Investigation of vitamin B_{12} biosynthesis by known and newly identified bacterial strains

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List of Abbreviations

AdoCbl	adenosylcobalamin
ALA	δ-aminolevulinic acid
CE	capillary electrophoresis
CL	chemiluminescence
CNCbl	cyanocobalamin
D-A-CH	The Nutrition Societies of Germany, Austria, and Switzerland
DCW	dry cell weight
DFS	difluorosuccinic acid
DMBI	5,6-dimethylbenzimidazole
EFSA	European Food Safety Agency
E_4P	erythrose-4-phosphat
EMCP	ethylmalonyl-CoA pathway
FDA	Food and Drug Administration
FMN	flavin mononucleotide
GRAS	Generally Recognized as Safe
HPLC	high performance liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
LEV	levulinic acid
LOD	limit of detection
LOQ	limit of quantification
MBA	microbiological assay
MCI	mild cognitive impairment
MetCbl	methylcobalamin
OHCbl	hydroxycobalamin
QPS	Qualified Presumption of Safety
SA	succinylacetone
TCA	tricarboxylic acid
THF	tetrahydrofolate

TIC	total ion current

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Fig. 50 Possible mechanisms explaining the absence of the inhibitory effect upon addition of vitamin B_{12}-b-monocarboxylic acid and vitamin B_{12}-c-lactam. (A) the agent is not able to bypass the cell membrane and enter the cell, the transport of the agent through the cell membrane occurs but it is not able to bind the vitamin B_{12}-dependent enzyme (B), is used by the cell as a coenzyme (C) or is converted to vitamin B_{12} (D)

Fig. 51 Possible mechanism of the ALA - dehydratase inactivation: being structurally similar to ALA, the actual substrate of ALA - dehydratase, LEV and SA are used by the enzyme preventing the formation of porphobilinogen, thus, blocking further vitamin B_{12} biosynthesis

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Zusammenfassung

Vitamin B_{12} (Cobalamin) ist ein essenzieller Nährstoff, der bei vielen entscheidenden Prozessen im menschlichen Körper eine wichtige Rolle spielt. Aufgrund dieses grundlegenden Bedarfs nimmt die Nachfrage nach Vitamin B_{12} und damit auch das Interesse an effizienten Vitamin B_{12} Quellen zu.

Die Bedeutung von Vitamin B_{12} wurde in den 1920er Jahren deutlich, nachdem zwei amerikanische Mediziner, Minot und Murphy, die Rolle eines so genannten "extrinsischen Faktors" bei der Behandlung der perniziösen Anämie nachgewiesen hatten. Nach weiteren Jahren der Forschung gelang Woodward und Eschenmoser in 1972 die vollständige chemische Synthese von Cobalamin. Aufgrund der sehr komplexen chemischen Struktur von Vitamin B_{12} umfasste das beschriebene Syntheseverfahren jedoch etwa 70 Schritte, was die chemische Synthese wirtschaftlich unvorteilhaft macht. Aus diesem Grund wird heute ausschließlich die mikrobielle Synthese für die industrielle Vitamin B_{12} Herstellung eingesetzt. Obwohl mehrere Vitamin B_{12}-produzierende Bakterienarten bekannt sind, gibt es Unterschiede in der Art der Verbindung, die von den verschiedenen Stämmen synthetisiert wird. *Propionibacterium freudenreichii* (früher bekannt als *P. shermanii*) ist beispielsweise für seine Fähigkeit bekannt, die aktive Form von Vitamin B_{12} zu synthetisieren, während die Produktion von Pseudovitamin B_{12} für unterschiedliche *Lactobacillus*-Arten nachgewiesen wurde. Die beiden Formen unterscheiden sich in ihrer chemischen Struktur, wobei Pseudovitamin B_{12} als Kofaktor für menschliche Enzyme nicht nutzbar ist. Aus diesem Grund ist es wichtig, die Art des von den Mikroorganismen synthetisierten Cobalamins zu bestimmen, wenn Vitamin B_{12} für die menschliche Ernährung hergestellt werden soll.

Heute gibt es viele verschiedene Methoden für die Analyse von Vitamin B_{12}, die sich in ihrer Empfindlichkeit und ihrer Fähigkeit zur Bestimmung von Vitamin B_{12} Formen unterscheiden. Der klassische mikrobiologische Assay (MBA) ist dafür bekannt, zwischen der aktiven Vitamin B_{12}-Form und dessen inaktiven Analoga nicht unterscheiden zu können, während HPLC-basierte Methoden die erforderliche Spezifität besitzen.

In dieser Arbeit wurde eine empfindliche und zuverlässige LC-MS/MS-Methode zur Identifizierung und Quantifizierung von Vitamin B_{12} entwickelt, die nicht nur die Quantifizierung von Vitamin B_{12}, sondern auch eine klare Unterscheidung zwischen der aktiven und inaktiven Form ermöglicht. Die entwickelte Methode wurde erfolgreich für den quantitativen Vergleich der Syntheseleistung bekannter Vitamin B_{12}-Produzenten und für die Analyse der aktiven Vitamin B_{12}-Produktion in neu identifizierten

Bakterienstämmen eingesetzt. In dieser Arbeit wurden verschiedene Strategien zur Identifizierung neuer produzierender Bakterienstämme entwickelt, welche unter aeroben Bedingungen hohe Mengen von aktivem Vitamin B_{12} produzieren. Unter den identifizierten Stämmen erwiesen sich *Terrabacter* sp. DSM 102553 und *Hyphomicrobium* sp. DSM 3646 als die Organismen mit der höchsten Cobalamin-Syntheseleistung. Die Cobalamin-Synthese mit den identifizierten Bakterien wurde in verschiedenen Medien untersucht und kostengünstige Medien, die eine hohe Vitamin B_{12} Ausbeute ermöglichen, wurden identifiziert. Hohe Vitamin B_{12}-Konzentrationen, die mit den identifizierten Stämmen in einfachen Kultivierungsexperimenten in günstigen Medien erreicht wurden, eröffnen neue Möglichkeiten für eine kosteneffektive biotechnologische Vitamin B_{12}-Produktion.

Abstract

Vitamin B_{12} (cobalamin) is an essential nutrient playing an important role in many crucial processes in the human body. Due to such essential requirement, the demand for vitamin B_{12} and thereby interest in alternative efficient vitamin B_{12} sources is growing.

The importance of vitamin B_{12} became clear in the 1920s after two American physicians, Minot and Murphy, demonstrated the role of their so-called “extrinsic factor” in the treatment of pernicious anemia. After years of further research, the complete chemical synthesis of cobalamin was achieved by Woodward and Eschenmoser in 1972. However, due to the very complex chemical structure of vitamin B_{12}, the described synthesis procedure included about 70 steps, which makes it economically disadvantageous. For this reason, microbial synthesis is used as the exclusive strategy for industrial vitamin B_{12} production nowadays.

Although there are several vitamin B_{12} - producing bacterial species known, there are differences in the type of the compound synthesized by different strains. For example, *Propionibacterium freudenreichii* (formerly *P. shermanii*) is well known for its ability to synthesize the active form of vitamin $B_{12,}$ while production of a vitamin B_{12} analogue called pseudovitamin B_{12} was shown for *Lactobacillus* species. The two forms differ in their chemical structure, which makes pseudovitamin B_{12} inactive as a cofactor for human enzymes. Hence, if the goal is to produce vitamin B_{12} for human nutrition purposes, it is important to determine the type of the cobalamin synthesized by the microorganisms.

Various methods of vitamin B_{12} analysis have been described, which differ in their sensitivity and ability to discriminate between the vitamin B_{12} forms. The classical microbiological assay (MBA) is known to respond not only to vitamin B_{12} but also to its analogues, while HPLC - based methods possess the required specificity.

In this work, a sensitive and reliable LC-MS/MS method for the identification and quantification of vitamin B_{12} was developed which not only allows vitamin B_{12} quantification, but also enables clear discrimination between its active and inactive form. The developed method was successfully applied for the quantitative comparison of the well - known vitamin B_{12} - producing species and for the analysis of the active vitamin B_{12} production in the newly selected candidate strains. Different strategies for the identification of new producing bacterial strains were proposed in this work which resulted in the description of bacteria with the ability to selectively produce high levels of active vitamin B_{12} under

aerobic conditions. Among the identified strains, *Terrabacter* sp. DSM 102553 and *Hyphomicrobium* sp. DSM 3646 proved to be the most promising cobalamin-producing strains. Cobalamin synthesis with the identified bacteria in different media was investigated, which resulted in identification of low-cost media enabling accumulation of high vitamin B_{12} yields. The relatively high vitamin B_{12} concentrations achieved with the strains in simple cultivation experiments performed in low-cost media open new opportunities for a cost-effective biotechnological vitamin B_{12} production.

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1. Introduction

The term “vitamine” was firstly proposed by Casimir Funk in 1912 and includes different organic compounds of various chemical structure. Vitamins are essential for the maintenance of metabolism but most of them cannot be produced by human body, which is why they should be supplied from the diet. Vitamins are present in food in very small amounts and are therefore classified as micronutrients. Due to the lack of a precise definition, different substances have been referred to as vitamins at different times. Up today, there are 13 vitamins known and they are divided into two groups: fat-soluble and water-soluble vitamins. Fat-soluble vitamins can be accumulated in the body with fatty tissue or in the liver (Stevens 2021), while water-soluble vitamins cannot be stored in significant quantities and are excreted from the body with the urine in case of excess. This explains the higher prevalence of hypovitaminosis of water-soluble vitamins in comparison to fat-soluble vitamins.

Vitamin B_{12} (cobalamin) is a water - soluble vitamin involved in such key processes as DNA synthesis (Weyden et al. 1973), fatty acid (Finadza and Audisio 1982) and amino acid metabolism (Charkey et al. 1950). A long-term insufficient vitamin B_{12} intake can have strong health effects leading to severe disorders like megaloblastic anemia, leukopenia, thrombocytosis and many other clinical manifestations (Langan and Goodbred 2017). Vitamin B_{12} deficiency is a worldwide problem and especially elderly people, pregnant women and vegans are at a particular risk of vitamin B_{12} deficiency. Despite such essential requirement, human body is not capable of vitamin B_{12} production, that is why it must be obtained from the food sources or vitamin supplements. Due to the very complex chemical synthesis of vitamin B_{12}, microbial fermentation is applied for the commercial production of vitamin B_{12}, which is restricted to two species: *P. freudenreichii* (formerly known as *P. shermanii)* and *Pseudomonas denitrificans* (Martens et al. 2002). Nevertheless, the rising popularity of veganism leads to a growing interest in alternative bacterial strains which can be used as efficient vitamin B_{12} sources and might help to cover the increasing vitamin B_{12} demand.

This thesis focuses on the identification of new microbial species and spontaneous mutants, which have not been previously described as vitamin B_{12} producers. Firstly, the extensive literature review will concentrate on the essential role of vitamin B_{12}, the severe health risks associated with its deficiency and will summarize the information about the known producing strains and demonstrate challenges in vitamin B_{12} analysis. Secondly, the development and validation of a new LC-MS method for the sensitive

and reliable identification and quantification of the active vitamin B_{12} is described. Subsequently, new strategies for the identification of spontaneous mutants and new strain candidates for cobalamin production are introduced. Finally, the identified new strain candidates are described and their growth behavior and ability to produce vitamin B_{12} are characterized.

1.1 Milestones in the history of vitamin B_{12}

The history of vitamin B_{12} began in 1926 when George Minot and William Murphy showed that the patients suffering from pernicious anemia, a blood deficiency leading to the production of too low levels of red blood cells, can be treated with a special diet containing abundant amounts of liver (Minot and Murphy 1983). For this discovery, Minot, Murphy and Whipple received the Nobel Prize in 1934. The mechanism of the treatment remained unclear until the American physiologist William Castle demonstrated the inability of the pernicious anemia patients to prepare and obtain certain compounds, which he called “extrinsic factor” from their diet (Berk and Castle 1948). After years of research and many attempts to identify this dietary component, two independent groups of researchers succeeded to purify it from the liver in 1948: the team of Karl Folker from the Merck company in the USA (Rickes et al. 1948b) and Smith and Parker at Glaxo laboratories in the UK (Smith 1948) were able to isolate the anti-pernicious anemia factor which was then called vitamin B_{12}. The isolation of the pure vitamin B_{12} gave rise to the growing interest and new attempts for its structure elucidation in the 1950s. The crystal structure of the cyano-form of vitamin B_{12} has been successfully solved in 1956 by the group of Dorothy Hodgkin (Hodgkin et al. 1956). Adenosylcobalamin was the next vitamin B_{12} form, whose structure could be elucidated in 1961 (Lehnert and Hodgkin 1961). For these achievements Dorothy Hodgkin was awarded with the Nobel Prize in 1964. For today, four different forms of cobalamin are known as the active vitamin B_{12} as well as a great number of vitamin B_{12} analogues.

1.2 Structure and diversity of cobamides

Vitamin B_{12} has a very complex chemical structure and belongs to a large family of cobamides, organometallic cofactors that contain cobalt. The molecule of a cobamide consists of three main parts: the corrinoid ring with the central cobalt atom and the upper and lower ligands. Since different

combinations of the upper and lower ligands are possible, more than 30 naturally occurring cobamides has been described up today (Kennedy and Taga 2020). The lower ligand is the site of most cobamide diversity and such variety of cobamides is due to the different biosynthetic origins of lower ligand bases combined with various processes of its incorporation in the cobamide molecule (Crofts 2013). Involvement of cobamides in a wide range of metabolic reactions, their distinct metabolic role and flexibility of many microorganisms against different cobamides are the reasons for the great variety of natural cobamides (Kennedy and Taga 2020). In contrast to the microorganisms, human enzymes are very selective towards the cobamide forms and the type of the ligand can influence the biological activity of cobalamin.

In case of vitamin $B_{12,}$ the upper ligand can be represented by adenosyl-, methyl-, hydroxo- or cyano-group in the molecules of adenosylcobalamin (AdoCbl), methylcobalamin (MetCbl), hydroxycobalamin (OHCbl) and cyanocobalamin (CNCbl), respectively (Fig. 1). AdoCbl and MetCbl are the actual coenzymes which can be directly used by the human body as cofactors. Both forms are very light-sensitive and are oxidized to OHCbl when they are exposed to light. For this reason, the more stable CNCbl is the form which is mainly produced industrially (Martens et al. 2002). Nevertheless, OHCbl and CNCbl can be converted into the active coenzymes in the human body, which is why all four forms are referred to as the active vitamin $B_{12.}$ On the contrary, the type of the lower ligand has a great influence on the activity of vitamin B_{12}.

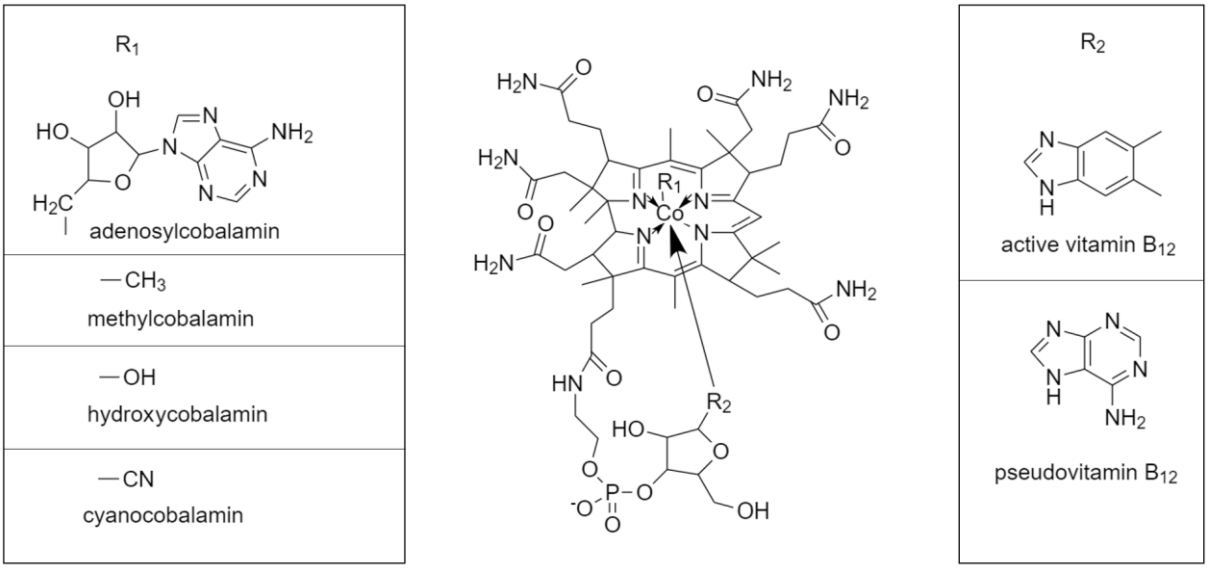

Fig. 1 Possible structures of different forms of active vitamin B_{12} and its inactive analogue. R_1: alternative upper ligands represented in the four active forms of vitamin B_{12}. R_2: structures of DMBI and adenine present in the active and pseudo form of cobalamin, respectively

The active vitamin B_{12} carries 5,6-dimethylbenzimidazole (DMBI) as the lower ligand, while cobamides with other lower ligands are generally called biologically inactive B_{12} analogues. Various microorganisms can produce different B_{12} analogues, where DMBI is replaced by other benzimidazoles, purines or phenolic compounds (Crofts 2013). One of the best-known inactive vitamin B_{12} analogues is called pseudocobalamin, an analogue with adenine as lower ligand (Fig. 1), which cannot be converted into the active form and be used as a cofactor by the human enzymes. The very complex structure of vitamin B_{12} requires many enzymatic steps for its biosynthesis, which is why more than 30 genes are involved in the biosynthetic pathway of vitamin B_{12} (Roth et al. 1993).

1.3 Biosynthesis of vitamin B_{12}

Microorganisms have developed different strategies for vitamin B_{12} biosynthesis. *De novo* synthesis can occur via aerobic, or oxygen-dependent way, which has been mostly studied in *P. denitrificans*, while *Salmonella* Typhimurium*, Bacillus megaterium, and P. freudenreichii* use the anaerobic pathway (Fang et al. 2017; Martens et al. 2002). The genes encoding the enzymes are prefixed as *cob* and *cbi* for the aerobic and anerobic pathway, respectively. Moreover, in the aerobic pathway cobalt is inserted several steps later and oxygen is required for the corrinoid ring formation in comparison to the anaerobic pathway, where cobalt insertion takes place earlier and oxygen is not necessary (Fig. 2). Nevertheless, both routes include many common steps and similar reactions.

First, the precursor δ-aminolevulinic acid (ALA) is formed via the C4 or C5 pathway. C5 pathway is the most common in bacteria, while C4 pathway is spread only in mammals, fungi and purple nonsulfur microorganisms (Zhang et al. 2015). After the condensation of two molecules of ALA to porphobilinogen, four molecules of porphobilinogen are cyclized to form uroporphyrinogen III. Subsequent methylation of uroporphyrinogen III leads to the formation of precorrin-2, the step when the two pathways diverge. Hydrogenobyrinic acid is formed from precorrin-2 in a number of subsequent reactions in the aerobic

pathway, while formation of cobyrinic acid from sirohydrochlorin occurs in the anaerobic pathway via several methylation steps.

After that, both pathways converge with the formation of cob(II)yrinic acid a, c-diamide, which is converted to adenosylcobyric acid via further adenylation and amidation steps.

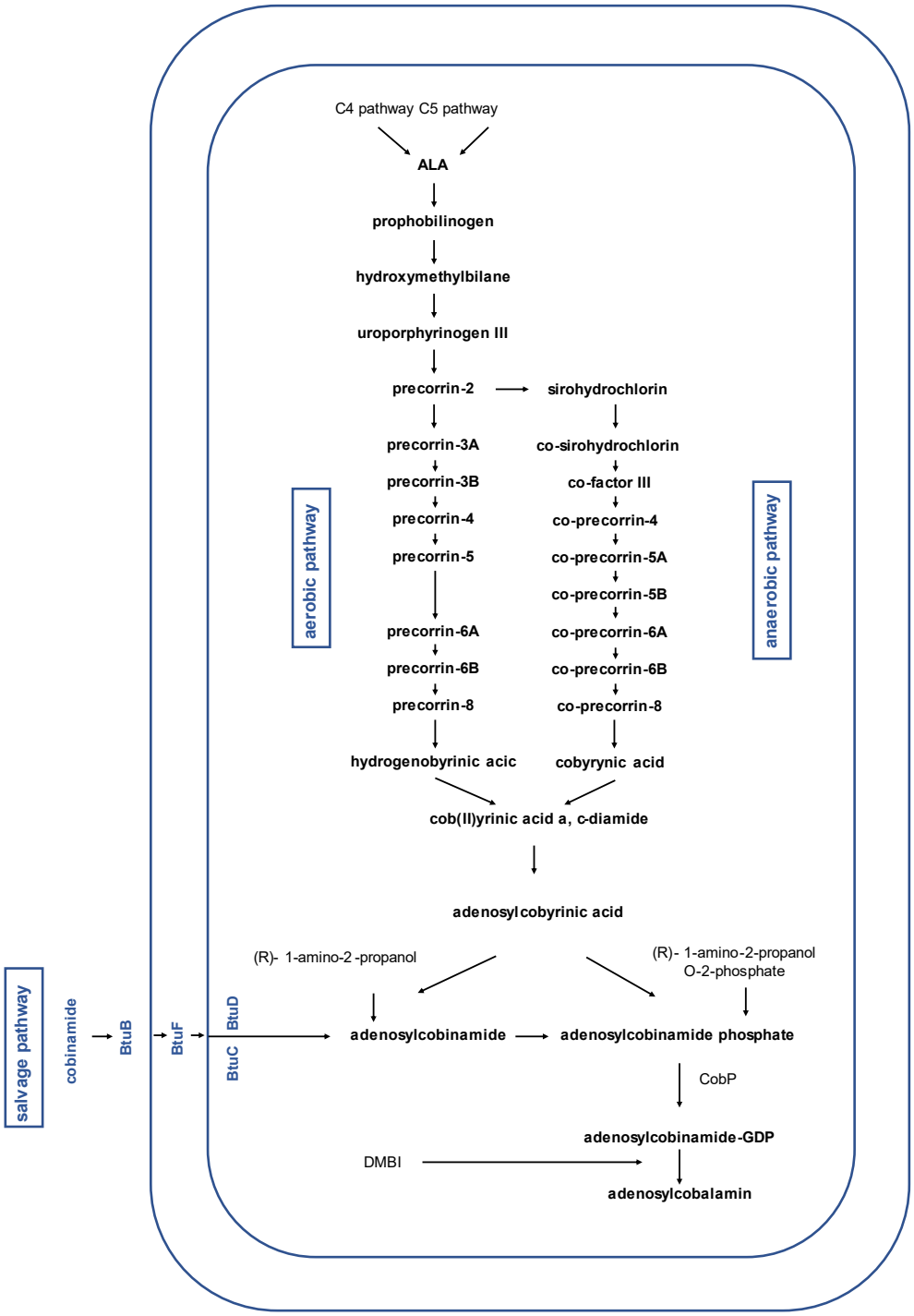

Fig. 2 Schematic overview of the main reactions of cobalamin biosynthesis. The main steps and intermediates of the aerobic, anaerobic and salvage pathway are listed

(R)- 1-Amino-2-propanol O-2-phosphate is directly attached to the adenosylcobyric acid in the anaerobic pathway, while attachement of (R)-1-amino-2-propanol takes place to form adenosylcobinamide, which is then phosphorylated to adenosylcobinamide phosphate in the aerobic pathway. The last steps include the synthesis of adenosylcobinamide-GDP via a further phosphorylation step performed by CobPenzyme possessing ATP:AdoCbi (adenosylcobinamide) kinase and GTP:AdoCbi-P guanylyltransferase activity (Fang et al. 2017). Finally, the incorporation of the lower ligand takes place to form the complete adenosylcobalamin molecule.

