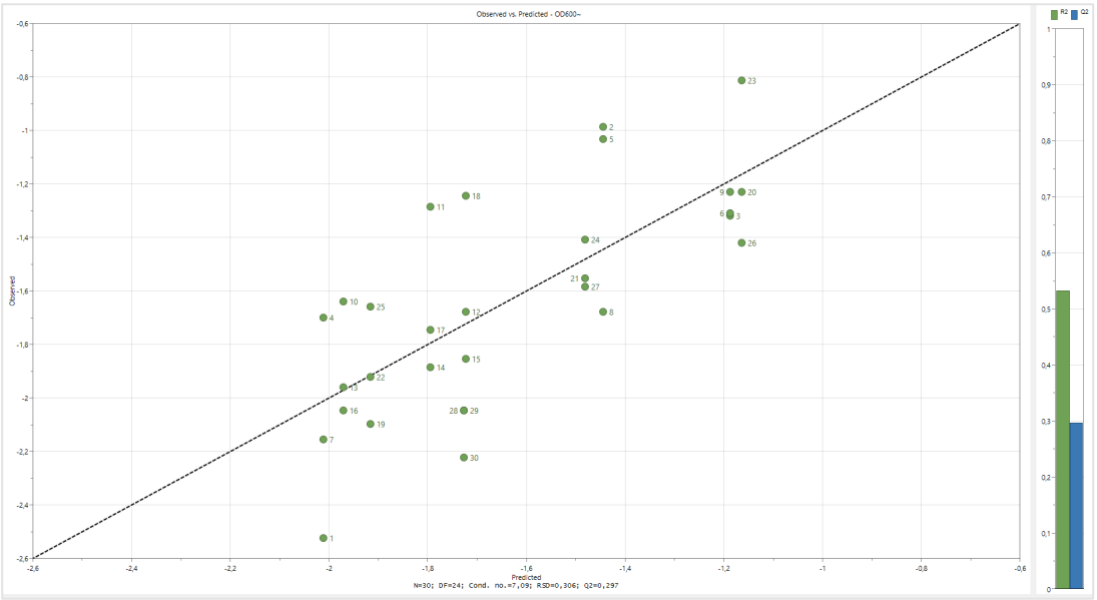

Figure 15: Observed vs. Predicted plot showing the distribution of the output variables of the 30 samples and a very high degree of freedom (24) indicating a non-ideal model

4 An alternative to Brock basal

As the DoE suggested that the mineral composition of Brock basal can be adjusted to the needs for cultivation of *M. sedula*, a set of chemically defined media (Resurge™ CD PAK) were examined. Those media were originally developed for CHO (Chinese Hamster Ovary) cells in order to enhance production of recombinant proteins.[117] This series of media contains 5 different mixtures of chemically defined, but mostly unknown components with the explicit exception of L-Glucose and L-Glutamine. The cultivations in this study were performed in 5 different replicates using 2 g/L of each of the media alone (CD1-CD5). Additionally, 1 g/L tryptone were added as C-source because at that particular timepoint the knowledge of the growth kinetics of cultures supplemented with CAAs still lacked. As a control, *M. sedula* cells were cultivated in Brock basal medium with 1 g/L tryptone. As displayed in Figure 16, the cultures with CD4 and CD5 grew slightly better and to higher final cell densities than the one with Brock basal, while those with CD1-CD3 did not grow at all. The reason for that was not further investigated, but either the CD media 1-3 are completely different to CD4 and CD5 or there are only one to a few components which make the difference. Nevertheless, since those media are commonly not used and very expensive compared to Brock basal, they probably will not be used in everyday practice.

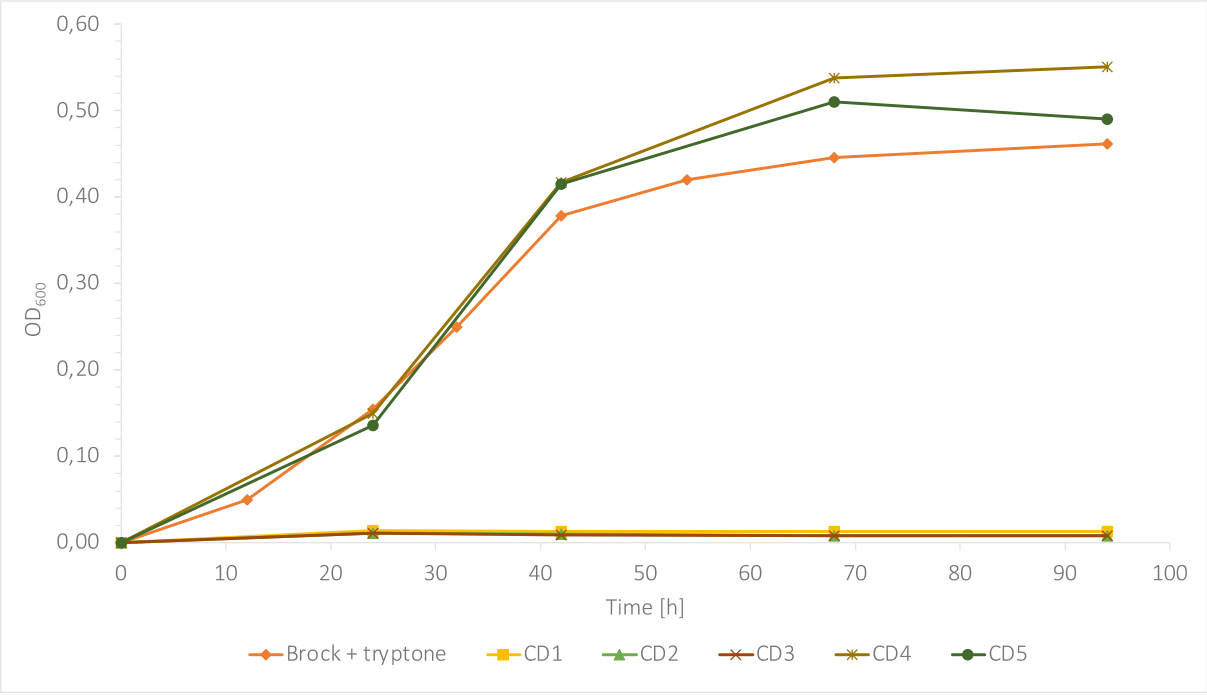

Figure 16: M. sedula cells grown on diverse basal salt media supplemented with 1 g/L tryptone