Starting from the adenosylcobinamide, all described steps are also present in the salvage pathway, an alternative energy-efficient way to obtain cobalamin (Zhou et al. 2021). This pathway is shorter and includes 13 genes. In this pathway, vitamin B_{12} synthesis does not occur *de novo* but rather adenosylcobalamin is derived from cobinamide. Salvage pathway is common in the microorganisms capable of cobalamin uptake into the cell, which requires a transporter system in the cell membrane. In Gram-negative bacteria, the TonB-dependent transporter BtuB performs the transport of cobalamin precursor cobinamide across the outer membrane, while the ABC transporter BtuCDF enables the further transport across the cytoplasmic membrane. The majority of Cbl-auxotroph microorganisms depend on the uptake of cobalamin or cobinamide from external sources (Rempel et al. 2018), which shows the important role of the salvage pathway for the microorganisms which are not able to synthesize vitamin B_{12} *de novo* but still need it as a cofactor for essential enzymes.

1.4 Cobalamin as cofactor for various enzymes

Bacterial vitamin B_{12}-dependent enzymes participate in a great number of reactions and processes essential for their metabolism. MetCbl-dependent enzymes catalyze methylation reactions and methionine synthase from *Escherichia coli* (MetH), which catalyzes the formation of methionine from homocysteine via CH_3-H_4-folate demethylation, is the best studied enzyme among them (Banerjee and Ragsdale 2003). The enzyme consists of four modules responsible for binding and activation of CH_3-H_4-folate and homocysteine and their presentation to MetCbl, binding of cobalamin and reductive activation of the protein (Matthews 2009). The reaction catalyzed by methionine synthase occurs in two steps: the methyl group of MetCbl is abstracted by homocysteine to form methionine and an enzyme-

bound cob(I)alamin, while the abstraction of the methyl group of CH_3-H_4-folate leads to the reconstitution of the bound MeCbl and closes the catalytic circle (Gruber et al. 2011).

Other MetCbl-dependent enzymes include mainly various methyltransferases involved in acetogenesis and methanogenesis. In acetogenic bacteria, MetCbl-dependent methyltransferase plays a key role in the Wood–Ljungdahl pathway, which they use to convert CO_2 to cellular carbon (Ragsdale 2008). Similar to their role in homocysteine methylation, enzyme-bound corrinoids are used as methyl group carriers between methanol and the thiol coenzyme M, a coenzyme required for methyl-transfer reactions in the metabolism of methanogens (Gruber et al. 2011).

To the AdoCbl-dependent group belong various isomerases catalyzing structural rearrangements of their substrates, which include glutamate mutase, 2-methyleneglutarate mutase, methylmalonyl-CoA mutase, isobutyryl-CoA mutase, D-α-lysine-5,6-aminomutase, 1,2-diol dehydratase, glycerol dehydratase, ethanolamine amino lyase and ribonucleotide reductase (Lyatuu 2012). The majority of the isomerases use a common mechanism of rearrangements shown in Fig. 3. In the first step, the cleavage of the Co-C bond of AdoCbl occurs leading to the formation of 5′-deoxyadenosyl radical, which serves as actual catalyst. The reactive radical abstracts an H atom from the substrate and the formation of the 5′-deoxyadenosine and the substrate radical complex takes place, which is then rearranged into the product radical complex. After the reabstraction of the H atom from this complex the rearranged product is released and AdoCbl is regenerated from 5′-deoxyadenosyl radical.

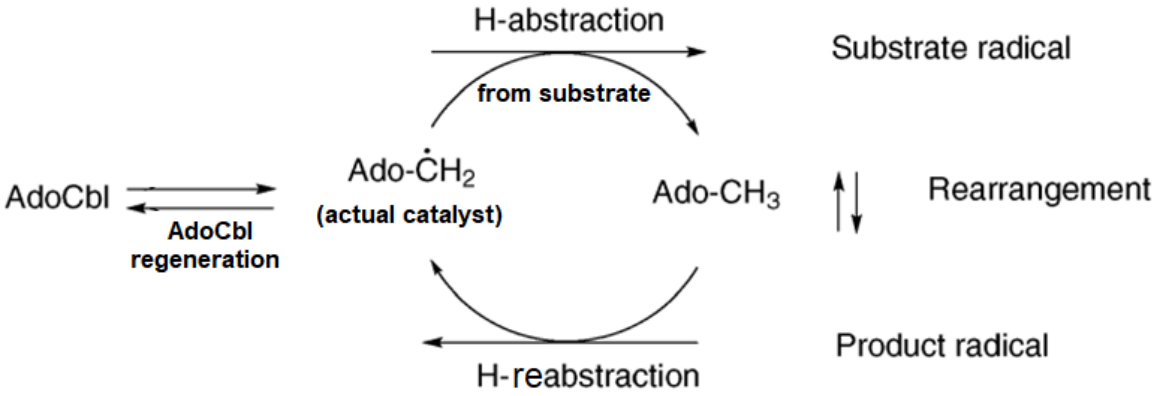

Fig. 3 Mechanism of AdoCbl-dependent rearrangement reactions commonly used by most isomerases (modified from Gruber et al. 2011)

The only exception from this mechanism are the ribonucleotide reductases, which do not use the 5′-deoxyadenosyl radical for the direct abstraction of the H atom from the substrate but rather generate an enzyme-bound radical for the reaction with the substrate (Gruber et al. 2011). Ribonucleotide reductases catalyze the reduction of the ribonucleotides to the corresponding deoxyribonucleotides (Tauer and Benner 1997), thus, playing a very important role in the process of DNA synthesis.

In the native vitamin B_{12} forms the central cobalt cation has an oxidation state of +3, while recent studies have shown that in abiotic systems in the presence of strong reducing agents cob(III)alamin can be reduced to 4-coordinated cob(I)alamin, a highly reactive form without axial ligands (Ji et al. 2017). These findings enabled the identification of the third group of the cobalamin-dependent enzymes, the cob(I)alamin-dependent reductive dehalogenases. In these reactions, the role of cob(I)alamin differs from the role of the native B_{12} forms in the AdoCbl-dependent and the MetCbl-dependent reactions (Banerjee and Ragsdale 2003). Cob(I)alamin serves as electron donor and the electron transfer on the chlorinated substrate leads to the release of a chloride ion (Matthews 2009). Although there is little known about dehalogenases and their reaction mechanism, microorganisms possessing such activity attract a lot of attention due to their possible application in remediation of polyhalogenated pollutants (Giedyk et al. 2015).

In contrast to the use of several cobalamins by bacterial enzymes, only MetCbl and AdoCbl can act as cofactors in humans and their activity is restricted to two enzymes: methionine synthase and methylmalonyl-CoA mutase. Human cytosolic MetCbl-dependent methionine synthase is very similar to MetH from *E. coli*, consists also of 4 domains and shares 55% amino acid sequence identity with the bacterial enzyme (Kräutler 2012). The enzyme catalyzes the generation of methionine via the transfer of the methyl group to homocysteine with parallel demethylation of 5-methyltetrahydrofolate (5-methyl-THF) to tetrahydrofolate (THF) (Fig. 4), thus playing the key role in amino acid metabolism and also indirectly participating in nucleotide synthesis (Nielsen et al. 2012).

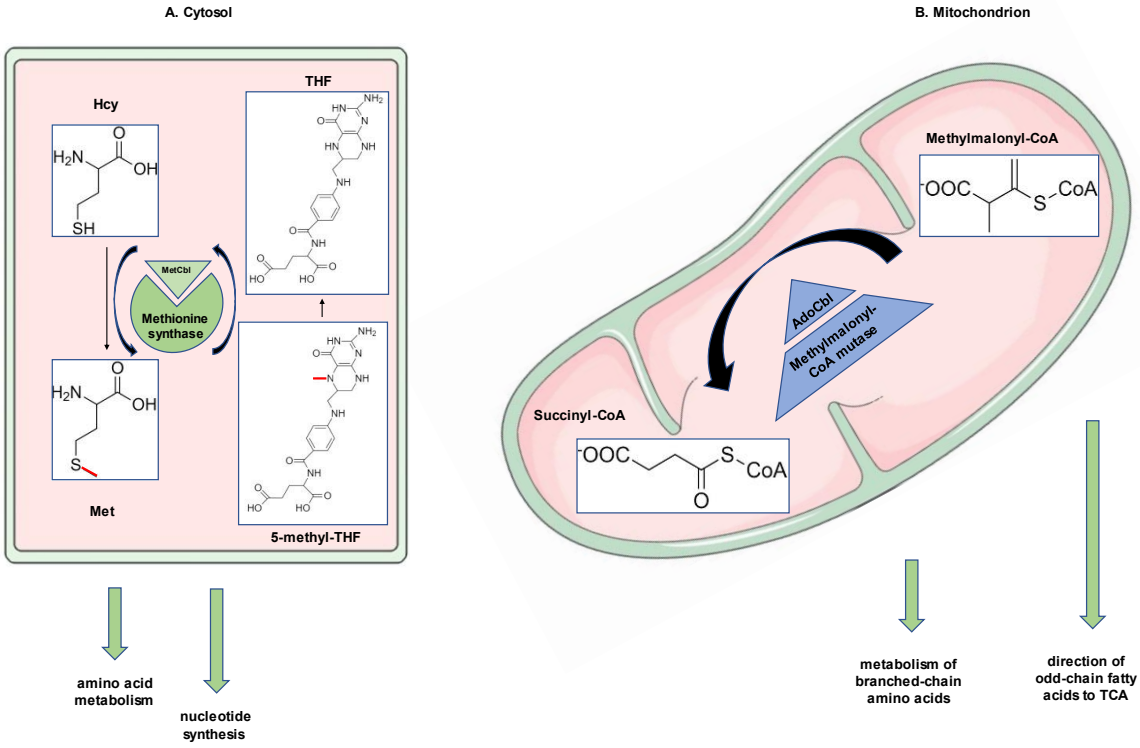

Fig. 4 Reactions catalyzed by the human vitamin B_{12}-dependent enzymes: (A) Methionine synthase uses MetCbl as cofactor for the synthesis of methionine in cytosol and (B) AdoCbl acts as cofactor for methylmalonyl-CoA mutase during succinyl-CoA formation in mitochondrion

In mitochondria, AdoCbl serves as cofactor for the methylmalonyl-CoA mutase in the formation of succinyl-CoA from methylmalonyl-CoA. The substrate of this reaction is derived from propionyl-CoA, a substance formed from the catabolism of branched-chain amino acids, while succinyl-CoA, the product of this reaction, is the key molecule in the (TCA).

1.5 Causes and symptoms of vitamin B_{12} deficiency

Due to the very important roles which cobalamin plays in human metabolism, it is necessary to obtain enough vitamin B_{12} from the food sources. The required values for vitamin B_{12} intake for adults of 2.4 µg per day were estimated by the Institute of Medicine already in 1998, nevertheless, based on the recent data of several vitamin B_{12} status biomarkers studies (Bor et al. 2006; Bor et al. 2010), this value

was raised to 4 µg per day recently according to D-A-CH (Ströhle et al. 2019). A long-term vitamin B_{12} intake below this recommended amount can lead to vitamin B_{12} deficiency which, in its turn, can have severe health consequences. Although it is a worldwide problem, there are several groups, which are at a particular risk of vitamin B_{12} deficiency.

Vegetarian and vegan diets have developed into modern nutritional trends in recent years. These diets become more appealing nowadays due to the health and environmental benefits and ethical reasons. Nevertheless, elimination of animal products from the diet is also often associated with health concerns. Vegans and vegetarians are at a particular risk of vitamin B_{12} deficiency since this vital nutrient naturally originates mainly from the foods of animal origin (Watanabe et al. 2013). According to Pawlak et al. 2014, vitamin B_{12} deficiency is prevalent in up to 86.5% of adult vegans.

Another vitamin B_{12} risk group are pregnant women. Due to the high demand for B_{12} during pregnancy, the recommended daily allowance for B_{12} increases to 6.0 µg per day (Rashid et al. 2021) and the prevalence of vitamin B_{12} deficiency has been reported in 25% of pregnant women (Sukumar et al. 2016).

Age-related changes alter the ability of the body to absorb the nutrients from the food sources, therefore atrophic gastritis-associated malabsorption and pernicious anemia are the main causes of vitamin B_{12} deficiency (Wong 2015) prevalent in up to 20% of elderly population (Andrès et al. 2004).

Although the cobalamin deficit in the mentioned groups is caused by different reasons, they may all be susceptible to symptoms of vitamin B_{12} deficiency.

One of the most common early morphological expressions of vitamin B_{12} deficiency is megaloblastic anemia (Briani et al. 2013), a dysfunction in which the bone marrow produces megaloblasts, unusually large, structurally abnormal, immature red blood cells. Although the precise mechanism of the megaloblastic changes is still not known, it is thought that nuclear-cytoplasmic asynchrony is the result of defective DNA synthesis, the faster synthesis of cytoplasmatic components leads to the higher cytoplasm accumulation in the abnormal cells, while unbalanced cell growth and impaired cell division are the reasons for the formation of immature cells (Green and Datta Mitra 2017). Since the methyl-form of vitamin B_{12} is the key cofactor in the regeneration of THF (Fig. 4), which is then used for thymidine and, therefore, DNA synthesis, the role of vitamin B_{12} deficit in the development of megaloblastic anemia becomes clear.

The clinical manifestations of vitamin B_{12} deficiency have effects on multiple systems and organs and can vary greatly in severity depending on the degree and duration of deficiency (Hunt et al. 2014). Skin and mucous membrane changes such as skin hyperpigmentation, skin pallor or glossitis are common signs of vitamin B_{12} disorders (Langan and Goodbred 2017). Such symptoms as fatigue or distal sensory impairment also belong to the mild deficiency consequences (Hunt et al. 2014).

Neurological manifestations of vitamin B_{12} deficiency include a broad spectrum of symptoms such as cognitive impairment, symmetric paresthesias or subacute combined degeneration of the spinal cord (Ammouri 2019). Mild cognitive impairment (MCI) is a neurocognitive disorder which may be an early disease stage for dementia (Moore et al. 2015). Earlier data demonstrated that there is a positive correlation between the decreased levels of vitamin B_{12} and the degree of impairment of cognitive function and memory in elderly people (Goodwin 1983). Symmetric paresthesia, a peripheral neuropathy which affects the legs more than the arms, is observed in 25% of patients suffering from the lack of B_{12} and is the most common neurologic symptom of vitamin B_{12} deficiency (Ammouri 2019). The best characterized neurologic manifestation of Cbl deficiency that has commonly been referred to as subacute combined degeneration of the spinal cord is a myelopathy characterized by such disorders as spastic paraparesis, extensor plantar response, impaired perception of position and vibration and sometimes peripheral nerve or optic nerve involvement (Kumar 2014).

1.6 Microbial vitamin B_{12} synthesis

Although vitamin B_{12} plays such important role in human body, humans are not capable of vitamin B_{12} production. This ability is common only for some bacteria and archaea (Martens et al. 2002). Chemical synthesis of vitamin B_{12} described by Woodward and Eschenmoser (Eschenmoser and Wintner 1977) includes about 70 synthesis steps, which makes it very complicated, costly and challenging for the industrial vitamin B_{12} production. Due to the complexity of chemical synthesis, the industrial vitamin B_{12} production is performed exclusively via microbial synthesis (Martens et al. 2002).

Already in 1959, Perlman has described several cobalamin-producing species belonging to the genera *Aerobacter, Agrobacterium, Alcaligenes, Azotobacter, Bacillus, Clostridium, Corynebacterium, Flavobacterium, Mycobacterium, Propionibacterium, Pseudomonas, Rhizobium, Streptococcus, Xanthomonas, Streptomyces* (Perlman 1959). Vitamin B_{12} production with various *Streptomyces*

species was demonstrated already 70 years ago (Erf and Wimer 1949; Hall et al. 1953). Synthesis of vitamin B_{12} in *Priestia megaterium* (previously known as *B. megaterium*) has been reported in early 1970s (Gaicy 1973). Vitamin B_{12} production and its role in the symbiosis with the plant host has been investigated in the genus *Sinorhizobium* (syn. *Ensifer*) (Campbell et al. 2006; Taga and Walker 2010). Recently, *E. coli* has also been used for the heterologous expression of cobalamin biosynthesis pathway and vitamin B_{12} synthesis was demonstrated for the recombinant strains (Fang et al. 2018; Ko et al. 2014).

Nevertheless, industrial vitamin B_{12} production is restricted only to two species: *P. denitrificans*, which is predominantly used for the industrial production of vitamin B_{12} (Martens et al. 2002) and *P. freudenreichii* well-known for its Generally Recognized as Safe (GRAS) status and the ability to synthesize the active form of vitamin B_{12} (Deptula et al. 2015).

In addition to the species already known as vitamin B_{12} producers, the number of further reports on new producing strains or metabolically engineered microorganisms is increasing.

1.6.1 *Streptomyces* species

First studies on the ability of *S. griseus* to synthesize vitamin B_{12} providing evidence that the crystalline substance isolated from this strain was identical with vitamin B_{12} obtained from liver were reported in the late 1940s (Rickes et al. 1948a). This finding was later supported with further reports on the positive response of pernicious anemia patients to the treatment with vitamin B_{12} isolated from *S. griseus* (Erf and Wimer 1949; Pappworth 1950). Later investigations have also demonstrated vitamin B_{12} synthesis with *S. olivaceus* (Hall et al. 1953; Maitra and Roy 1960) and *S. aureofaciens* (Rosová and Zelinka 1968). However, the European Food Safety Authority classified *Streptomyces* species as inappropriate as sources of food and feed additives due to their ability to produce antibiotics (European Food Safety Authority 2012), which makes vitamin B_{12} synthesis with *Streptomyces* undesirable.

1.6.2 *P. megaterium* (previously *B. megaterium*)

While investigating cobalamin biosynthesis-deficient mutants of *B. megaterium*, Wolf and Brey 1986 also reported production of 12 µg/L vitamin B_{12} in the culture of the wild type strain. A new perspective for the improvement of these amounts by means of metabolic engineering was opened after the finding of Raux et al. 1998. They elucidated a 16 kb DNA fragment from *B. megaterium* showing a very high

similarity to the previously identified *S. typhimurium* cobalamin biosynthetic gene cluster and characterized the involved genes. The combination of knowledge about the genetic background of vitamin B_{12} biosynthesis with the available genetic tools resulted in the 39.8-fold increase in the intracellular cobalamin concentration in the improved strain compared to the wild type in the study performed by Biedendieck et al. 2010. Another investigation described cloning of the cobalamin operon without control elements which resulted in the enhancement of vitamin B_{12} production with *B. megaterium* (Moore et al. 2014). The levels of vitamin B_{12} achieved with the improved strain were more than 25 times higher than those in the control culture reaching up to 220 µg/L of vitamin B_{12}.

1.6.3 *Sinorhizobium (Ensifer)* species

S. meliloti belongs to the Gram-negative bacteria and can be either free-living soil microorganisms or establish symbiosis with the legume hosts. Investigations of the symbiosis between the microorganism and the plant host contributed to the discovery of the ability of *S. meliloti* to synthesize vitamin B_{12} particularly. Campbell et al. 2006 described an unusual transposon mutant unable to grow in minimal media or to establish a symbiosis with alfalfa, which could be compensated by addition of vitamin B_{12}. Characterization of the mutant has shown that transposon insertion occurred in the gene homologous to the *bluB* gene of *Rhodobacter capsulatus*, which indicated biosynthesis of B_{12} in *S. meliloti*. Further investigation has shown that the symbiosis could be established only due to the function of the cobalamin-dependent ribonucleotide reductase, which also confirmed the ability of *S. meliloti* to produce vitamin B_{12} (Taga and Walker 2010).

A new *S. meliloti* strain was isolated and characterized by Dong et al. 2016. In this investigation, vitamin B_{12} synthesis was confirmed with HPLC-MS/MS and production of 140 ± 4.2 mg/L vitamin B_{12} was achieved in the culture of *S. meliloti* 320.

Recently, vitamin B_{12} production has also been reported with an engineered strain of *E. adhaerens* (Xu et al. 2022). In this study, the key genes of cobalamin synthesis in *E. adhaerens* were identified and various promoters of different strength were tested. Combined expression of the strong promoter and cobalamin genes resulted in the creation of a high-producing recombinant strain. Cobalamin levels of 143.8 mg/L achieved with the improved strain in shaking flasks were 41% higher than those in the culture of the original strain. Moreover, further increase of cobalamin production was achieved using fed-batch fermentation reaching up to 171.2 mg/L of vitamin B_{12}.

1.6.4 Metabolic engineering and vitamin B_{12} production in *E. coli*

Being a very well characterized model microorganism, *E. coli* is often used as a microbial cell factory for the synthesis of target products. Ko et al. 2014 used metabolic engineering for the creation of an *E. coli* strain carrying cob operon genes responsible for vitamin B_{12} production in *P. denitrificans* ATCC 13867. The recombinant strain was capable of vitamin B_{12} production, but the achieved cobalamin amounts of 0.21 ± 0.02 µg/g dry cell weight were 13 times lower than amounts produced with the initial *P. denitrificans* strain. Nevertheless, in comparison to *P. denitrificans* which produces cobalamin via the aerobic pathway, the engineered *E. coli* strain was capable of vitamin B_{12} production under both aerobic and anaerobic conditions.

However, since the microbiological assay known to sometimes yield false positive results was used in that report, Fang et al. 2018 concluded, that there was no unambiguous evidence of cobalamin production provided in the study. For this reason, they described another engineered *E. coli* strain expressing 28 genes of cobalamin synthesis from *R. capsulatus, Brucella melitensis, S. meliloti 320, S. typhimurium, and Rhodopseudomonas palustris*. Production of vitamin B_{12} as well as of the intermediates of its biosynthetic pathway was confirmed by the means of LC-MS. However, the amounts of vitamin B_{12} produced with the obtained strain were low, which is why further metabolic engineering and optimization of fermentation conditions were performed and finally production of 307 µg/g dry cell weight was reported.

1.6.5 *P. denitrificans*

There are several reports describing large-scale vitamin B_{12} production with *P. denitrificans.* Synthesis of 52.9 ± 0.6 µg/mL of vitamin B_{12} in 120-m^3 fermenter under optimal betaine feeding conditions was described by Li et al. 2008. In another study, combination of maltose syrup and corn steep liquor was suggested as cost-effective fermentation medium for *P. denitrificans* and production of 198.2 ± 4.6 mg/L of vitamin B_{12} was reported (Xia et al. 2015). Most famous is *P. denitrificans* for its application in the industrial production of vitamin B_{12} and more than 80% of the world production of vitamin B_{12} is covered with this microorganism (Martens et al. 2002). Industrial production is currently achieved with genetically modified strains of *P. denitrificans* producing above 200 mg/L of vitamin B_{12} (Martens et al. 2002; Balabanova et al. 2021).

1.6.6. Fortification of food products with vitamin B_{12} by *P. freudenreichii*

Although vitamin B_{12} is synthesized exclusively by microorganisms, animals can store it in the liver and muscles or secrete into the milk due to their gut microbial activities (Watanabe and Bito 2018), which is why vitamin B_{12} is naturally found in the foods of animal but not of plant origin. In the case of plant products food fortification for the enrichment with vitamins produced by synthesizing strains can be a potential way to increase the nutritional value of such food products (Burgess et al. 2009). Despite the widespread application of genetically engineered strains for the industrial production of vitamin B_{12}, the use of genetically modified organisms in food production is still controversial.

P. freudenreichii is well-known for the ability to synthesize predominantly the active vitamin B_{12} (Deptula et al. 2017). The high selectivity with respect to production of active vitamin B_{12} was shown to be the result of the BluB/CobT2 fusion enzyme activity (Deptula et al. 2015). The BluB enzyme is responsible for the formation of the lower ligand DMBI via release of erythrose-4-phosphat (E4P), while CobT2 facilitates the activation and the subsequent incorporation of DMBI into the cobalamin molecule.

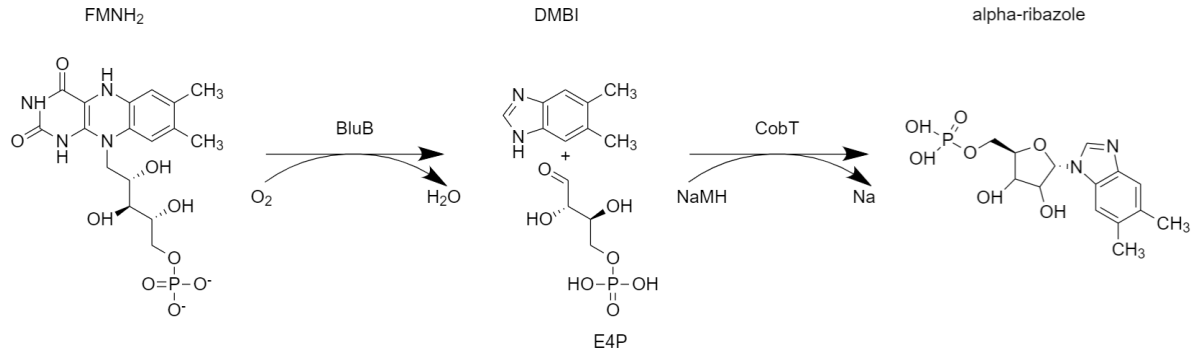

Fig. 5 Synthesis and activation of the lower ligand DMBI into α-ribazole phosphate catalyzed by the fusion enzyme BluB/CobT2. In the first step, oxygen-dependent formation of DMBI from the reduced flavin mononucleotide ($FMNH_2$) is catalyzed by BluB. Secondly, DMBI is activated into α-ribazole-phosphate by CobT (modified from Deptula et al. 2015)

Since oxygen is essential for the formation of DMBI, which facilitates the production of active vitamin B_{12} (Deptula et al. 2015; Deptula et al. 2017), a two-step cultivation is widely applied for the aerotolerant anaerobe *P. freudenreichii*. In the first step, anaerobic cultivation is performed for the biomass accumulation, which is then followed by aerobic cultivation necessary for vitamin B_{12} formation.

Due to the capability of active vitamin B_{12} production and the GRAS status allowing the application in food manufacturing, *P. freudenreichii* is the most attractive microorganism for vitamin B_{12} production in food industry and food fortification. Moreover, the ability of *P. freudenreichii* to synthesize vitamin B_{12} under food-like growth conditions or in the food-based media was proved in many reports (Table 1).

Table 1 B_{12} contents in food-based matrices fortified with *P. freudenreichii*

Matrix used for fermentation	**Obtained B_{12} content**	**Reference**
Kefir	up to 13.79 ± 0.95 mg/100 mL	van Wyk et al. 2011
Medium with waste frying sunflower oil as a carbon source	1.6 ± 0.12 µg/mL	Hajfarajollah et al. 2015
Cheese-like DL-sodium lactate medium	124.8 ± 34.7 ng/mL	Deptula et al. 2017
Industrial-type whey-based liquid medium	120 ± 10.4 ng/mL	Deptula et al. 2017
Barley flour (6% w/v)/wheat aleurone matrix (15% w/v)	10 ng/g	Chamlagain et al. 2018
Malted barley flour matrix (33% w/v)	37 ng/g (fresh weight)	
Durum flour (30% w/v)	33 ± 4 ng/g	Xie et al. 2018
Whole wheat flour (30% w/v)	87 ± 10 ng/g	
Wheat bran (20% w/v)	155 ± 17 ng/g (dry weight)	
Liquid acid protein residue of soybean as culture medium	0.6 mg/g cells	Assis et al. 2020

Due to the growing popularity of veganism, the interest for fortified plant-based foods as alternative vitamin B_{12} sources is continuously growing leading to the increasing number of new investigations on the fortified products and new strains appropriate for the food fortification.

L. reuteri CRL 1098 was reported to be successfully applied for the preparation of a novel soymilk beverage (Molina et al. 2012). No vitamin B_{12} was detected in the unfermented soymilk, while the administration of fermented soymilk prevented the development of vitamin B_{12} deficiency symptoms in

female mice and in their offsprings examined in the study. Soy-yogurt was used for the fortification with *L. reuteri* ZJ03 in another investigation (Gu et al. 2015). The obtained product contained 18 µg/ 100 mL of vitamin B_{12}, so that 14 mL of this drink can cover the daily demand for vitamin B_{12} for the vegetarians and elderly people. Recently, a new cobalamin-producing strain, *Acetobacter pasteurianus* DSM 3509, was characterized by Bernhardt et al. 2019. The authors reported up to 7.3 ng/mL of vitamin B_{12} in the culture of *A. pasteurianus* showing that this strain can be promising as a source of cobalamin supplements for the production of vegetable-based foods.

1.6.7 Methylotrophic microorganisms

Methylotrophs are a versatile group of bacteria associated with plants. Their characteristic feature is the ability to use C1-compounds synthesized by plants as energy and carbon sources for the growth. This can be achieved via the serine cycle elucidated by Quayle and coworkers (Large et al. 1961, 1962a, 1962b, 1963) which requires continuous regeneration of glyoxylate. This occurs in many organisms in the widespread glyoxylate cycle, while some serine cycle-methylotrophs lack the isocitrate lyase, an essential enzyme of the glyoxylate pathway (Anthony 1982). In this case an alternative pathway for glyoxylate regeneration, the ethylmalonyl-CoA pathway (EMCP), is operated which was elucidated by Erb et al. 2007. This pathway includes two vitamin B_{12}-dependent enzymes (Fig. 6), the ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase (Smejkalová et al. 2010), which indicates intracellular cobalamin synthesis in methylotrophs.

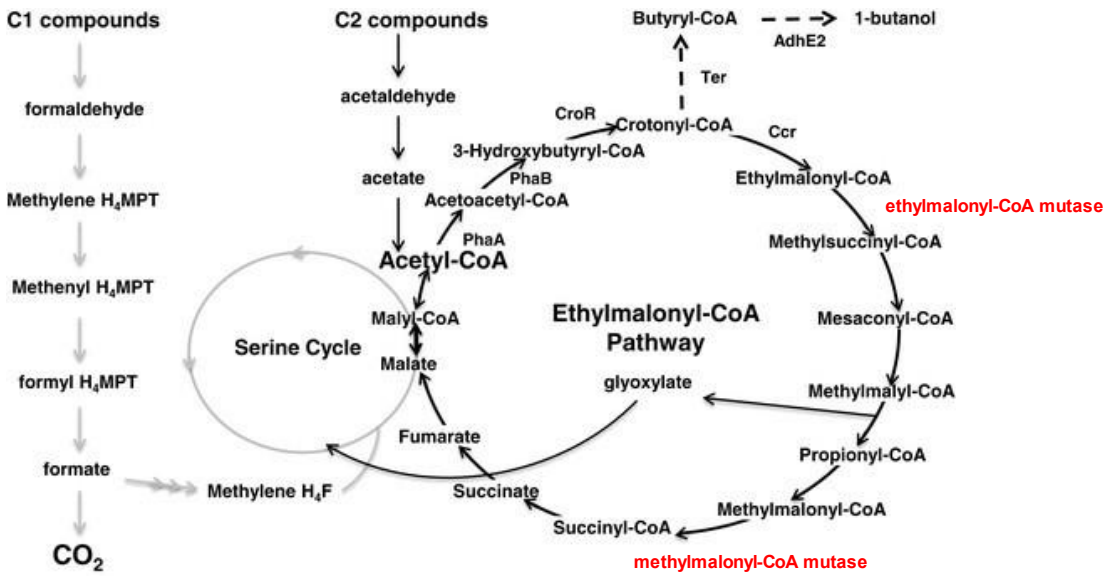

Fig. 6 C1 metabolism in *Methylorubrum extorquens* AM. Formate is synthesized from the C1 compounds via several enzymatic steps and used for the formation of methylenetetrahydrofolate, which enters the serine cycle. The resulting acetyl-CoA is assimilated and converted to glyoxylate in the ethylmalonyl-CoA pathway. The EMCP vitamin B_{12} - dependent enzymes are highlighted in red (modified from Hu and Lidstrom 2014)

Although an early report on the ability of *M. extorquens* (formerly *Protaminobacter ruber, Methylobacterium extorquens*) was published already in 1977 (Sato et al. 1977), it remained unclear for a long time whether the ability to produce vitamin B_{12} is widespread in methylotrophs. In 2002, Miyamoto et al. investigated the activity of a cobalamin-dependent methylmalonyl-CoA mutase in *M. extorquens* and measured parallelly the amount of AdoCbl in the cells. Further reports providing evidence for the cobalamin production in methylotrophic bacteria, especially in the genus *Methylobacterium* were made by Trotsenko et al. 2001; Danilova et al. 2004; Ivanova et al. 2006. Finally, the complete genome sequence of *M. aquaticum* was published in 2015 showing the presence of genes of cobalamin synthesis in the genome (Tani et al. 2015).

Nevertheless, in all mentioned reports vitamin B_{12} was determined with the help of the microbiological assay using *E. coli* as test organism, which is known to be sensitive also to vitamin B_{12} analogues (Ford 1959).

1.7 Methods of vitamin B_{12} analysis

Analysis of vitamin B_{12} is a very challenging procedure for several reasons. Firstly, being a micronutrient, vitamin B_{12} is synthesized by microorganisms or is present in food sources in minor amounts, which is why the analytical methods should be very sensitive. Secondly, due to the variety of different analogues of the active vitamin B_{12}, it is always important to define the type of the corrinoid during the analysis. Nowadays there are many different methods for vitamin B_{12} analysis described including MBA, radioisotopic assay, spectrophotometric methods, fluorimetric determination, capillary electrophoresis (CE), chemiluminescence (CL) assay, biosensor assay, high-performance liquid chromatography (HPLC), HPLC coupled with mass spectrometry (MS) (Kumar et al. 2010).

1.7.1 Microbiological assay

MBA is the classical method which has been used for vitamin B_{12} analysis over decades. The test principle is based on the inability of some microorganisms to grow without vitamin B_{12}. After addition of vitamin B_{12} as a standard or of the analyzed sample containing vitamin B_{12} the microorganism grows until the vitamin is depleted. The growth of the microorganism is detected as turbidity and the amount of vitamin B_{12} is directly proportional to the turbidity. For today, *E. coli* mutant 113, *L. leichmannii* 326, *L. delbrueckii, Euglena gracilis, Ochromonas malhamensis, Arthrobacter* Lochhead 38 have been described as microorganisms used in MBA (Kumar et al. 2010).

Microbiological assay using *E. coli* 113 has already been described in the early 1950s (Chiao and Peterson 1953). Unfortunately, a later study has shown that *E. coli* 113 is not able to differentiate between vitamin B_{12} and its analogues and, besides vitamin B_{12}, can also utilize benzyl-vitamin B_{12III}, pseudovitamin B_{12} and MMHP, an analogue of pseudovitamin B_{12} containing 2-methyl-mercapto-6-hydroxypurine in its nucleotide for the growth (Ford 1959).

First attempts to use *L. leichmannii* 326 (ATCC4797) (BacDive 2021) for vitamin B_{12} MBA were made by Skeggs et al. 1948 and Capps et al. 1949, who have demonstrated the growth response of *L. leichmanii* to the added vitamin B_{12}. Nevertheless, already Skeggs et al. 1948 have shown that this microorganism can also respond to thymidine and ascorbic acid, while the growth-promoting activity of various reducing agents was also demonstrated in a further study (Hoffmann et al. 1949). Pseudovitamin B_{12} has been shown to be 0.66 as active as vitamin B_{12} for the growth-promoting activity in another bacterium belonging to the family *Lactobacillaceae*, *L. delbrueckii* ATCC 7830 (Berman et al. 1956).

E. gracilis variant *bacillaris* was described as test microorganism for the analysis of vitamin B_{12} in human serum by Ross 1950. First investigations have shown that vitamin-free casein hydrolysate, carbon-treated peptone, methionine, ascorbic acid, pteroylglutamic acid, a variety of purine and pyrimidine bases or xanthopterin and thymidine could not replace vitamin B_{12} in its growth-promoting activity for *E. gracilis* (Robbins et al. 1950; Hutner and Provasoli 1949). Nevertheless, a further study proved that this assay cannot surely indicate the presence of vitamin B_{12}: other substances such as α- and β-ribazole, ammonia cobalichrome and especially pseudovitamin B_{12} having no activity for humans were shown to be utilized by *E. gracilis* (Robbins et al. 1952).

Lochhead and Thexton described a bacterium belonging to the genus *Arthrobacter*, which needs vitamin B_{12} for growth in 1955. The requirement of *Arthrobacter* Lochhead 38 for vitamin B_{12} was described to

be similar to the flagellate *O. malhamensis* (Goldberg et al. 1957) which was known to possess a similar specificity in vitamin B_{12} requirement to that of higher animals (Ford 1953). Nevertheless, a later report showed the ability of 5-methythioadenosine to completely replace the vitamin B_{12} requirement of *O. malhamensis* (Sugimoto and Fukui 1976).

1.7.2 Spectrophotometric methods

Spectrophotometric methods for vitamin B_{12} determination also belong to the classical analytical procedures. There are two ways for spectrophotometric determination of vitamin B_{12}: either by direct measurement of the absorbance of its aqueous solution or indirectly by cobalt determination (Bruno 1981).

In 1958 Bruening et al. developed a method for the direct determination of vitamin B_{12} in pharmaceutical products which was based on the measurement of radiant energy absorption of the cyanocobalamin. Although the reported method was rapid and easy to handle, the results of the measurement were influenced if other interfering substances were present.

Spectrophotometric methods of vitamin B_{12} analysis via cobalt determination were reported by Bruno 1981 and Medina-Escriche et al. 1987. The results obtained from the measurements performed with the described methods confirmed the quantitative recovery of cobalt. However, both techniques required a complicated procedure of sample preparation for cobalt extraction. Moreover, since the determination of vitamin B_{12} occurs via cobalt, the presence of other cobalt containing compounds can lead to higher values (Bruno 1981), which makes the sensitivity of these methods questionable.

1.7.3 Radioisotopic assay

First radioisotopic dilution assays have been developed in the 1960s (Rothenberg 1961; Lau et al. 1965; Mathews et al. 1967) and applied mainly for the measurement of vitamin B_{12} in serum and tissues. The principle is based on the competition between the vitamin B_{12} and a known amount of ^{57}Co-labeled vitamin B_{12} for the serum and intrinsic factor which serve as binding agents. Despite the simple procedure of vitamin extraction from the tissues and a high recovery (Frenkel et al. 1976), greater simplicity, rapidity and reproducibility in comparison to MBA (Lau et al. 1965), Kumar et al. 2010 reviewed such disadvantages of radioisotopic dilution assays as possible binding of biologically inactive cobalamin analogues as well as high costs of the available test kits.

1.7.4 Fluorimetric assay

Mori et al. 1992 proposed a fluorimetric method for the determination of cobalt, which can also be applied for the indirect determination of vitamin B_{12}. The principle is based on the ability of cobalt to catalyze the fluorescence reaction between p-hydroxy-2-anilinopyridine and hydrogen peroxide as an oxidizing agent. In this method, the difference of relative fluorescence intensities at the emission wavelength of 450 nm is measured between the reagent solutions containing and lacking cobalt. Since this assay is based on the determination of cobalt, the ability of the method to differentiate between the active form of vitamin B_{12} and the analogues is questionable.

An assay based on the spectral characteristics of B_{12} was suggested by Li and Chen, who proposed to determine vitamin B_{12} by measuring the fluorescence emission of its aqueous solution at $\lambda_{ex}/\lambda_{em}$ 275/305 nm (Li and Chen 2000). The method was applied to determine vitamin B_{12} in pharmaceutical preparations and turned out to be very sensitive. Unfortunately, there is no data on the method selectivity since the proposed analysis procedure has not been tested with the inactive vitamin B_{12} analogues.

1.7.5 Capillary electrophoresis

Lambert et al. 1992 described a high-performance capillary method for the analysis of different vitamin B_{12} forms and analogues. The method performed well and the separation of OHCbl, AdoCbl, MetCbl, CNCbl and CN-cobinamide was achieved. However, comparison of the developed technique with a high-performance liquid chromatography method has demonstrated a low sensitivity of high-performance capillary method. Further attempts were made by Baker and Miller-Ihli 2000, who described a new method based on capillary electrophoresis inductively coupled plasma mass spectrometry. The introduced technique enabled the separation of four active vitamin B_{12} forms and provided excellent selectivity and fast analysis of the samples. Nevertheless, it still lacked the sensitivity of microbiological assay and radioimmunoassay techniques. Finally, Chen and Jiang 2008 developed a sensitive capillary electrophoresis inductively coupled plasma mass spectrometry method for the analysis cyanocobalamin, hydroxycobalamin and inorganic cobalt and were able to achieve detection limits of 0.3, 0.2, and 1.7 ng of Co per mL analyzed sample, respectively. The main advantages of the method are the high sensitivity and good separation of the investigated forms, but at the same time the described sample preparation procedure is laborious and time-consuming.

1.7.6 Chemiluminescent assay

Methods using a fully automated chemiluminescence B_{12} analyzer (Chiron Diagnostics, East Walpole, MA) have been described (Watanabe et al. 1998; Miyamoto et al. 2006). These methods measure luminescence which is the result of the competitive reaction between the acridinium ester labeled B_{12} derivative and the unlabeled vitamin B_{12} from the test sample for the intrinsic factor. With the increasing concentration of the unlabeled B_{12} in the test sample less labeled B_{12} can bind to the intrinsic factor which leads to its accumulation in the supernatant. The resulting increased chemiluminescence intensity is measured to assay vitamin B_{12} in the test sample. Although the described methods are rapid and easy to run, the use of not easily obtainable intrinsic factor is associated with high costs.