5 An alternative HPLC method for offline monitoring of amino acid uptake

As cultivations on CAAs were conducted with supplementation of Wolin's vitamin solution, the uptake of the different vitamin components was tried to be recorded. For that purpose, HPLC analysis of supernatants during cultivation was performed using a method described by Hofer et al. (2017)[118]. Analytes were measured using an Ultimate 3000 (Thermo Fisher Scientific, USA) system with a pump (LPG-3400SD), an autosampler with a 20 µL sample loop (CTC autosampler), a column oven (TCC-3000SD) and a diode array detector (DAD 3000).

For analyte separation, a reversed-phase column (Agilent Acclaim PA, 150x4.6 mm, 3 µm) was used in a column oven heated to 30°C. For the gradient in this method (Table 10), eluent A (25 mM KH_2PO_4, pH 3.5) and eluent B (ACN) with a flowrate of 1.0 mL/min were used. The gradient applied can be found in Table 10.

Table 10: Gradient of HPLC method analyzing vitamins/aromatic amino acids with a washing step in order to remove residues from the column and for baseline stabilization

	Separation gradient			Washing gradient			
t (min)	**0**	**15**	**16**	**0**	**5**	**6**	**10**
% A	0	20	20	20	60	0	0
% B	100	80	80	80	40	100	100

After the first analysis, it was recognized that this method was not suitable for this application due to vitamin concentrations used for cultivation being below the limit of detection. Nevertheless, we then found out that it still can be applied to our experiments for the analysis of aromatic amino acids. Compared to the method used for the amino acid uptake of all 20 proteinogenic amino acids, this method is not only less laborious in sample preparation and preparation of components needed for derivatization etc., but also shorter in time (26 minutes with the other method compared to 18 minutes with this one).

Although this method is not as powerful as the one analyzing all 20 proteinogenic amino acids, it is definitely a very useful tool to monitor cultivation processes in a less elaborate way. As an example of the application of this method, a comparison of inoculated cultures with *M. sedula* cells grown on Brock basal + 1 g/L CAAs and the Blanks (Brock basal) are shown in Figure 17. As an example analyte, phenylalanine was used.

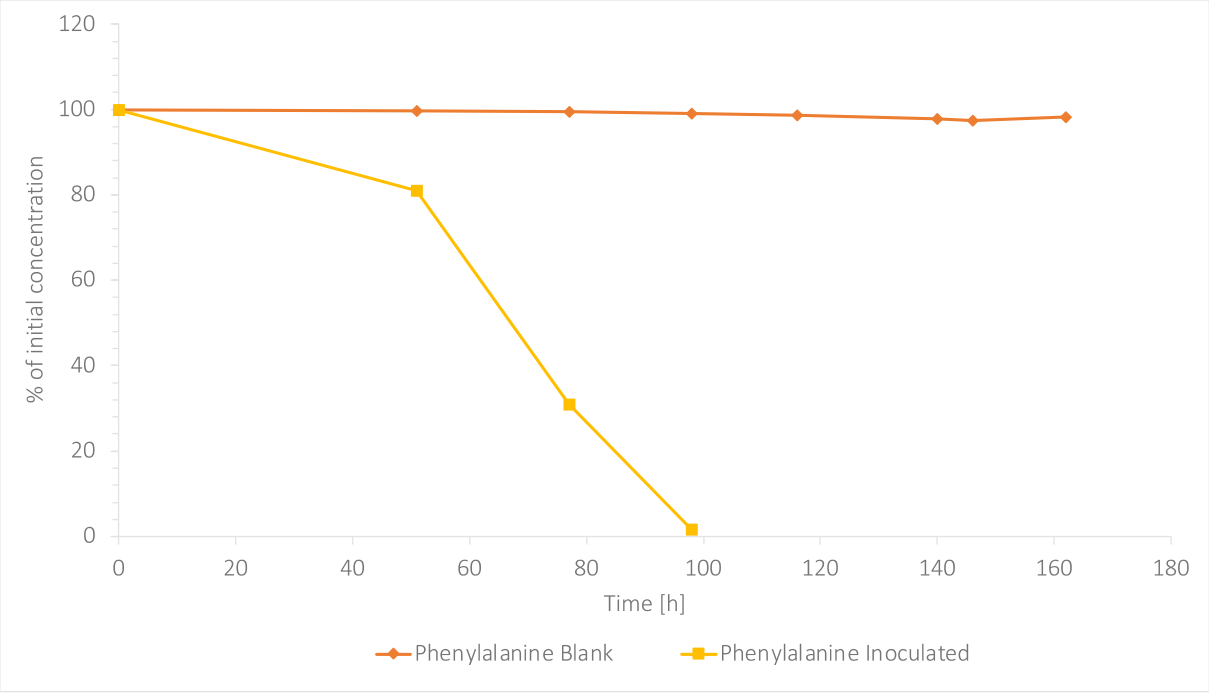

Figure 17: Comparison of evolution of phenylalanine concentration over time. M. sedula inoculated culture (orange) and blank medium (blue).

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