Different research groups have reported methods of chemiluminescence determination of vitamin B_{12} without using the expensive intrinsic factor (Song and Hou 2003; Akbay and Gök 2008; Kumar et al. 2009). In these assays, cobalt is released from the vitamin B_{12} molecule during acidification process and then used as catalyst in the chemiluminescence reaction between luminol and oxidizing agents. The main advantage of these methods is the very high sensitivity, as methods proposed by Song and Hou and Kumar et al. enable determination of picogram amounts of vitamin B_{12}. Nevertheless, since the methods are based on the catalytic activity of cobalt, the selectivity of these methods needs to be better investigated.

1.7.7 Biosensor assay

The diversity of riboswitches, genetic control elements used by many microorganisms for the regulation of their gene expression in response to the environmental and metabolic changes, was reviewed by Winkler and Breaker 2003, 2005. In the further research, Breaker with the colleagues also contributed to the identification and description of the AdoCbl riboswitch (Nahvi et al. 2004). Biosensor assays based on the ability of riboswitches to control gene expression are a relatively new technique. There are only few reports describing such biosensors for vitamin B_{12} determination and they are all based on the similar principle of measurement (Fig. 7).

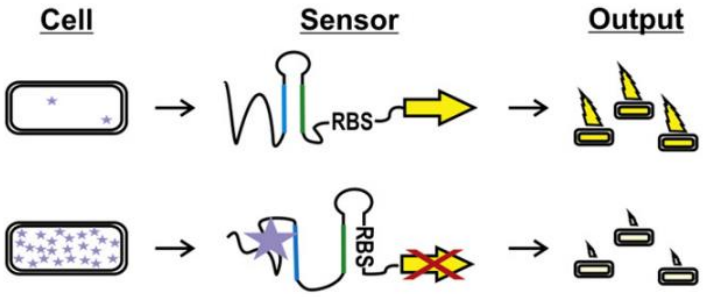

Fig. 7 Schematic representation of the biosensor-based assay principle. The expression level of the reporter gene after the analyte has bound to the riboswitch is compared to the expression level before binding. The signal decrease is proportional to the concentration of the target metabolite (Fowler et al. 2010)

The biosensor construct consists of the riboswitch and ribosome-binding site (RBS) placed upstream of the reporter gene. In case of low concentration of the target metabolite in the cell, the majority of riboswitches remains unbound and the translation of the reporter gene takes place resulting in the high signal output. If the concentration of the analyte in the cell increases, its binding to the riboswitch occurs leading to changes in its conformation. Conformational changes prevent the translation of the reporter gene mRNA, which results in the decreased signal output.

Fowler et al. (2010) described three biosensor constructs, where AdoCbl-responsive riboswitch that regulates vitamin B_{12} transport in *E. coli* is used as a control element in front of different reporter genes: β-galactosidase, firefly luciferase and DsRed-Express red fluorescent protein. The described riboswitch demonstrated a high sensitivity being able to detect AdoCbl at high picomolar media concentrations and showing a saturated response when AdoCbl was present in low-mid nanomolar concentrations, while the potential to scale up the assay to multi-well plate format makes it applicable for high-throughput screenings.

A couple of years later, Zhu et al. 2015 described a vitamin B_{12} biosensor consisting of the fragment containing a predicted riboswitch and RBS from *P. freudenreichii* spp. *shermanii* DSM 20270 and green fluorescent protein as reporter molecule. The biosensor turned out to be sensitive and selective showing no response to pseudovitamin B_{12}.

In 2017, Polaski et al. reported on the development of biosensors containing wild type and mutated variants of *env*8Cbl-IIa riboswitch and mNeon as fluorescent reporter gene. The main advantage of

these biosensors was the ability to sensitively discriminate between the adenosyl- and methyl-form of vitamin B_{12}.

Despite so many advantages of the biosensor assay, the main drawback is that they do not directly detect the target molecule, but rather measure the level of expression of a reporter gene, which can be influenced by varying environmental conditions (Fowler et al. 2010). Moreover, like MBA these assays depend on the growth of the test microorganism, which makes the analytical procedure time-consuming.

1.7.8 HPLC methods

HPLC-UV

HPLC systems with UV detectors have been used for the analysis of vitamin B_{12} already many years ago (Iwase and Ono 1997) and nowadays there are numerous HPLC methods for the determination of vitamin B_{12} (Heudi et al. 2006; Campos-Gimenez et al. 2008; Guggisberg et al. 2012). These HPLC methods have many common characteristics: they all use reverse-phase C18 columns, water and acetonitrile as mobile phase, characteristic wavelength of 361 nm for the UV-detection and require a multistep extraction and immunoaffinity column purification of vitamin B_{12} from the sample prior the measurement. Although the provided methods offer such advantages as selectivity, repeatability and intermediate reproducibility, the main drawback is the mentioned complicated and time-consuming sample preparation procedure. Moreover, the sensitivity of the HPLC methods can be increased if an MS detector is used for vitamin B_{12} determination.

LC-MS

MS is often used for the qualitative determination of vitamin B_{12} (Santos et al. 2007; Chamlagain et al. 2015; Bernhardt et al. 2019). The very important advantage of this technique is that due to the differences in the chemical structures it is possible to identify characteristic fragmentation patterns and differentiate between the active and inactive forms of vitamin B_{12}.

There have been also several reports on methods for vitamin B_{12} quantification via LC coupled with MS, which have been applied for the analysis of vitamin B_{12} in various food products (Zironi et al. 2013; Zironi et al. 2014; Szterk et al. 2012) and substitutes (Lee et al. 2015), multivitamin tablets (Luo et al. 2006) and plasma samples (Schwertner et al. 2012). Luo et al. 2006 described a rapid method for the detection and quantification of the total vitamin B_{12} amount in the analyzed sample, which demonstrated high

specificity and the ability of the method to discriminate B_{12} from potential interfering substances. A good separation and discrimination between different water-soluble vitamins obtained from the multivitamin tablets was achieved by Lu et al. 2008. Various sample preparation procedures, which enabled total vitamin B_{12} quantification as well as discrimination between CNCbl and OHCbl were shown in the study performed by Szterk et al. 2012. The ability to discriminate between these two vitamin B_{12} forms in the plasma samples was also demonstrated for the method described by Schwertner et al. 2012. The limits of detection and samples analyzed with the mentioned methods are listed in Table 2:

Table 2 Comparison of the reported LC-MS based methods for vitamin B_{12} analysis and their detection limits

Method	Analyzed sample	Required sample amount	Analysis time	LOD	Reference
HPLC-ESI-MS	multimineral tablets and milk powder	15 - 30 g	15 min	2 ng/g	Luo et al. 2006
UPLC–ESI-MS–MS	fortified milk powder and rice powders	1 g	10 min	0.006 µg/L	Lu et al. 2008
HPLC-MS	beaf sirloin, tenderloin, chuck, top round and liver	5 - 50 g	32 min	0.06-0.16 ng/20 µL injection (LOQ)	Szterk et al. 2012
LC-MS-MS	plasma samples	100 µL	10 min	0.1-1.0 mol/L	Schwertner et al. 2012
UPLC-MS	dairy products	5 g	5 min	2 ng/g (LOQ)	Zironi et al. 2013
LC-MS/MS	infant and toddler milk formulas	1 g	40 min	0.03 µg/L	Lee et al. 2015

The listed data show that the described methods demonstrate high sensitivity, can be applied for the analysis of various samples and many of them enable a fast analytical procedure. In spite of all

advantages, the complicated sample preparation like in the case of HPLC-UV methods remains the major drawback.

1.8 Aims of the study

The main aim of the study was to get insights in vitamin B_{12} synthesis performed by different producing strains and to identify those capable of active vitamin B_{12} production. Due to the variety of different vitamin B_{12} analogues, the first aim of the study was to establish a reliable and sensitive method, enabling discrimination between the active and inactive forms. The developed method will be applied for the comparison of the strains well known from the literature in order to identify efficient vitamin B_{12} - producing microorganisms. Further, different searching strategies will be proposed which can be applied for the identification of novel cobalamin-producing strains. The identified candidates will be characterized for their ability to produce the active vitamin B_{12}. Finally, the ability to produce vitamin B_{12} in the different media will be compared in order to find the best medium appropriate for a low-cost cobalamin production with the newly identified microorganisms.

2. Materials and methods

2.1 Bacterial strains

All strains used in this work for the investigation of vitamin B_{12} biosynthesis were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Cultivation of all strains used in this work was performed in the media recommended by DSMZ or previously described in literature for every specific strain. Bacterial strains and the corresponding cultivation media used for the analysis of vitamin B_{12} production by are listed in Table 3.

Table 3 Bacterial strains and media used in the study

Strain	Cultivation medium	Vitamin B_{12} production medium
E. meliloti DSM 23808	Medium 1	-
E. meliloti DSM 6047	Medium 1	-
E. meliloti DSM 23913	Medium 1	-
E. meliloti DSM 23914	Medium 1	-
E. meliloti DSM 30135	Medium 1	-
E. meliloti DSM 30136	Medium 1	-
E. meliloti DSM 23809	Medium 1	
E. meliloti DSM 6048	Medium 1	
B. megaterium DSM 509	Medium 1	
B. megaterium DSM 2894	Medium 1	
P. denitrificans DSM 1650	Medium 1	
P. freudenreichii DSM 20270	Medium 91	
M. extorquens DSM 1338	Mineral methanol medium (Peyraud et al. 2009)	
Terrabacter sp. DSM 102553	Medium 513, Medium 184 (ATCC)	Medium 513, 2x medium 513, mineral M9 medium

Terrabacter sp. DSM 102554	Medium 513	
Calidifontibacter indicus DSM 22967	Medium 92	
Raineyella antarctica DSM 100494	Medium 92	
Yimella lutea DSM19828	Medium 65	
Blastococcus sp. DSM 44272	Medium 714	
Xanthobacter autotrophicus DSM 1618	Medium 1	Mineral methanol medium (Peyraud et al. 2009)
X. autotrophicus DSM 432	Medium 1	Mineral methanol medium (Peyraud et al. 2009)
Hyphomicrobium sp. DSM 3646	Medium 162	Mineral methanol medium (Peyraud et al. 2009)
Pseudonocardia dioxanivorans DSM 44775	Medium 553	Mineral methanol medium (Peyraud et al. 2009)
Ruegeria pomeroyi DSM 15171	Medium 974 (Salgado et al. 2014)	Mineral medium with 0.5% ethanol (v/v) (Peyraud et al. 2009)
L. reuteri DSM 20016	Medium 11	
E. coli DSM 18039	Medium 1	-

2.2 Media

All media were prepared in ddH_2O (double-distilled water), 1.5% (w/v) agar was added when solid media were used. If not stated otherwise, all complex media and single components of the mineral media were autoclaved at 121 °C for 20 min.

Nutrient broth (medium 1)

Peptone	5.0 g
Meat extract	3.0 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.0

MRS medium (medium 11)

Casein peptone, tryptic digest	10.0 g
Meat extract	10.0 g
Yeast extract	5.0 g
Glucose	20.0 g
Tween 80	1.0 g
K_2HPO_4	2.0 g
Na-acetate	5.0 g
$(NH_4)_3$ citrate	2.0 g
$MgSO_4$ x 7 H_2O	0.2 g
$MnSO_4$ x H_2O	0.05 g
ddH_2O	add 1000.0 mL

pH is adjusted to 6.2 - 6.5

GYM *Streptomyces* medium (medium 65)

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.2 and 2.0 g of $CaCO_3$ are added if solid medium is used

***Propionibacterium* broth (medium 91)**

Casein peptone, tryptic digest	10.0 g
Yeast extract	5.0 g
Na-lactate	10.0 g

ddH_2O	add 1000.0 mL

pH is adjusted to 7.0 - 7.2

Trypticase soy yeast extract broth (medium 92)

Trypticase soy broth	30.0 g
Yeast extract	3.0 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.0 - 7.2

***Hyphomicrobium* medium (medium 162)**

KH_2PO_4	1.36 g
Na_2HPO_4	2.13 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4$ x 7 H_2O	0.2 g
$CaCl_2$ x 2 H_2O	6.0 mg
$FeSO_4$ x 7 H_2O	3.0 mg
$MnSO_4$ x H_2O	1.0 mg
Na_2MoO_4 x 2 H_2O	1.5 mg
Methylamine hydrochloride	3.38 g
Yeast extract	0.1 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.4 with NaOH

PP-medium (medium 513)

Polypeptone (mixture of casein peptone and meat peptone)	10.0 g
Yeast extract	2.0 g
$MgSO_4$ x 7 H_2O	1.0 g
ddH_2O	add 1000.0 mL

pH of medium is adjusted to 7.0

2xPP-medium (modified medium 513)

Polypeptone (mixture of casein peptone and meat peptone)	20.0 g
Yeast extract	4.0 g
$MgSO_4$ x 7 H_2O	2.0 g
ddH_2O	add 1000.0 mL

pH of medium is adjusted to 7.0

GPHF-medium (medium 553)

Glucose	10.0 g
Peptone from Casein	5.0 g
Yeast extract	5.0 g
Beef extract	5.0 g
$CaCl_2$ x 2 H_2O	0.74 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.2

GEO-medium (medium 714)

Yeast extract	1.0 g
Glucose	1.0 g
Starch, soluble	1.0 g
$CaCO_3$	1.0 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.2

1/2 YTSS medium (medium 974)

Yeast extract	2.0 g
Tryptone	1.2 g
Sea salts	20.0 g

ddH_2O	add 1000.0 mL

pH is adjusted to 7.0

Glucose asparagine agar (ATCC medium 184)

K_2HPO_4	0.5 g
Asparagine	0.5 g
Glucose	15.0 g
ddH_2O	add 1000.0 mL

pH is adjusted to 7.0

M9 mineral medium

For 1 L M9 mineral medium add to 867 mL sterile water:

Amount	Stock concentration	Component	Final concentration
100 mL	M9 salt solution (10x)	Na_2HPO_4	33.7 mM
		KH_2PO_4	22.0 mM
		NaCl	8.55 mM
		NH_4Cl	9.35 mM
20 mL	20% glucose	glucose	0.4%
1 mL	1M $MgSO_4$	$MgSO_4$	1 mM
0.3 mL	1M $CaCl_2$	$CaCl_2$	0.3 mM
1 mL	biotin (1mg/mL)	biotin	1µg
1mL	thiamine (1mg/mL)	thiamine	1 µg
10 mL	trace elements (100X)	trace elements	1x

Stock solutions

M9 salt solution (10x)

Na_2HPO_4 x $2H_2O$	75.2 g/L
KH_2PO_4	30 g/L
NaCl	5 g/L
NH_4Cl	5 g/L

The salts are dissolved in 800 mL of ddH_2O and the pH is adjusted to 7.2 with NaOH. ddH_2O is added to a final volume of 1 L

20% (w/v) glucose

For 500 mL stock solution add 100 g glucose to 440 mL water

Biotin (1 mg/mL)

For 50 mL stock solution 50 mg of biotin are dissolved in 45 mL water. Small aliquots of 1N NaOH are added until the biotin has dissolved. ddH_2O is added to a final volume of 50 mL. The solution is sterilized through a 0.22-µm filter. 1 mL aliquots are prepared and stored at -20°C

Thiamine (1 mg/mL)

For 50 mL stock solution 50 mg of thiamin-HCl are dissolved in 45 mL water. ddH_2O is added to a final volume of 50 mL. The solution is sterilized through a 0.22-µm filter. 1 mL aliquots are prepared and stored at -20°C

Trace elements solution (100X)

Component	Mass concentration	Molar concentration	Final concentration in medium
EDTA	5 g/L	13.4 mM	0.13 mM
$FeCl_3 \times 6H_2O$	0.83 g/L	3.1 mM	0.03 mM
$ZnCl_2$	84 mg/L	0.62 mM	6.2 µM
$CuCl_2 \times 2H_2O$	13 mg/L	76 µM	0.76 µM
$CoCl_2 \times 2H_2O$	10 mg/L	42 µM	0.42 µM
H_3BO_3	10 mg/L	162 µM	1.62 µM
$MnCl_2 \times 4H_2O$	1.6 mg/L	8.1 µM	0.08 µM

5 g of EDTA are dissolved in 800 mL ddH_2O and the pH is adjusted to 7.5 with NaOH. Then $FeCl_3 \times 6H_2O$, $ZnCl_2$ and the liquid stocks of the other components are added in the quantities mentioned below. ddH_2O is added to a final volume of 1 L. The solution is sterilized through a 0.22-µm filter

$FeCl_3$ (anhydrous)	498 mg
$ZnCl_2$	84 mg
0.1 M $CuCl_2$ x $2H_2O$	765 µL
0.2 M $CoCl_2$ x $6H_2O$	210 µL
0.1 M H_3BO_3	1.6 mL
1 M $MnCl_2$ x $4H_2O$	8.1 µL

Alternative C-sources

2.5 g of glycerol or arabinose are dissolved in 8 mL ddH_2O, water is added to a final volume of 10 mL. 2 mL of the obtained stock solution are added per 100 mL medium.

Mineral methanol medium (Peyraud et al. 2009)

For 1 L mineral methanol medium add to 493 mL sterile water:

0.07 M Phosphate buffer pH 7.1	300 mL
Methanol	5 mL
Mineral salts	200 mL
$CaCl_2$ stock (x1000)	1 mL
Trace elements (x1000)	1 mL

0.07 M Phosphate buffer pH 7.1

K_2HPO_4	8 g/L
NaH_2PO_4 x $2H_2O$	3.6 g/L

Mineral salts

NH_4Cl	8.1 g/L
$MgSO_4$ x $7H_2O$	1 g/L

$CaCl_2$ Stock (x1000)

0.075 g of $CaCl_2$ x $2H_2O$ are dissolved in 50 mL ddH_2O. The solution is sterilized through a 0.22-µm filter. 1 mL aliquots are prepared and stored at -20°C

Trace elements (x1000)

$Na_2EDTA \times 2H_2O$	1.5 g
$ZnSO_4 \times 7\ H_2O$	0.45 g
$CoCl_2 \times 6\ H_2O$	0.3 g
$MnCl_2 \times 4\ H_2O$	0.1 g
H_3BO_3	0.1 g
$Na_2MoO_4 \times 2\ H_2O$	0.04 g
$FeSO_4 \times 7\ H_2O$	0.3 g
$CuSO_4 \times 5\ H_2O$	0.03 g
ddH_2O	add 100 mL

Ethanol as sole carbon source

In the growth experiments with ethanol as carbon source 5 mL of ethanol are added instead of methanol per 1 mL medium.

2.3 Chemicals, buffers and solvents

Chemicals and solvents used for the LC-MS/MS analysis of vitamin B_{12} are listed in Table 4.

Table 4 Chemicals and solvents used for the LC-MS/MS analysis

Component	Manufacturer
CNCbl analytical standard	Merck, Darmstadt, Germany
Methanol, HPLC grade	VWR, Darmstadt, Germany
Acetonitrile, HPLC grade	VWR, Darmstadt, Germany
Formic acid	Carl Roth, Karlsruhe, Germany
Water, HPLC grade	VWR, Darmstadt, Germany

Commercially available vitamin B_{12} supplements used in the study for the validation of the developed LC-MS/MS analysis method are listed in Table 5.

Table 5 Commercial vitamin B_{12} supplements used for the LC-MS/MS method validation

B_{12} supplement	**Manufacturer**
Doppelherz B_{12} Vita-Energie drink bottles	Queisser Pharma, Flensburg, Germany
B_{12} drops Ankermann	Wörwag Pharma, Böblingen, Germany
B_{12} Vita-Kick drink ampoules	Tetesept Pharma, Frankfurt, Germany
B_{12} drink bottles, Vitasprint	GlaxoSmithKline Consumer Healthcare, Munich, Germany
Doppelherz vitamin B_{12} mini tablets	Queisser Pharma, Flensburg, Germany

Acetate buffer

For the preparation of the acetate buffer used for vitamin B_{12} extraction and purification 4.1 g of Na-acetate is dissolved in 950 mL of ddH_2O. Then the pH is adjusted to 4.5 with acetic acid. ddH_2O is added to a final volume of 1 L and the pH is checked to be 4.5.

Vitamin B_{12} analogues and derivatives

Vitamin B_{12} analogues and derivatives used in the search for mutant screening agent were either purchased or custom synthesized (Table 6).

Table 6 Chemicals used in the search for mutant screening agent

Chemical	**Manufacturer**
Succinylacetone	Merck, Darmstadt, Germany
Levulinic acid	Merck, Darmstadt, Germany
Vitamin B_{12}-monocarboxylic acid	ABCR, Karlsruhe, Germany
Vitamin B_{12}-c-lactam	GenoSynth, Berlin, Germany
Vitamin B_{12}-dodecylamine	GenoSynth, Berlin, Germany
Difluorosuccinic acid	VWR, Darmstadt, Germany

2.4 Equipment

Devices used in the experiments performed in the study are listed in Table 7.

Table 7 Devices used in the study

Device	Description	Manufacturer
LC-MS	LC-MS 8045	Shimadzu, Kyoto, Japan
Centrifuge	Centrifuge 5415 R	Eppendorf, Hamburg, Germany
Centrifuge	MiniSpin plus table centrifuge	Eppendorf, Hamburg, Germany
Incubator	HT Minitron	Infors, Bottmingen/Basel, Switzerland
Microbioreactor	BioLector® MB Microbioreactor	m2p-labs, Baesweiler, Germany
Speedvac	Rotary Vacuum Concentrator RVC 2-25 CDplus	Martin Christ, Osterode, Germany

SPE-columns

Purification of corrinoid compounds from the cell extracts was performed using BAKERBOND spe™ C18 columns JB7020-03 from J. T. Baker (VWR, Darmstadt, Germany)

2.5 Databases and Software

NCBI (National Center for Biotechnology Information, United States) non-redundant protein database was used for the identification of new vitamin B_{12}-producing strains. LabSolutions Analysis Software (Shimadzu) was applied for the data acquisition and analysis of the LC-MS data. Visualization and statistical analysis of the data obtained in this study was performed using OriginPro® (OriginLab, Northampton, MA, USA) data analysis software

2.6 Microbiological methods

2.6.1 Cultivation of microorganisms in BioLector microbioreactor

The pre-cultures of the investigated strains were grown in sterile culture tubes for 48 h and used for the inoculation of 1 mL cultures at the starting OD_{600} of 0.1. The cultivation was carried out in a BioLector® MB system (m2p-labs, Germany) in MTP-48 FlowerPlates® with pH optodes at 30 °C, 1000 rpm and

95% humidity. The growth of the cultures was monitored online by scattered light intensity measurement. In the experiments on the search for the mutant screening agent, the respective vitamin B_{12} analogues or derivatives were added directly in each well at the beginning of the experiment.

2.6.2 Cultivation of *P. freudenreichii* DSM 20270 for vitamin B_{12} production

A two-step cultivation was applied for the production of vitamin B_{12} with *P. freudenreichii* DSM 20270 (Chamlagain et al. 2018; Deptula et al. 2017). Briefly, the pre-cultures were inoculated from the glycerol culture stocks (-80 °C) in 20 mL medium and incubated in septum flasks anaerobically for 3 days at 30 °C. The production cultures were inoculated in 50 mL medium and incubated at 30 °C for 72 h under anaerobic conditions, followed by 96 h of aerobic incubation, when septum flasks were opened under sterile conditions and the cultures were transferred into the 100 mL Erlenmeyer flasks. Aerobic cultivation was performed at 180 rpm.

2.6.3 Cultivation of *Bacillus* strains for vitamin B_{12} production

A two-step cultivation was applied for the production of vitamin B_{12} with *B. megaterium* DSM 509 and *B. megaterium* DSM 2894. The pre-cultures were incubated in the sterile culture tubes for 2 days at 30 °C. The production cultures were inoculated in 100 mL medium and incubated in Erlenmeyer flasks at 30 °C for 24 h at 180 rpm. The cultures were centrifuged (3.150 × g, 30 min), the cell pellet was transferred into septum flasks with 90 mL of fresh oxygen-free medium and incubated anaerobically for 72 h.

2.6.4 Cultivation of other microorganisms for vitamin B_{12} production

50 mL production cultures of the investigated bacteria were propagated in liquid media in sterile 300 mL Erlenmeyer flasks at 30 °C and 180 rpm in a shaking incubator under aeration. Growth on solid media was carried out for 48-120 h at 30 °C.

2.7 Biochemical methods

2.7.1 Vitamin B_{12} production, extraction and purification

For the cobalamin analysis from the solid media the cells obtained after the incubation were scratched with the spatula from the agar surface and the whole biomass was used for cobalamin extraction. For

the cobalamin analysis from the liquid cultures, 25 mL of the broth were harvested by centrifugation at 3.150 × g for 30 min. The cobalamin was extracted in the cyano-form and the cell pellets were resuspended in 10 mL of acetate buffer containing 100 µL of 1% KCN. After the incubation in a water bath at 98 °C for 30 min the samples were cooled on ice for 30 min and centrifuged again. Vitamin B_{12} was purified from the obtained supernatants using BAKERBOND spe™ C18 columns according to the manufacturer's instructions. The extracts were then syringe filtered (0.2 µm), dried at 60 °C in the speedvac and resuspended in 100 µL of deionized H_2O.

2.8 Analytical methods

2.8.1 LC-MS/MS method for the analysis of cobalamins

Analysis of the samples was performed with a triple quadrupole LCMS-8045 system. A Luna® Omega 3 µm PS C18 100 Å Column (Phenomenex, Aschaffenburg, Germany) was operated with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The following LC gradient program was employed: 0-3 min 18-32% B, 3-3.1 min 32-95% B, 3.1-4.1 min 95% B, 4.1-4.3 min 95-18% B, 4.3-7 min 18% B. The flow rate was 0.4 mL/min and the column temperature was maintained at 40 °C. The MS analysis was carried out in positive ion mode using electrospray ionization (ESI) under following parameters: nebulizing gas flow 3 L/min, drying gas flow 10 L/min, interface temperature 300 °C, desolvation line temperature 250 °C and heat block temperature 400 °C. The mass spectrometer was run in multiple reaction monitoring (MRM) mode for cobalamin (MRM (+) m/z 678.40 → m/z 146.95, m/z 678.40 → m/z 359.10) and pseudocobalamin (MRM (+) m/z 672.75 → m/z 136.05, m/z 672.75 → m/z 348.05). The injection volume was 1 µL and the quantification of vitamin B_{12} in the samples was performed using a calibration curve obtained from a set of cyanocobalamin standards, which were diluted from the stocks stored in the dark at -80 °C. Since pseudovitamin B_{12} is commercially not available as analytical standard, the cell extract of *L. reuteri* DSM 20016 containing pseudovitamin (Santos et al. 2007) was used as reference material. Vitamin B_{12} amounts were calculated per 100 mL culture and per g dry cell weight (DCW).

2.8.2 Method optimization

The effect of the solvent flow rate and the used C18 column was investigated. The flow rates of 0.25 – 0.4 mL/min and C18 columns Luna Omega 1.6 µm Polar C18 100 Å and Luna Omega 3 µm PS C18 100 Å were compared. The columns were evaluated for the peak height and the number of theoretical plates, which was calculated according to the following formula:

$$N = 5.54 \times \left(\frac{t_R}{W_{0.5h}}\right)^2,$$

where N is the number of theoretical plates, t_R is the retention time and $W_{0.5h}$ is the width of the peak at half height. Mass spectrometry method parameters (collision energies, dwell times and exact m/z values) were optimized with the LabSolutions Analysis Software. The ion source and mobile phase condition, concentration of formic acid in the mobile phase were investigated for their influence on the CNCbl signal.

2.8.3 Method validation

The instrumental detection limit (LOD) was determined as the lowest concentration of cyanocobalamin standard corresponding to the first peak that can be integrated and distinguished from zero using the signal-to-noise (S/N) approach (S/N ratio ≥ 3). The limit of quantification (LOQ) was defined as the lowest concentration of cyanocobalamin that can be determined with an acceptable repeatability (relative standard deviation between the samples under 10%) using the signal-to-noise (S/N) approach (S/N ratio ≥ 3).

The linearity of the method was estimated over the concentration range of 20 – 2000 nM of CNCbl, a set of 10 concentrations of the standard solution was used, each solution was injected in triplicate. The determination coefficient R^2 was calculated to estimate the linearity.

To evaluate the selectivity of the method, the chromatograms of vitamin B_{12}-free acetate buffer and *E.coli* DSM 18039 cell extracts were compared with the chromatograms of the respective samples spiked with cyanocobalamin standard. The blank and spiked samples were prepared following the previously developed vitamin B_{12} extraction and purification protocol and then analyzed for the presence of cyanocobalamin.

Commercially available vitamin B_{12} supplements were used for assessing the accuracy of the method. Five different samples containing various amounts of cyanocobalamin were spiked with a known amount

of cyanocobalamin standard and the accuracy was estimated through the recovery rates which were calculated as described by Campos-Gimenez et al. 2008:

$$\frac{Cs-Cn}{Ca} \times 100,$$

where Cs is the concentration of vitamin B_{12} found in the spiked sample, Cn is the concentration of vitamin B_{12} in the native sample, and Ca is the concentration of cyanocobalamin added in the spiked sample.

The within-day repeatability was evaluated in the range of 100 – 1000 nM by analysing five different samples, each solution was injected in triplicate. The intermediate reproducibility (between-day) was determined by analysing a calibration row of five different samples in the range of 100 – 1000 nM during three consecutive days

The robustness of the method was evaluated through the introduction of small deliberate variations in method parameters. Three analytical parameters were selected and small variations were introduced into the original values of the method (Table 8).

Table 8 Initial parameters and introduced variations for the robustness evaluation of the developed method

Parameter	Original value	Variation
Flow rate, mL/min	0.4	0.41
Column temperature, °C	40	35
Formic acid in mobile phase, %	0.2	0.22

The same cyanocobalamin standard solution was measured in triplicates under original and changed method conditions to determine the influence of each parameter and the obtained peak areas and the retention times were compared. To determine the influence of each parameter on the final result, the mean value of the triplicate measurements obtained under the original conditions was compared to the mean value of the triplicate measurements obtained under the altered conditions and the relative standard deviation was calculated.

2.9 Bioinformatic methods

2.9.1 Identification of BluB/CobT2 homologues

The BluB/CobT2 protein sequence from *P. freudenreichii* was used for the identification of bacterial homologues by means of BLAST (National Center for Biotechnology Information). The blastp tool was applied for the search in the protein sequence database excluding the genus *Propionibacterium* (taxid:1743), the maximum number of sequences was set to 1000. ClustalW online tool was used for the alignment of the identified protein sequences.

2.9.2 Identification of strains with possible EMCP function

To identify microorganisms that possess a functional EMCP, bacterial genome sequences were analyzed for the presence of a crotonyl-CoA carboxylase/reductase (Ccr)-encoding gene. To exclude strains that additionally contain the glyoxylate pathway, only bacteria containing no isocitrate-lyase (Icl)-encoding gene were considered. The genome dataset used as starting point was created in house from all bacterial genomes available at the Microbial Nucleotide Blast homepage (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) at the time of investigation. If sequences similar to the *M. extorquens* AM1 Ccr protein sequence with e-values lower than e-value of 10^{-43} could be identified within a tblastn analysis with the standard tblastn parameter values, the presence of the EMCP was concluded. Sequences with similarity to Icl of *Escherichia coli* with a boundary e-value of 10^{-14} served as marker genes for the glyoxylate shunt. If the candidate carried crotonyl-CoA carboxylase/reductase and lacked isocitrate lyase, they were included in the further analysis.

2.9.3 Statistical analysis

If not stated otherwise, all experiments were repeated in biological triplicates. Data are presented as the mean value ± standard deviation. Two-sample t-test was used to determine the significance of the difference between the means. Values were considered significant at $p < 0.05$.

3. Results

3.1 Development, optimization and validation of the LC-MS/MS method for vitamin B_{12} analysis

3.1.1 Selection of the appropriate column and optimization of mass spectrometric and chromatographic parameters

Two reversed phase columns were tested for their possible application for vitamin B_{12} analysis: Luna Omega 1.6 µm Polar C18 100 Å with a polar modified particle surface and Luna Omega 3 µm PS C18 100 Å with a positive surface modification (Fig. 8).

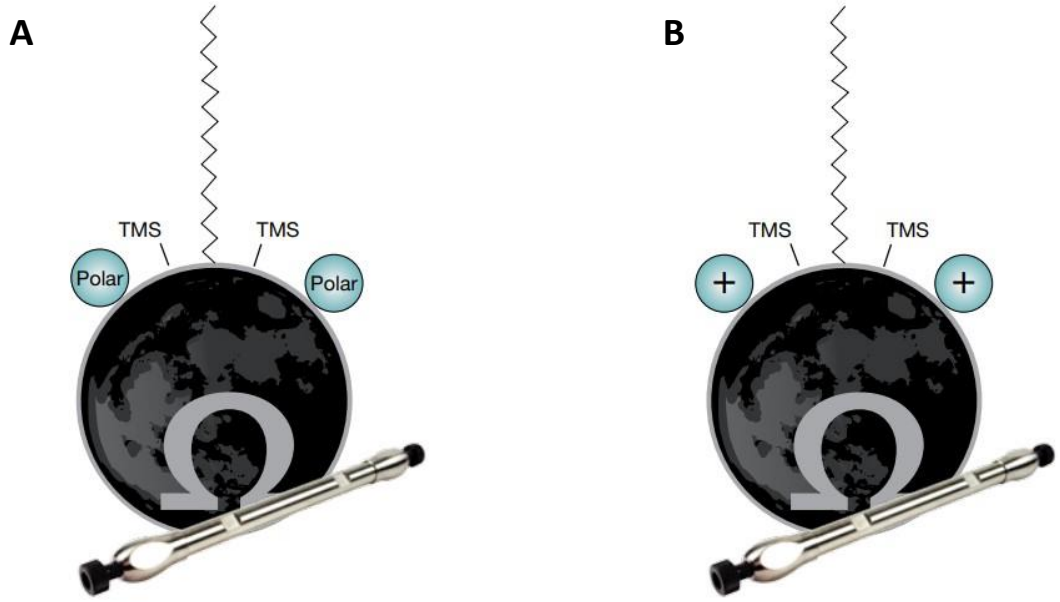

Fig. 8 Comparison of the stationary phase surface of the columns (A) Luna Omega 1.6 µm Polar C18 100 Å and (B) Luna Omega 3 µm PS C18 100 Å (Phenomenex®)

As Fig. 9 shows, the peak at 2.23 min produced with Luna Omega 3 µm PS C18 100 Å was two times taller than that at 2.42 min obtained with Luna Omega 1.6 µm Polar C18 100 Å. Although the obtained peak areas of 223230 and 224945 were comparable for Luna Omega 3 µm PS C18 100 Å and Omega 1.6 µm Polar C18 100 Å, the latter column demonstrated a shoulder peak. Moreover, the number of theoretical plates on the Luna Omega 3 µm PS C18 100 Å column was 2.85 times higher than on Luna

Omega 1.6 µm Polar C18 100 Å. Therefore, Luna Omega 3 µm PS C18 100 Å was used as the analytical column for all further tests.

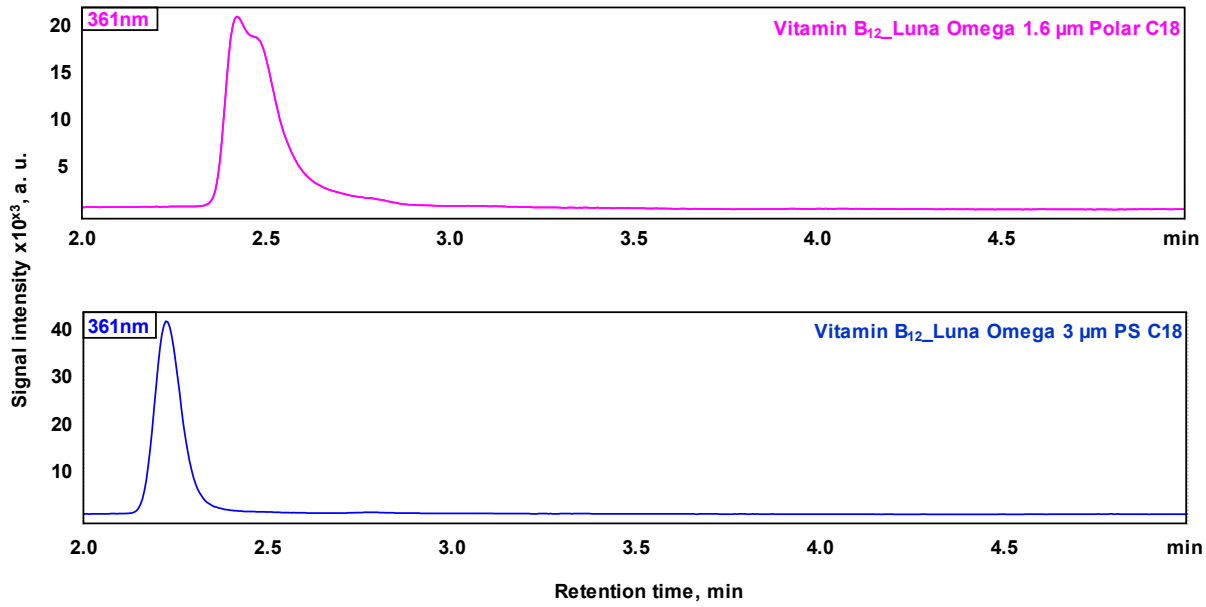

Fig. 9 Selection of the appropriate C18 column. Demonstrated vitamin B_{12} peaks were detected with PDA detector at the characteristic wavelength of 361 nm

After column selection, development and optimization of the chromatographic parameters was performed. Since the LC-MS/MS method was targeted to be applied for the vitamin B_{12} analysis in bacterial cells, it was aimed to create a representative system applicable for cell matrices which can enable to avoid a cross talk between the analyzed CNCbl and other vitamin B_{12} forms naturally produced by the microorganisms. For this reason, AdoCbl and MetCbl standards were also included in the analysis and chromatographic separation of the three vitamin B_{12} forms was performed. In order to differentiate between the cobalamins according to their masses and to assign the observed peaks to the corresponding analyzed forms, positive full-scan was performed in the range m/z 500-2000, which included the masses of the simple and double-charged ions of all analyzed forms.

In the first step, a linear gradient was applied in order to find out which composition of mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1% formic acid) is necessary to eluate the analytes from the column: 0-1 min 5% mobile phase B, 1-7 min 95% mobile phase B, 7-8.5 min 95% mobile phase B, 8.5-8.6 min 5% phase B, 8.6-10 min 5% mobile phase B. For the purpose of better visualization, the described LC-program is shown in Appendix 1A.

As Fig. 10A shows, only two peaks were observed, although a mixture of CNCbl, AdoCbl and MetCbl was analyzed. Since the recorded chromatogram was obtained using the scan method, the mass spectrometric data were analyzed, which have shown that the retention time of CNCbl and AdoCbl both corresponded to the peak at 4.3 min, while the second peak at 4.7 min was identified as MetCbl (Appendix 2). Moreover, there was also no baseline separation between the two peaks observed. Therefore, the goal of the further optimization step was to obtain three well-separated peaks during the shortest separation time. Since the first peak in Fig. 10A occurs at approx. 4 min, the second run was started at 15% of phase B to elute the analytes from the column earlier and to make the analytical procedure faster. For this purpose, the following LC-program was used: 0-0.1 min 15% mobile phase B, 0.1-5 min 50% mobile phase B, 5-5.1 min 95% mobile phase B, 5.1-6.5 min 95% phase B, 6.5-6.6 min 15% mobile phase B, 6.6-8.2 min 15% mobile phase B (Appendix 1B). As Fig. 10B shows, this allowed to shorten the method, since the analyte peaks were eluted from the column earlier than in the method shown in Fig. 10A. Moreover, due to the flatter gradient between 15 and 50% of phase B two peaks were observed for CNCbl and AdoCbl.

However, the optimal separation of these first two peaks was still not achieved, hence an even flatter gradient was applied in the “interesting region” between 1 and 4 min. In addition, the method was started at 18% B to further shorten the analytical procedure. This resulted in the following LC-program: 0-0.1 min 18% mobile phase B, 0.1-4.5 min 38% mobile phase B, 4.5-4.6 min 95% mobile phase B, 4.6-5.6 min 95% phase B, 5.6-5.7 min 15% mobile phase B, 5.7-7.2 min 15% mobile phase B (Appendix 1C). The applied method allowed to achieve a better separation of the MetCbl peak from the other two cobalamin forms (Fig. 10C) than in previously demonstrated steps, and a baseline separation between the CNCbl and AdoCbl peaks was observed.

In the last step, final optimization was performed and the gradient was made even steeper for a better separation of CNCbl and AdoCbl peaks: 0-0.1 min 18% mobile phase B, 0.1-4.0 min 32% mobile phase B, 4.0-4.1 min 95% mobile phase B, 4.1-5.1 min 95% phase B, 5.1-5.2 min 18% mobile phase B, 5.2-6.8 min 18% mobile phase B (Appendix 1D). The improved results of the obtained LC-method are shown in Fig.10D.

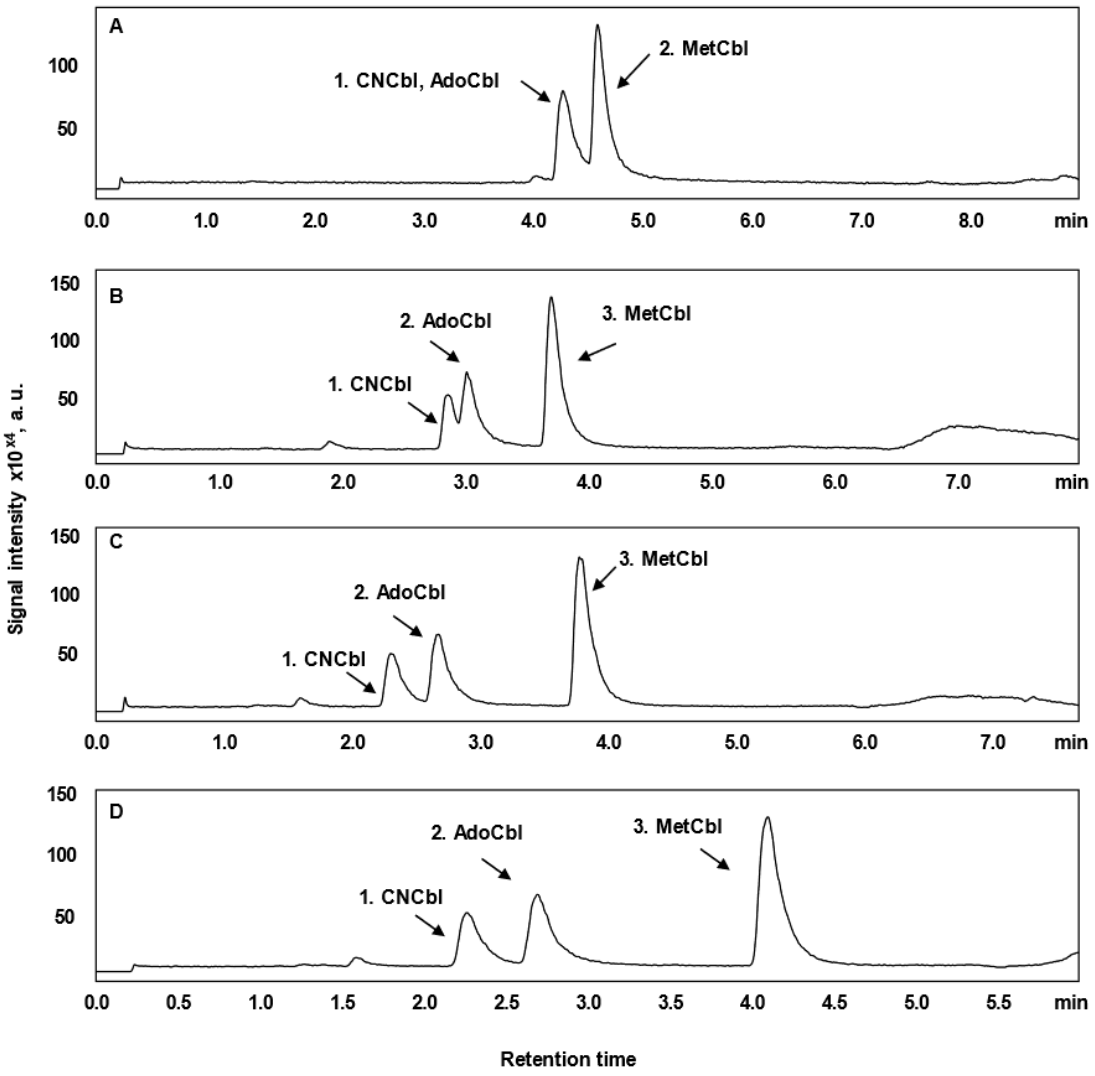

Fig. 10 Stepwise optimization of LC-parameters for separation of AdoCbl, CNCbl and MetCbl, details are described in the text. Cobalamin forms corresponding to the observed peaks are marked with asterisks

After development and optimization of the LC-method, optimization of mass spectrometric method parameters was performed. Since further vitamin B_{12} detection was performed by means of mass spectrometry, optimization of the method parameters for the single and double charged molecular ions in the MRM mode was performed with the LabSolution Software for each transition to identify peaks with the highest abundance. After optimization, the results obtained for the single and double charged molecular ions were compared.

The peak area and signal abundance obtained for the double charged ion were approximately 25 times higher than those obtained for the single charged ion (Fig. 11). For this reason, the masses

corresponding to the double charged ion were chosen for the further analyses: m/z 678.4 was identified as the parent mass ion with m/z 146.95, m/z 359.10, m/z 456.75 as fragment ions.

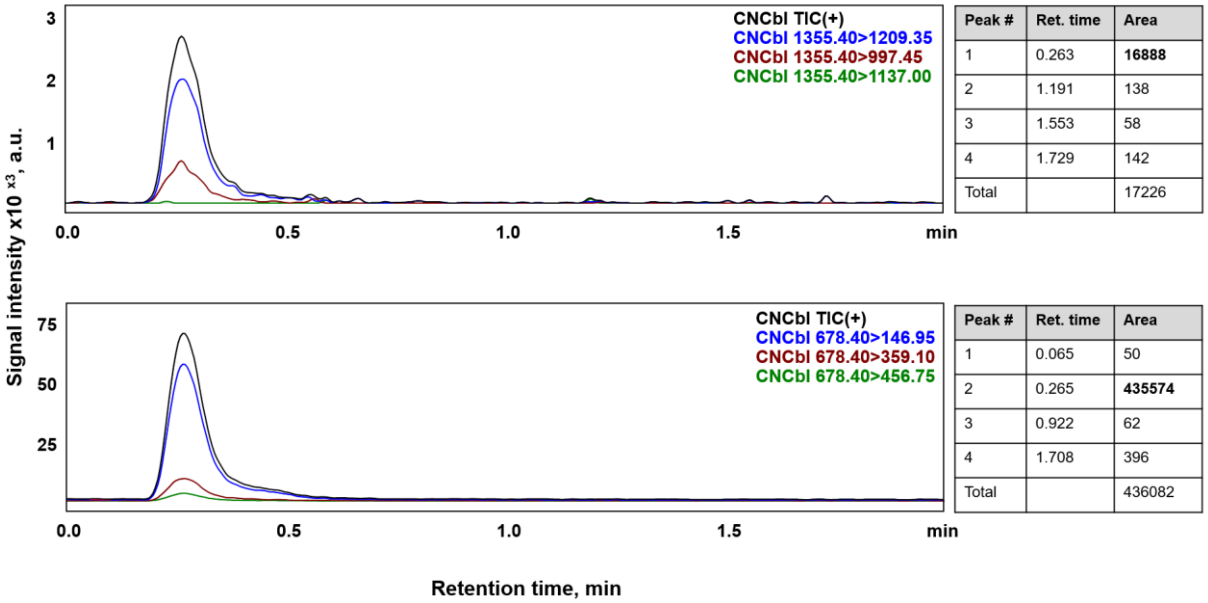

Fig. 11 Method optimization for the single and double charged cobalamin ions. Peak areas corresponding to the cobalamin peaks are shown in bold

After the selection of appropriate column and ion masses providing the most intensive signal, optimization of the flow rate for the established method was performed. Between flow rates of 0.25 and 0.4 mL/ min tested with the chosen column and identified precursor and product ion masses the flow rate of 0.4 mL/ min provided the fastest analytical procedure without a loss of chromatographic performance (Fig. 12).

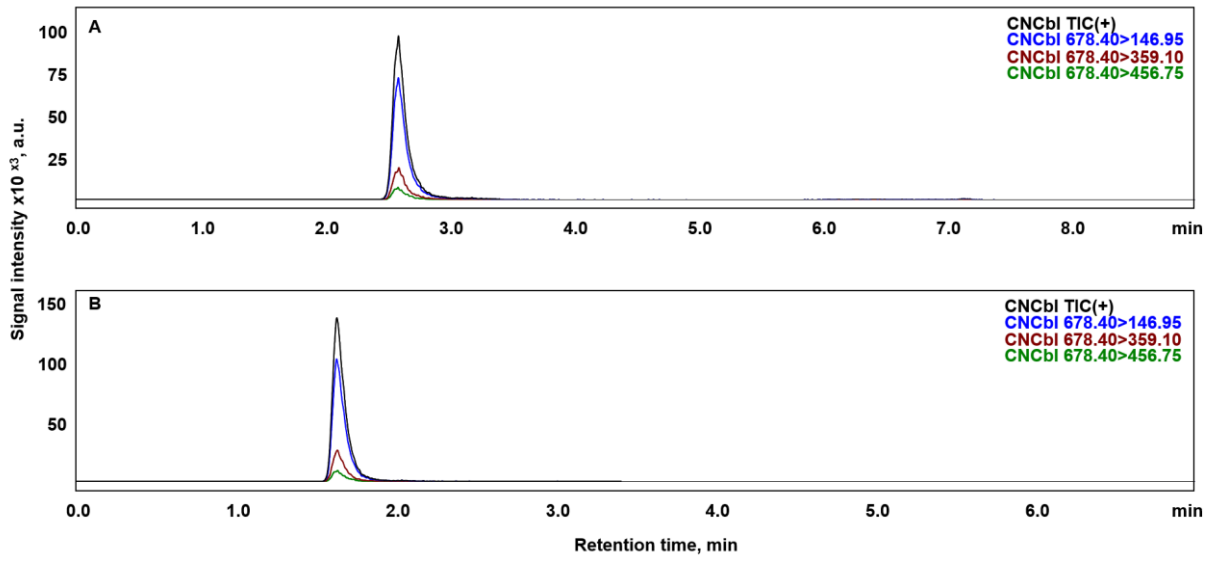

Fig. 12 Optimization of the flow rate for the developed method. Demonstrated peaks were obtained at the low rate of 0.25 mL/min (A) and 0.4 mL/min

The CNCbl peak at 2.6 min obtained when the method was run at 0.25 mL/min demonstrated a peak area of 752492, while CNCbl peak with an area of 724726 was detected at 1.6 min when the system was operated at 0.4 mL/min. Therefore, it was chosen as optimal flow rate for the further analysis.

3.1.2 Development of the method for the identification of the active and pseudovitamin B_{12}

Since in case of the active vitamin B_{12} the double charged ion demonstrated remarkably higher peak area in comparison to the single charged ion, the masses corresponding to double charged ion of pseudovitamin B_{12} were used for method development. All other method parameters were kept constant and applied from the method for active vitamin B_{12} identification.

As the active and inactive forms differ in their respective ligands (Fig. 1), their fragmentation patterns are characteristic and can be used to differentiate between the two forms. This resulted in the identification of two characteristic spectra corresponding to the active vitamin B_{12} and pseudocobalamin (Fig. 13). The spectrum of the cyanocobalamin reference compound demonstrated a characteristic peak with the retention time of 1.77 min and parent ion with m/z 678.40 and fragment signals with m/z 146.95 $[DMBI + H]^+$, m/z 359.10 $[DMBI + sugar + PO_3 + H]^+$ and an unidentified fragment with m/z 456.75. The peak identified in the extract of pseudocobalamin-producing *L. reuteri* demonstrated the retention time of 1.4 min and a parent ion with m/z 672.75 and fragment signals with m/z 136.05 $[adenine + H]^+$ and m/z 348.05 $[adenine + sugar + PO_3 + H]^+$ (Fig. 12).

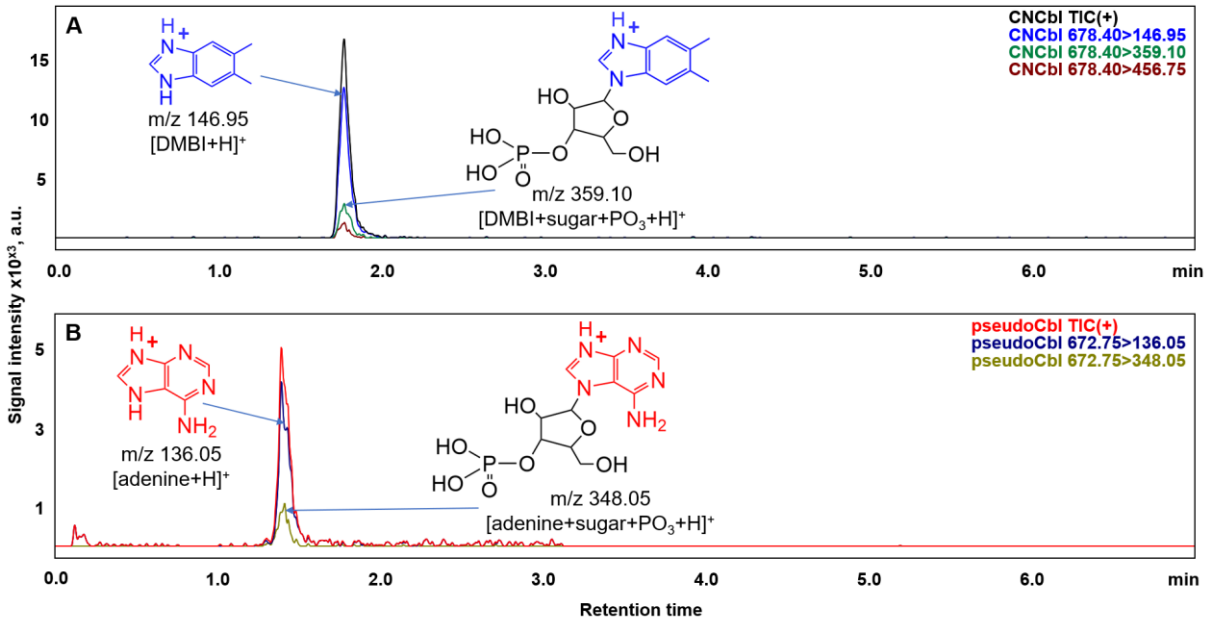

Fig. 13 MRM analysis showing characteristic fragment and parent ion masses and retention times present in the LC-MS/MS chromatograms of (A) vitamin B_{12} and (B) pseudovitamin B_{12}. The structures of the fragments corresponding to the mass spectral peaks are assigned with arrows

3.1.3 Identification of the factors influencing vitamin B_{12} signal intensity

During the development of the LC-MS/MS method it was observed that the CNCbl standard samples of the same concentration provided a stable signal when they were measured on the same day, while strong deviations were observed between the samples measured on different non-sequential days. Fig. 14 shows the results of three measurements obtained for the samples measured on three different days. The peak area of 500 nM CNCbl sample obtained in the second measurement was 1.6 times and 1.3 times higher than those from the first and third day of measurement, respectively. Subsequently, this phenomenon was investigated in detail to identify factors with an influence on the cobalamin signal intensity.

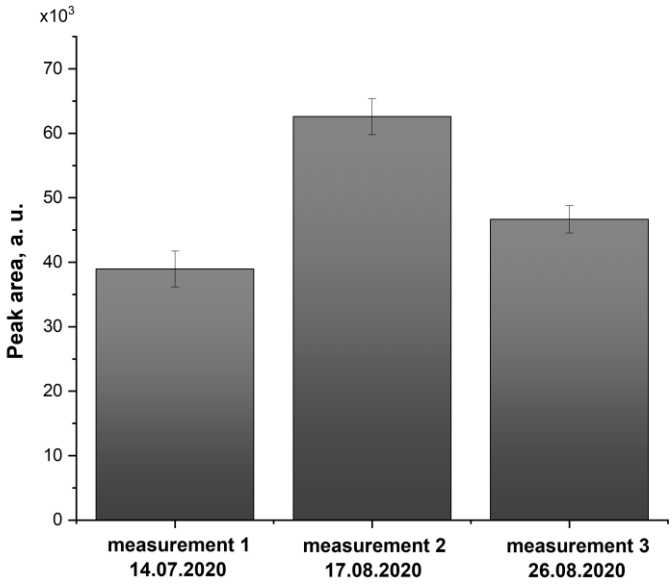

Fig. 14 Strong differences observed between measurements conducted on different days. The bar charts show the peak areas obtained from the 500 nM CNCbl standard measured on different days, error bars are standard deviations of the within-day technical triplicates

The influence of the ion source and mobile phase condition, concentration of formic acid in the mobile phase were investigated as the possible factors affecting the CNCbl signal intensity. For this purpose, a set of standards of various concentrations (100, 250, 500, 750 and 1000 nM) were prepared freshly from the stock and the peak area corresponding to each concentration was determined. To investigate the influence of the standard storage period on the recorded signal intensity a set of standard samples of the same concentrations stored in the dark at 4 °C for three months was also measured and the obtained peak areas were compared with those obtained for the fresh standards (Fig. 15).

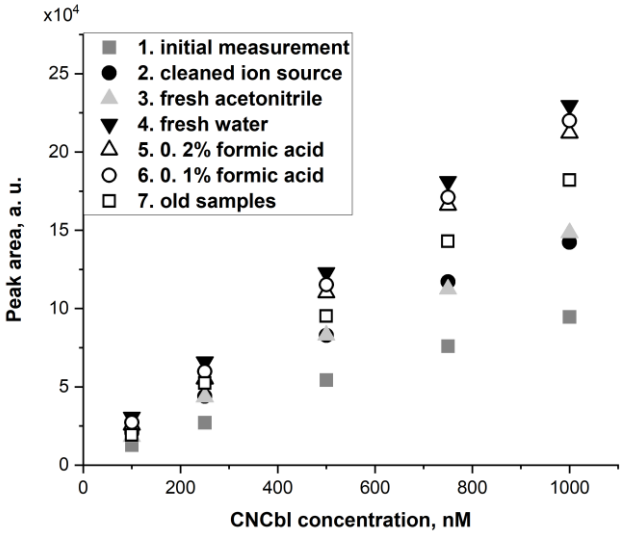

Fig. 15 Influence of the ion source, mobile phase, formic acid concentration in the mobile phase and sample storage period on the measured CNCbl signal in the concentration range between 100 and 1000 nM

The ion source condition was investigated as the first possible influence parameter. After the initial measurement was performed, the ion source was dismounted and cleaned according to the manufacturer instruction. After the sample row was measured again 1.5 times increase in peak area was detected for every analyzed concentration, which is why all following measurements were performed with the cleaned ion source.

The analysis of the mobile phase influence has shown that only water has a great effect on the CNCbl signal. As Fig. 15 shows, no peak area increase could be observed in the third measurement when fresh acetonitrile with 0.1% formic acid (mobile phase B) was used. On the contrary, the influence of the water storage time and conditions turned out to be great. Since water with 0.1% of formic acid used was used as a mobile phase A, the mixture was always prepared in a clear Schott glass bottle and usually applied for the measurement until the whole solvent was used. In the fourth measurement, when the mixture was prepared from the fresh analytic grade water bottle directly on the day of the measurement, an overall signal increase of 2.4 times compared to the initial measurement was detected. Moreover, a signal increase over 1.5 times in comparison to the third measurement (cleaned ion source, fresh acetonitrile) was observed, which is why fresh water was used for the following measurements.

0.1% of formic acid used in all previously described measurements in mobile phase was found to be the optimal concentration, since the increased formic acid concentration in measurement five caused slight decrease in the CNCbl signal, while the return to the initial concentration of 0.1% performed in the next measurement led to the overall signal increase. Since these conditions provided the most intensive signal during the measurement of fresh standards (measurement six), they were tested in combination with the three months old standards in order to estimate the influence of the standard storage period on the produced signal intensity.

When the system was kept under optimal conditions, even the three months old standards with possible degradation of the standard material provided higher peak areas (measurement seven) than those observed for the freshly prepared standards during the initial measurement. Nevertheless, significant differences in peak areas between the three months old (measurement seven) and fresh standards (measurement six) measured under optimal conditions were detected. Therefore, the ion source, the water storage period, formic acid concentration and the sample storage period were identified as the factors having a great influence on CNCbl signal.

After having identified the factors mostly influencing the obtained CNCbl signal, further investigations on the stability of CNCbl were performed. A standard row of the previously tested concentrations in the range of 100-1000 nM was prepared from the stock and serial measurements were performed on 4 consecutive days in order to investigate the stability of CNCbl standards (Fig. 16).

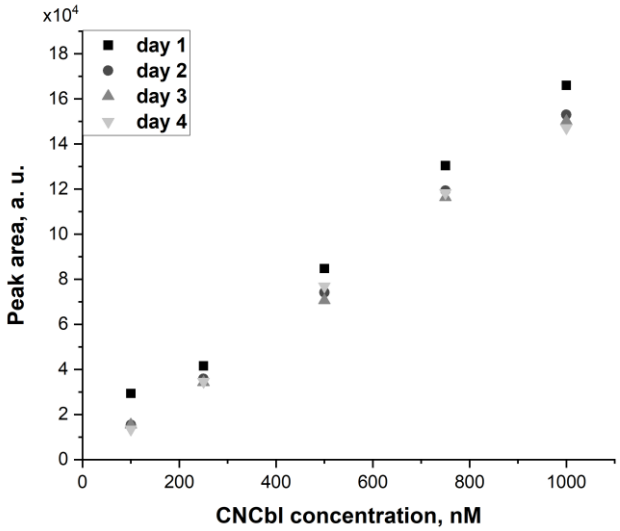

Fig. 16 CNCbl degradation in the standards. The standard row of five samples was prepared from the stock on day one, measured on four days and the changes in peak areas corresponding to each sample were observed

Although no significant peak area changes between day two and day four could be detected for all standards, a peak area decrease in comparison to day one was observed over the whole concentration range. A slight decline of 10 - 17% was measured over the range of 250 - 1000 nM vitamin B_{12}, while a drastic peak area decrease of over 55% was observed for the lowest concentration of 100 nM. To avoid standard degradation, the calibration standards were prepared freshly from the stock solution on the day of the analysis and used for quantification.

3.1.4 Validation of the developed LC-MS/MS method

Limit of detection (LOD), limit of quantification (LOQ), linearity, selectivity, accuracy, precision (repeatability and intermediate reproducibility) and robustness were selected as parameters for the validation of the developed LC-MS/MS method, the acceptance criteria are defined in Table 9.

Table 9 Acceptance criteria for the developed LC-MS/MS method of vitamin B_{12} analysis

Parameters	Acceptance criteria
LOD, nM	S/N ratio ≥ 3
LOQ, nM	S/N ratio ≥ 10
Linearity	R^2 >0.995 over the range of LOQ to 130%
Selectivity	no interfering peaks in the blanks in the target regions
Accuracy	90-110% recovery of CNCbl in the spiked samples
Precision: repeatability	RSD value of < 5.0% for peak area precision
Precision: intermediate reproducibility	RSD value of < 5.0% for peak area precision
Robustness	RSD value of < 5.0% for peak and retention time

LOD and LOQ. The instrumental LOD (S/N ratio ≥ 3) was determined to be 5 nM (Fig. 17B), lower concentrations did not produce peaks above the baseline noise level (Table 10, Fig. 17A).

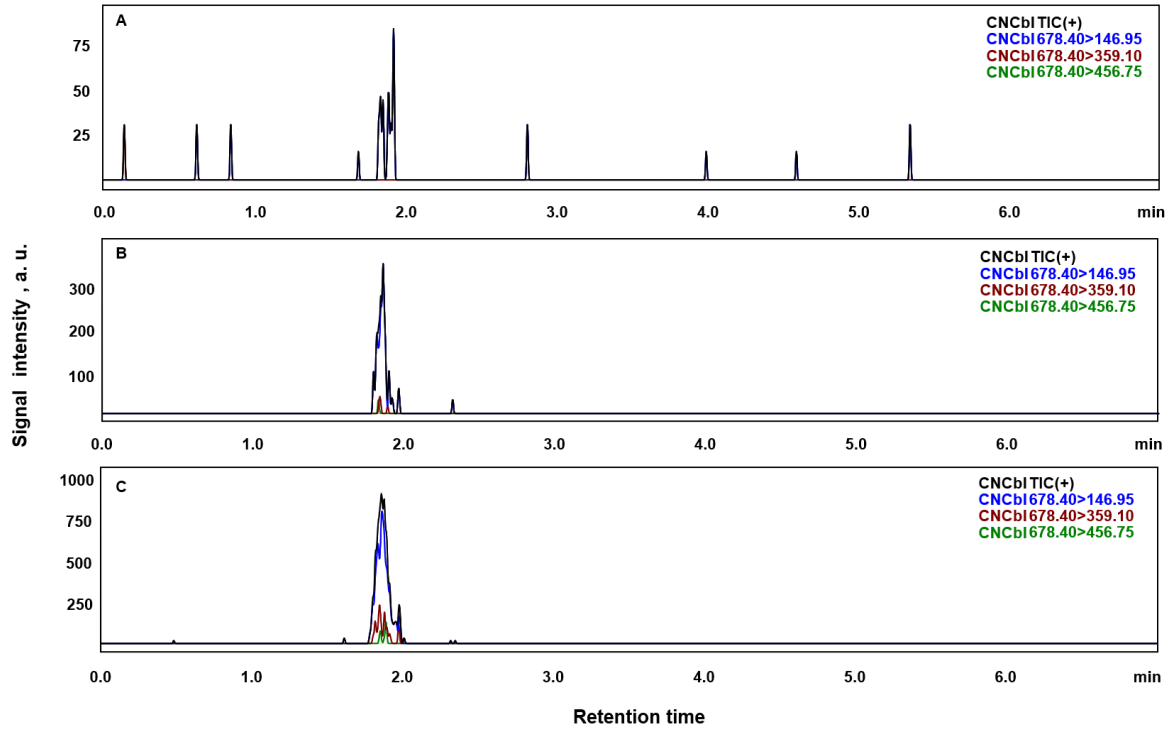

Fig. 17 Chromatograms obtained for different CNCbl concentrations. (A) 3 nM was identified to be under LOD, (B) concentration of 5 nM produced an integrable peak and was identified as LOD, (C) 15 nM was defined as LOQ

The acceptance criterium for LOQ (S/N ratio ≥ 10) was estimated as the lowest concentration with an acceptable reproducibility (relative standard deviation under 10%). LOQ was determined at 15 nM (Fig. 17C).

Table 10 Determination of the LOD and LOQ from CNCbl standard concentrations and their corresponding total ion current (TIC) peak areas expressed as the mean values of the measured triplicates and their standard deviations (SD) and relative standard deviations (RSD)

CNCbl concentration, nM	Peak area, a. u.	SD	RSD, %
1	ni*	-	-
3	ni	-	-
5	1605.7	365.5	22.8
8	2657	14.1	0.5

10	2867	328.8	11.5
15	5143.3	139.6	2.7
20	6329.7	407.9	6.5
25	8812.7	415.5	4.7
30	10076.3	161.6	1.6
40	14808	858.6	5.8
50	16917.7	392.1	2.3

*only noise signal was detected and the peak area could be not integrated (Fig. XY)

Linearity. The linearity of the method was estimated by plotting the TIC peak areas versus the corresponding standard concentrations. The developed method showed linearity over the concentration range between 20 and 2000 nM cyanocobalamin with an R^2 value of 0.9997 (Fig. 18).

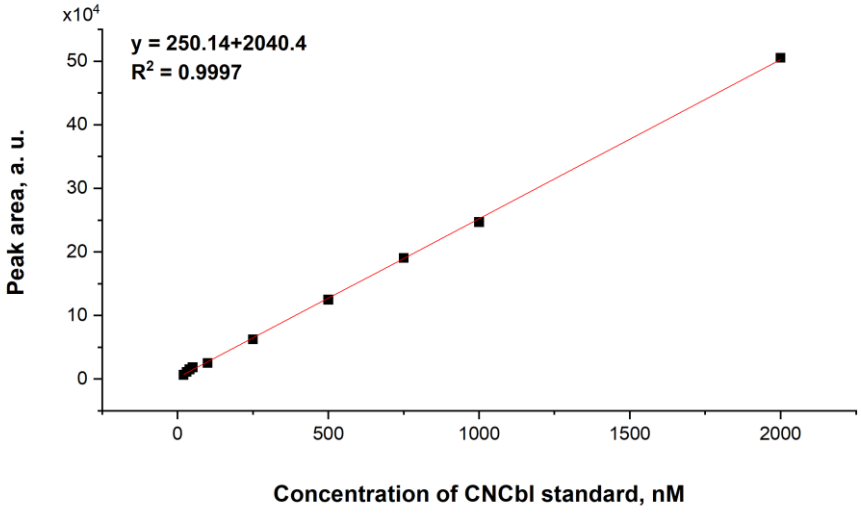

Fig. 18 Linearity plot of the developed method for vitamin B_{12} analysis. Calibration curve obtained from ten concentrations of the cyanocobalamin standard solution

Selectivity. The selectivity of the method was evaluated by comparing the chromatograms of the acetate buffer used for the extraction of cobalamin from the cells and cell matrix blanks with the buffer and cell matrix blanks spiked with cyanocobalamin standard. Since a cyanocobalamin-free cell extract for the cell matrix blank was required for this purpose, *E. coli* DSM 18039 was chosen as a matrix source. The comparison of the blanks with the spiked samples showed a high selectivity of the method, since no

peaks with characteristic masses and the retention time corresponding to cyanocobalamin could be detected in the blank chromatograms while cyanocobalamin was detected in all spiked samples (Fig. 19).

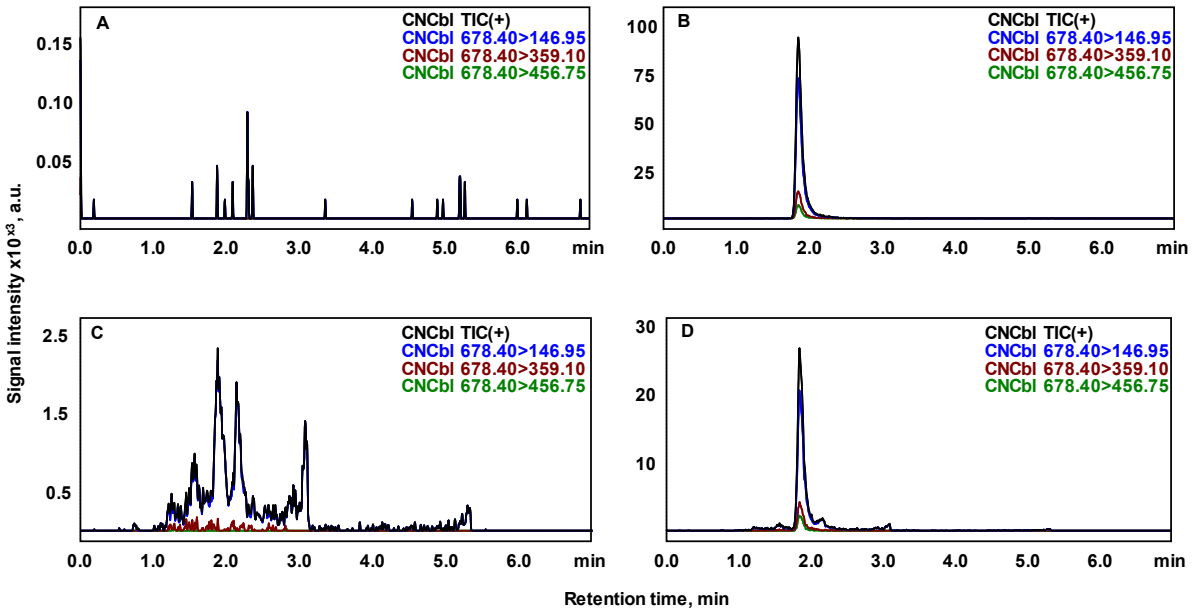

Fig. 19 Selectivity of the method to cyanocobalamin in the presence of the acetate buffer and cell matrix. Chromatograms corresponding to (A) buffer blank, (B) buffer spiked with CNCbl standard, (C) cell matrix blank obtained from the non-producing strain *E. coli* DSM 18039 and (D) cell matrix spiked with CNCbl standard are shown

Accuracy. The accuracy of the method was determined through recovery by spiking of five different commercially available vitamin B_{12} supplements with known amounts of CNCbl standard. The average recovery was estimated by determination of the peak areas of the samples before and after spiking. The method demonstrated high accuracy, since the average recovery from spiked samples was between 94.4% and 103.4% with an RSD under 5% (Table 11).

Table 11 Mean recovery in the samples of vitamin B12 supplements before and after spiking with CNCbl standard and their standard deviations (SD) and the relative standard deviations (RSD)

Vitamin B_{12} supplement	**Average recovery, %**	**SD**	**RSD, %**

B_{12} Vita-Energie drink bottles, Doppelherz	98.6	1.9	1.9
B_{12} drops, Ankermann	103.4	1.5	1.4
B_{12} Vita-Kick drink ampoules, tetesept	101.6	0.9	0.9
B_{12} drink bottles, Vitasprint	94.4	4.1	4.4
Vitamin B_{12} mini tablets, Doppelherz	103.2	2.1	2.0

Precision: repeatability. The repeatability of the method was evaluated by analyzing five standard samples of different concentrations measured in triplicates on the same day. The method demonstrated high repeatability showing relative standard deviation under 4% (Table 12).

Table 12 Within day repeatability of the method. Shown are concentrations of the used standards, the corresponding peak areas of TICs expressed as mean values of measured triplicates and their standard deviations (SD) and the relative standard deviations (RSD)

Standard concentration, nM	Mean peak area, a. u.	SD	RSD, %
100	24608.7	951.3	3.9
250	61931.3	1767.4	2.9
500	124047.0	3913.9	3.2
750	186133.3	1560.1	0.8
1000	245145.0	3354.6	1.4

Precision: intermediate reproducibility. The intermediate precision (within-laboratory day-to-day reproducibility) was determined by analyzing five standard samples of different concentrations on three consecutive days. The relative standard deviation of the intermediate precision value for all five concentrations was determined to be below 4% (Table 13).

Table 13 Day-to-day reproducibility of the method. Shown are concentrations of the used standards, the corresponding peak areas of TICs expressed as mean values of triplicates measured on different days and their standard deviations (SD) and the relative standard deviations (RSD)

Standard concentration, nM	Mean peak area, a. u.	SD	RSD, %

100	24235.3	803.8	3.3
250	61878.7	1699.2	2.7
500	125812.3	1596.5	1.3
750	188337.7	1846.5	1.0
1000	242772.3	3605.2	1.5

Robustness. The robustness of the method was evaluated through the introduction of deliberate variations in method and instrument parameters. The peak area and retention time mean values of the triplicate measurements obtained under the standard and altered conditions were compared. The flow rate, column oven temperature and formic acid concentration in the mobile phase were selected as test parameters (Table 14).

Table 14 Influence of the altered parameters on method robustness. Shown are the mean values of the TIC peak areas and retention times obtained under the standard and altered conditions and their relative standard deviations

Parameter		**Peak area x 10^4, a. u.**	**Mean, a. u.**	**RSD, %**	**Retention time, min**	**Mean, a. u.**	**RSD, %**
Flow rate, mL/min	0.4	128.8 ± 1.13	129.6 ± 1.15	0.9	1.84 ± 0.004	1.83 ± 0.02	1.3
	0.41	130.4 ± 5.12			1.81 ± 0.003		
Column temperature, °C	40	130.5 ± 2.95	130.19 ± 0.5	0.4	1.85 ± 0.0005	1.89 ± 0.05	2.5
	35	129.8 ± 4.88			1.92 ± 0.004		
Formic acid in mobile phase, %	0.2	131.2 ± 0.5	129.7 ± 2.0	1.6	1.85 ± 0.002	1.85 ± 0.0007	0.04
	0.22	128.3 ± 1.52			1.85 ± 0.002		

Among all tested parameters, the relative standard deviations observed after the introduced variations was below 2.5%.

The developed and validated method was used for all further qualitative and quantitative analyses performed in this work.

3.2 Quantitative comparison of vitamin B_{12} produced by previously described synthesizing strains

After the successful development of the sensitive vitamin B_{12} analytics, the method was applied for the analysis of vitamin B_{12} production in various bacterial strains well-known from the literature as vitamin B_{12} producers. Among the known bacterial strains, it was important to find the best producing organism. For this reason, the main goal of this investigation was to compare the vitamin B_{12} amounts produced by different synthesizing strains after the cultivation and preparation of the extracts under comparable conditions.

Overall, various bacterial species belonging to five different genera were selected (Fig. 20) and strains available at DSMZ at the time of the study were taken for the investigation.

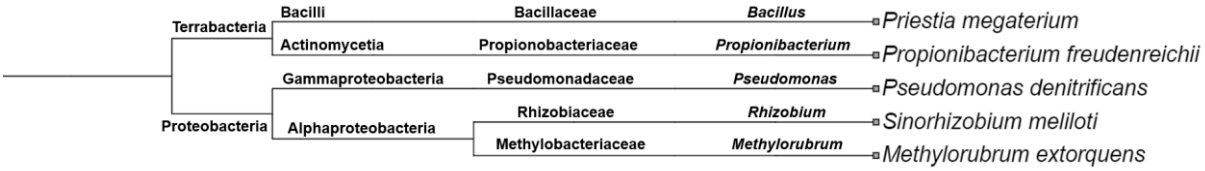

Fig. 20 Phylogenetic tree of the bacterial species analyzed for the ability to produce vitamin B_{12}

In case of *E. meliloti*, 9 different strains were available at DSMZ. To get a first impression of the growth behavior of these strains and to find the one demonstrating the fastest growth which can also accumulate high biomass and will be further used for vitamin B_{12} analysis, the growth curves of the strains in the mineral M9 medium were determined (Fig. 21).

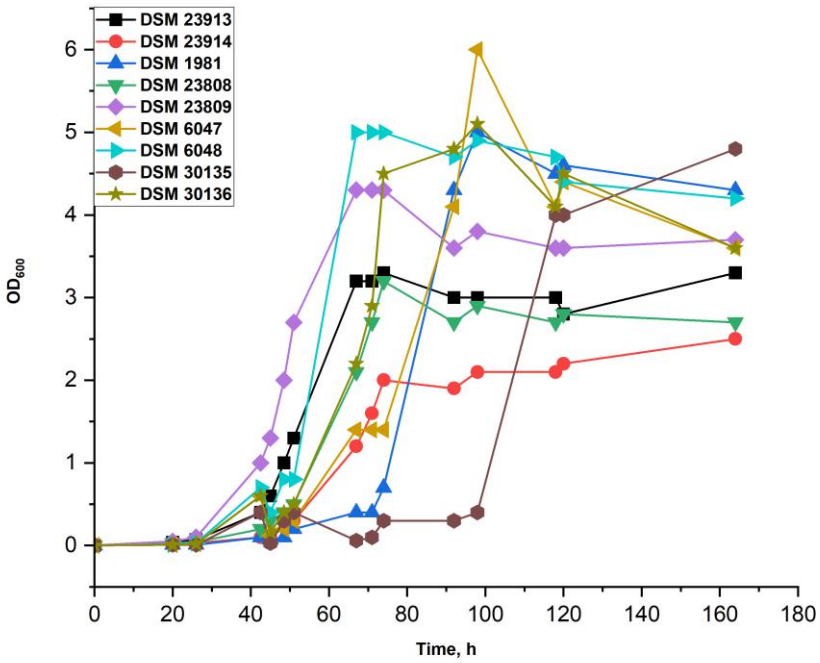

Fig. 21 Growth curves of *E. meliloti* strains available at DSMZ at the time of investigation. Single cultures were inoculated for this first preliminary investigation, the data points represent the results of the single measurements

Among the tested strains, the cultures of *E. meliloti* DSM 23809 and *E. meliloti* DSM 6048 demonstrated the shortest lag-phase and relatively high biomass accumulation. On the contrary, the maximum optical density measured in the cultures of *E. meliloti* DSM 23913, *E. meliloti* DSM 23914, *E. meliloti* DSM 23808 was relatively low. Although high optical density was observed in the cultures of *E. meliloti* DSM 6047, *E. meliloti* DSM 30135, *E. meliloti* DSM 30136, *E. meliloti* DSM 1981, these cultures demonstrated a longer lag-phase compared to the other cultures. For these reasons, *E. meliloti* DSM 23809 and *E. meliloti* DSM 6048 were selected for the further vitamin B_{12} analysis.

Altogether, seven vitamin B_{12}-producing strains previously described in literature and available at DSMZ at the time of the study were compared for the ability to produce vitamin B_{12}: *E. meliloti* DSM 23809 and *E. meliloti* DSM 6048, *B. megaterium* DSM 509 and *B. megaterium* DSM 2894, *P. denitrificans* DSM 1650, *M. extorquens* DSM 1338 and *P. freudenreichii* DSM 20270. In order to keep the cultivation conditions as similar as possible, all strains were propagated in the complex media recommended by DSMZ, only the minimal methanol medium described previously specifically for this strain was used for the production of vitamin B_{12} with *M. extorquens*. All strains were cultivated under aeration, except for

B. megaterium DSM 509, *B. megaterium* DSM 2894 and *P. freudenreichii* DSM 20270, when an anaerobic cultivation step was necessary for the production of vitamin B_{12} with these strains. Vitamin B_{12} extraction and purification was performed according to the same purification protocol and vitamin B_{12} content obtained from the cells was quantified using the previously developed LC-MS/MS method.

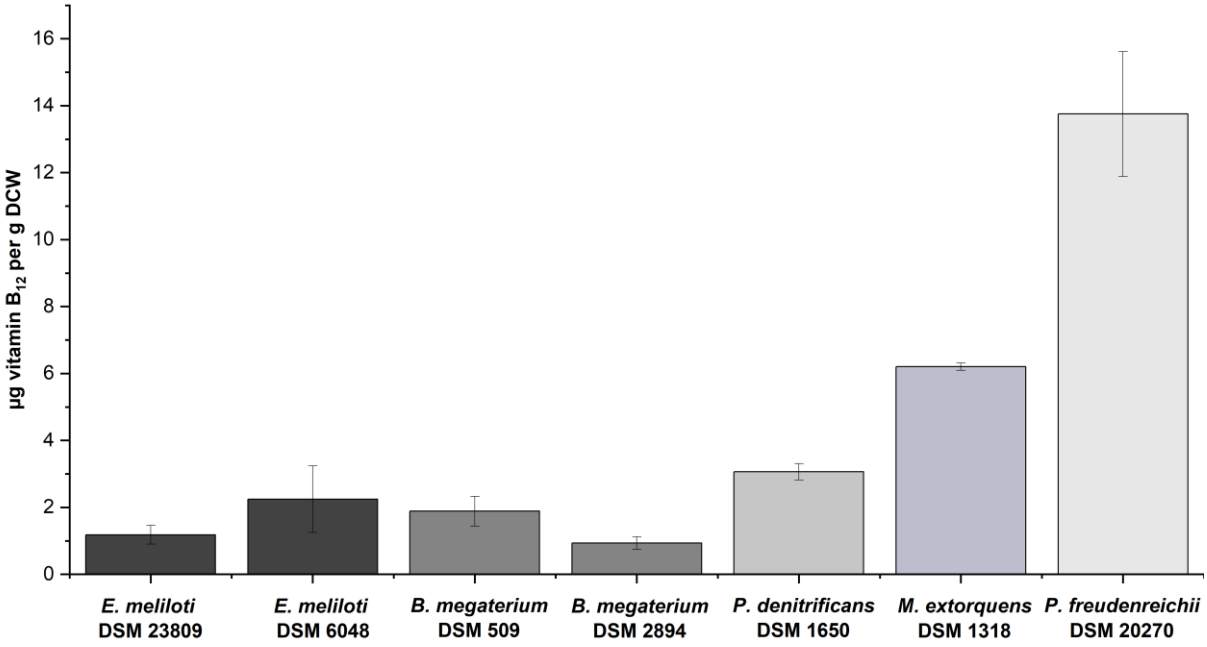

Fig. 22 Comparison of the vitamin B_{12} content produced by the selected known producers. The data are shown as the mean values and standard deviations of three technical replicates

As Fig. 22 shows, the amounts of vitamin B_{12} varied greatly between the investigated strains. The lowest amounts of 1.2 and 0.9 µg of vitamin B_{12} per g DCW were detected in the cultures of *E. meliloti* DSM 23809 and *B. megaterium* DSM 2894, respectively. Although higher amounts of 1.8 µg of vitamin B_{12} per g DCW and 2.2 µg of vitamin B_{12} per g DCW were measured in *B. megaterium* DSM 509 and *E. meliloti* DSM 6048, these amounts were still lower than 3 µg of vitamin B_{12} per g DCW detected in the culture of *P. denitrificans* DSM 1650. *M. extorquens* DSM 1338 and *P. freudenreichii* DSM 20270 were demonstrated to be the best producing strains, showing production of 6.2 and 13.8 µg of vitamin B_{12} per g DCW under the described conditions, respectively. In comparison to the vitamin B_{12} content detected in the culture of *P. denitrificans* DSM 1650, these results were 2.1 and 4.6 times higher, respectively.

Due to the high vitamin B_{12} amounts detected in *M. extorquens* DSM 1338 and *P. freudenreichii* DSM 20270, the strategies used by these strains for vitamin B_{12} synthesis were applied as a starting point for the identification of mutants and new candidates capable of vitamin B_{12} production.

3.3 Identification of mutants with enhanced vitamin B_{12} production

3.3.1 Development of the strategy for the selection of mutants capable of increased vitamin B_{12} production

Not only investigation of the vitamin B_{12} synthesis in the producing strains but also identification of mutants of these strains with the enhanced vitamin B_{12} production was a goal of this work. For this purpose, a strategy for the search of such mutants was proposed in this work (Fig. 23). Firstly, it was necessary to select bacterial strains which need vitamin B_{12} for their primary metabolism and are capable of its production. Vitamin B_{12} produced by the cells is used as a cofactor by the vitamin B_{12}-dependent enzymes essential for the cell metabolism and the cells are able to grow normally. In the next step, identification of the compounds which can either act as vitamin B_{12} antagonists or competitive inhibitors or analogues of vitamin B_{12} synthesis precursors should be identified. Then the cells should be treated with the identified compounds. Competitive inhibitors will bind vitamin B_{12}-dependent enzymes essential for the strain growth, while the analogues might replace the precursors of vitamin B_{12}, thus preventing vitamin B_{12} synthesis. This results in the inactivation of the essential enzymes leading to disruption of normal cell metabolism and cell death or at least growth reduction. Finally, selection of spontaneous mutants which are able to overcome this growth inhibitory effect and can grow in the presence of antagonists should be performed and checked for the produced amounts of vitamin B_{12}. Since spontaneous mutations in some cells can result in overproduction of vitamin B_{12}, it can replace the added agent and enable the mutant cells with the enhanced vitamin production to recover the enzyme activity and grow normally.

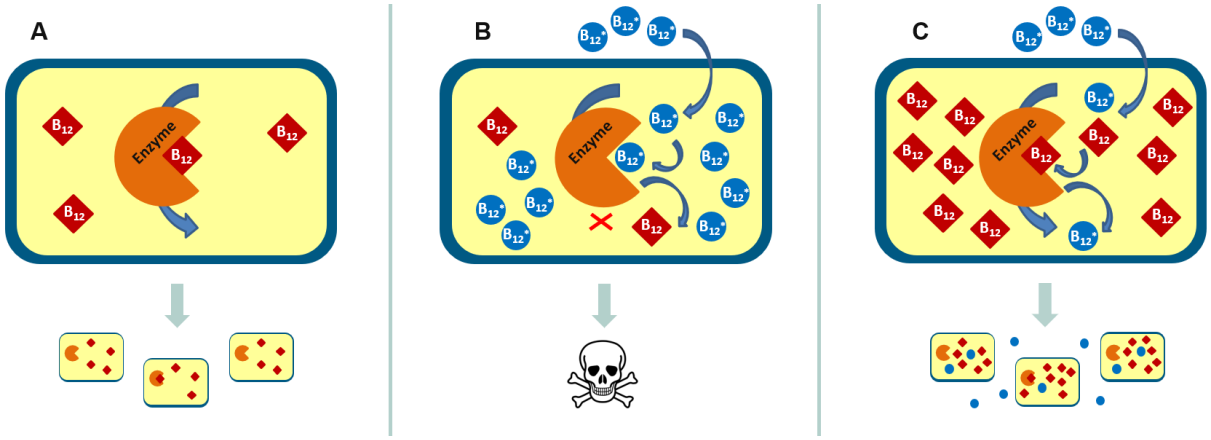

Fig. 23 Schematic representation of the strategy for the identification of the mutants with enhanced vitamin B_{12} production, B_{12}* stands for the vitamin B_{12} analogue or derivative. (A) Normal growth and reproduction of wild-type cells under standard conditions. (B) Added agent prevents biosynthesis or binds vitamin B_{12}-dependent enzymes which results in the cell death. (C) Vitamin B_{12} produced in high amounts is able to replace the added agent and reactivate the essential enzyme, the mutant cells are able to grow normally even upon inhibitor addition

To apply the introduced strategy for the search for the mutants with the enhanced vitamin B_{12} production, selection of the appropriate strain which meets the described criteria was performed in the first step.

3.3.2 Search for the wild-type microorganism matching the developed strategy for mutant identification

Since earlier research has shown that *M. extorquens* operates EMCP containing the vitamin B_{12}-dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase upon growth on methanol as sole carbon source, this microorganism was selected for further characterization. To validate the hypothesis that *M. extorquens* can be applied for the proposed mutant identification strategy, production of the active vitamin B_{12} in the cells during growth in minimal methanol medium was confirmed.

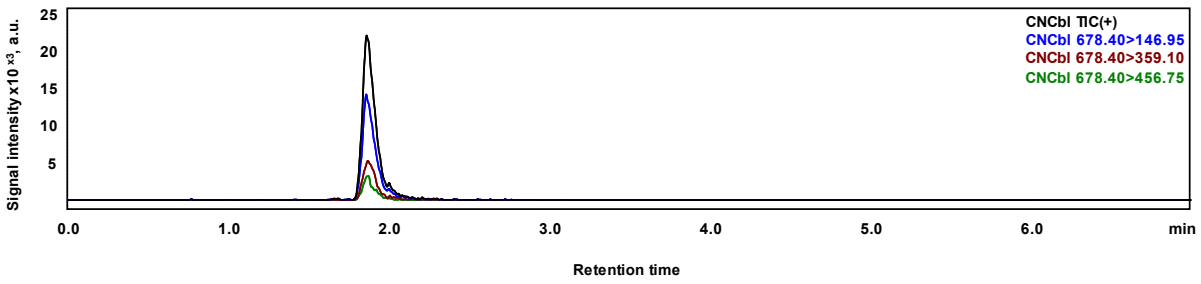

Fig. 24 Detection of vitamin B_{12} in the cell extract of *M. extorquens* DSM 1338. Shown is an MRM-chromatogram of the cell extract obtained from cultivation of *M. extorquens* in minimal medium with 0.5% (v/v) methanol

As Fig. 24 shows, the performed LC-MS/MS analysis of the compound purified from the cells demonstrated characteristic masses and retention time of cyanocobalamin. The detected parent ion signal with m/z of 678.40 and fragment ion signals with m/z 146.95 and m/z 359.10, which correspond

to $[DMBI + H]^+$ and $[DMBI + sugar + PO_3 + H]^+$, respectively, provided evidence of active vitamin B_{12} synthesis in *M. extorquens* DSM 1338.

For this reason, *M. extorquens* was chosen for the investigation of the proposed strategy and used in all further growth experiments with vitamin B_{12} derivatives and analogues.

3.3.3 Search for an appropriate selection agent

According to the proposed strategy, in the next step it was necessary to find a vitamin B_{12} analogue or derivative able to prevent the growth of *M. extorquens* on methanol as sole carbon source. For this purpose, analysis of available literature data was performed and compounds which have been previously described to act as vitamin B_{12} antagonists or inhibitors or prevent vitamin B_{12} synthesis in various organisms were chosen for further experiments. As a result, the following agents were selected for further growth experiments with *M. extorquens*: succinylacetone (SA) and levulinic acid (LEV) (Brumm and Friedmann 1981), vitamin B_{12}-monocarboxylic acid (Ford 1959), vitamin B_{12}-c-lactam (Matthews 1997; Matthews 1998; Stabler et al. 1991), vitamin B_{12}-dodecylamine (Bito et al. 2014). Since in *M. extorquens* the EMCP is necessary for glyoxylate regeneration during assimilation of C1-compounds in the serine cycle, it was expected that the identified agents would prevent *M. extorquens* growth only on methanol. For this reason, the influence of the chosen agents was tested parallelly in methanol and succinate medium and the agents causing growth inhibition only in methanol medium were selected for further investigation. Previous investigations on the growth behavior of *M. extorquens* have also demonstrated that difluorosuccinic acid (DFS) prevents the growth of the microorganism on methanol, while no growth inhibition by DFS was observed in the succinate medium (Pöschel et al. 2022), which is why DFS was also included in further tests.

Compounds with structural similarity to cobalamin tested as possible antagonists

As Fig. 25 shows, the structures of cyanocobalamin-b-monocarboxylic acid, cyanocobalamin-c-lactam and dodecylamine cyanocobalamin are very similar to that of cyanocobalamin (Fig. 1). Interestingly, other than in the well-known analogue pseudovitamin B_{12} (Fig. 1), these derivatives carry DMBI as the lower ligand in the structure like it is in the case of active vitamin B_{12}. Nevertheless, there are differences between the structures of the mentioned analogues and the cyanocobalamin molecule. A hydroxy group appears in the structure of cyanocobalamin-b-monocarboxylic acid instead of the amino group present

in the cyanocobalamin molecule. The presence of the lactam ring is the difference between the structures of vitamin B_{12} and its lactam analogue. The most striking difference between cyanocobalamin and its ribose 5‘-carbamate derivative is the presence of a long dodecylamine side chain in the structure of the latter.

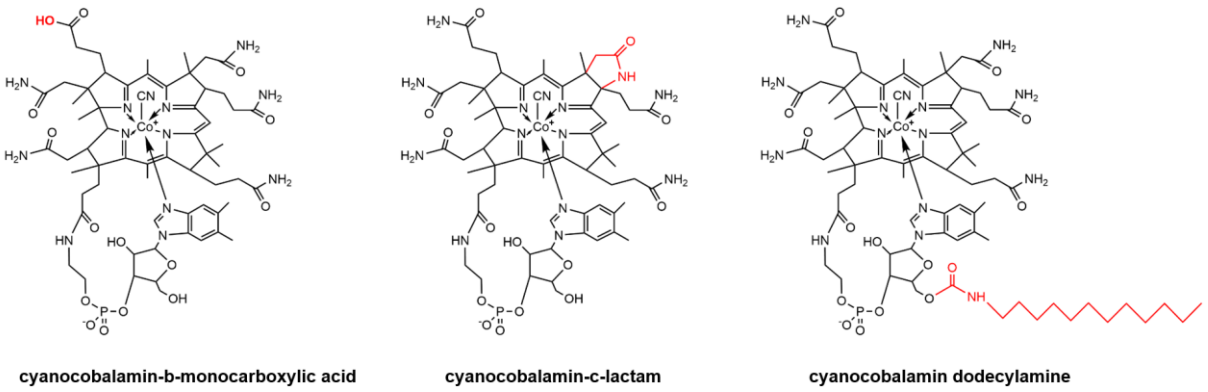

Fig. 25 Chemical structures of compounds tested as possible cyanocobalamin antagonists. Structural differences to cyanocobalamin are shown in red

Due to the structural differences between vitamin B_{12} and its derivatives shown in Fig. 25, it was decided to examine their influence on the growth of *M. extorquens* DSM 1338 in different media. Since cyanocobalamin-b-monocarboxylic acid, cyanocobalamin-c-lactam and dodecylamine cyanocobalamin resemble vitamin B_{12} in their structure, it was decided to test, whether these compounds can act as vitamin B_{12} inhibitors or antagonists according to the above-described strategy. In this case it was expected that the derivatives will bind ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase, vitamin B_{12}- dependent enzymes of EMCP, which is active in *M. extorquens* upon growth on methanol. This binding was expected to prevent the enzyme interaction with vitamin B_{12} and, therefore, inactivate these essential enzymes, which will lead to the cell death. Addition of vitamin B_{12} should compensate the inhibitory effect of the derivatives and restore normal growth of *M. extorquens* on methanol. On the contrary, no inhibitory effect of derivative addition was expected upon growth of *M. extorquens* on succinate.

To test this theory, the growth behavior of *M. extorquens* in the methanol and succinate medium upon addition of various concentrations of the vitamin B_{12} derivatives was compared and cyanocobalamin-b-monocarboxylic acid and cyanocobalamin-c-lactam were tested in the first step (Fig. 26). Since addition of 7.4 µM cyanocobalamin-c-lactam has already been reported to demonstrate cytotoxic effect in other

cell systems previously (Matthews 1998; Matthews 1997; Stabler et al. 1991), this concentration of cyanocobalamin-c-lactam and cyanocobalamin-b-monocarboxylic acid was selected to be applied to *M. extorquens* in this study.

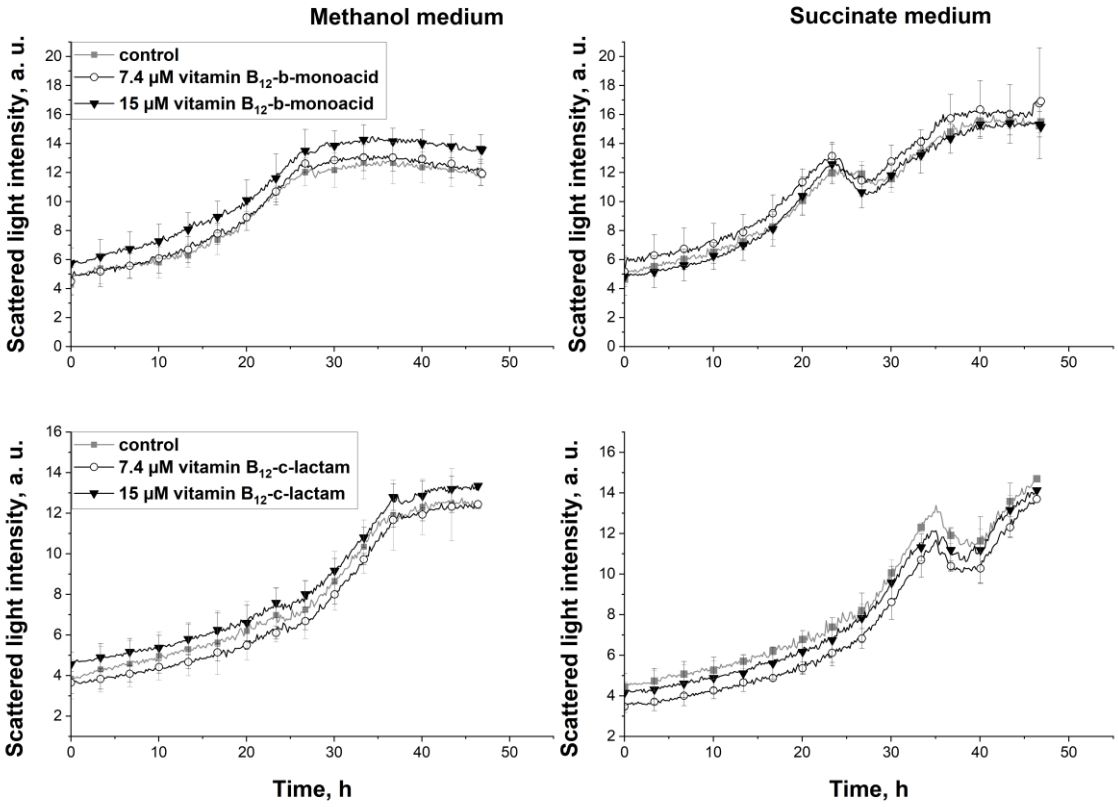

Fig. 26 Growth curves of *M. extorquens* DSM 1338 in methanol and succinate media under addition of 7.4 µM and 15 µM of vitamin B_{12}-b-monoacid or vitamin B_{12}-c-lactam. *M. extorquens* DSM 1338 grown in the same media without derivative addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

The results of the performed cultivations have shown that vitamin B_{12}-b-monoacid and vitamin B_{12}-c-lactam were not able to inhibit the growth of *M. extorquens* DSM 1338 neither in methanol nor in succinate medium. Moreover, a two-fold concentration increase to 15 µM vitamin B_{12}-b-monoacid or vitamin B_{12}-c-lactam did also not cause any inhibitory effect.

In the next step, the effect of dodecylamine cyanocobalamin addition was tested in order to check, whether it is capable of *M. extorquens* growth inhibition in methanol and succinate medium (Fig. 27).

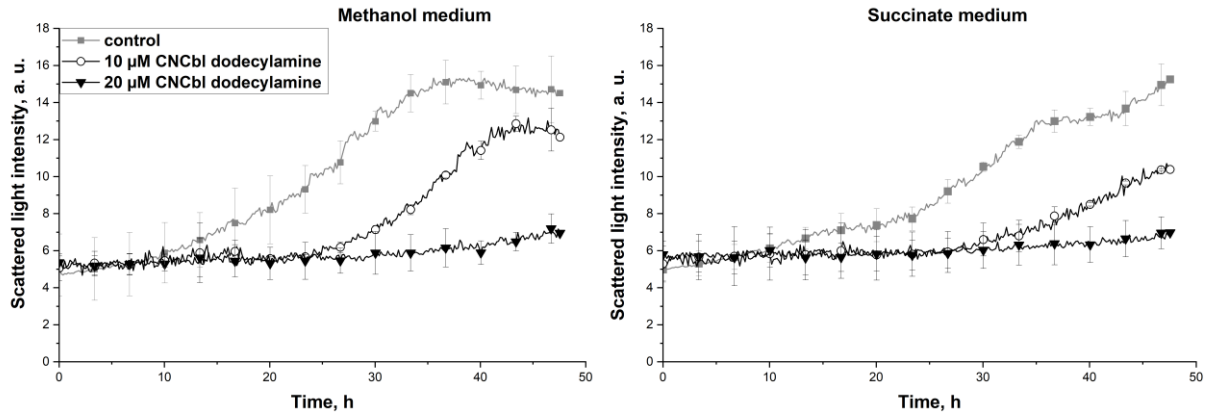

Fig. 27 Growth curves of *M. extorquens* DSM 1338 in methanol and succinate media under addition of 10 µM and 20 µM of cyanocobalamin dodecylamine. *M. extorquens* DSM 1338 grown in the same media without dodecylamine derivative addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

As the results of the experiment show, in comparison to the above tested derivatives, an inhibitory effect was observed and the growth inhibition of *M. extorquens* depended on the concentration of the added cyanocobalamin dodecylamine. Addition of 10 µM of the agent caused a remarkable *M. extorquens* growth inhibition in methanol medium, which was expressed in a prolonged lag-phase and the inability of the cultures to reach the maximum cell density comparable with the control cultures. When the concentration of the added CNCbl dodecylamine was increased to 20 µM, the growth of *M. extorquens* in methanol medium was almost stopped. Unexpectedly, the same trend was observed in the succinate medium. Nevertheless, it was decided to investigate the inhibitory effect of CNCbl dodecylamine upon vitamin B_{12} addition.

Since *M. extorquens* needs vitamin B_{12} only upon the growth on C1- substrates, further experiments were performed in methanol medium. According to the developed selection strategy, it was expected that the added vitamin B_{12} can replace CNCbl dodecylamine and, therefore, restore the normal growth of *M. extorquens*. To investigate this theory, various concentrations of vitamin B_{12} in its cyano form were added to the methanol medium upon simultaneous addition of CNCbl dodecylamine and the growth behavior of *M. extorquens* was examined.

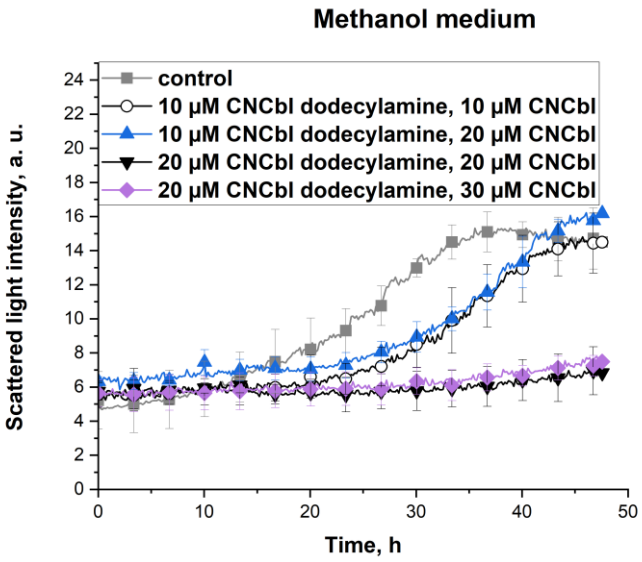

Fig. 28 Growth curves of *M. extorquens* DSM 1338 in methanol medium under addition of various vitamin B_{12} concentrations in the presence of CNCbl dodecylamine. *M. extorquens* DSM 1338 grown in the same medium without dodecylamine derivative addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

As Fig. 28 shows, the addition of vitamin B_{12} did not restore the normal growth of *M. extorquens.* In the presence of 10 µM of CNCbl dodecylamine, addition of 10 µM of vitamin B_{12} had no effect on the strain growth and the addition of the doubled amount of 20 µM of cyanocobalamin did not provide the growth of the cultures comparable with the control. Moreover, nearly no growth was observed upon addition of 20 µM of CNCbl dodecylamine even when 20 µM or 30 µM of vitamin B_{12} were supplied.

Due to these findings and the inhibitory effect observed in the succinate medium, cyanocobalamin dodecylamine was excluded from further investigations.

Compounds with structural similarity to cobalamin precursors tested as possible inhibitors of vitamin B_{12} synthesis

As Fig. 29 shows, unlike vitamin B_{12}-b-monacid, vitamin B_{12}-c-lactam or cyanocobalamin dodecylamine, LEV and SA resemble in their chemical structure δ-aminolevulinic acid, a precursor of vitamin B_{12} synthesis (Fig. 2).

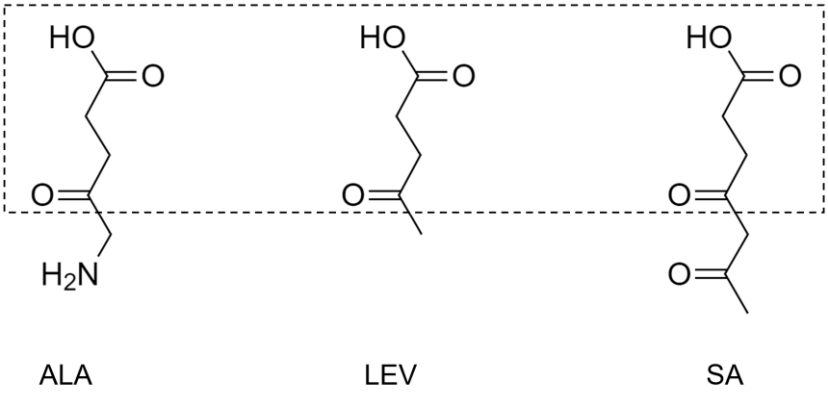

Fig. 29 Structure comparison of ALA, LEV and SA. The succinyl moiety similar in the structures of all compounds is marked with a dashed line

Due to these structural similarities, it was decided to investigate the ability of LEV and SA to replace ALA during cobalamin biosynthesis, thus, preventing vitamin B_{12} formation in *M. extorquens*. In this case, interruption of cobalamin synthesis should lead to the lack of vitamin B_{12} in the cell, B_{12}-dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase cannot work properly, which will lead to the cell death upon growth on methanol. To test this hypothesis, the influence of the addition of high concentrations of LEV or SA was examined (Fig. 30).

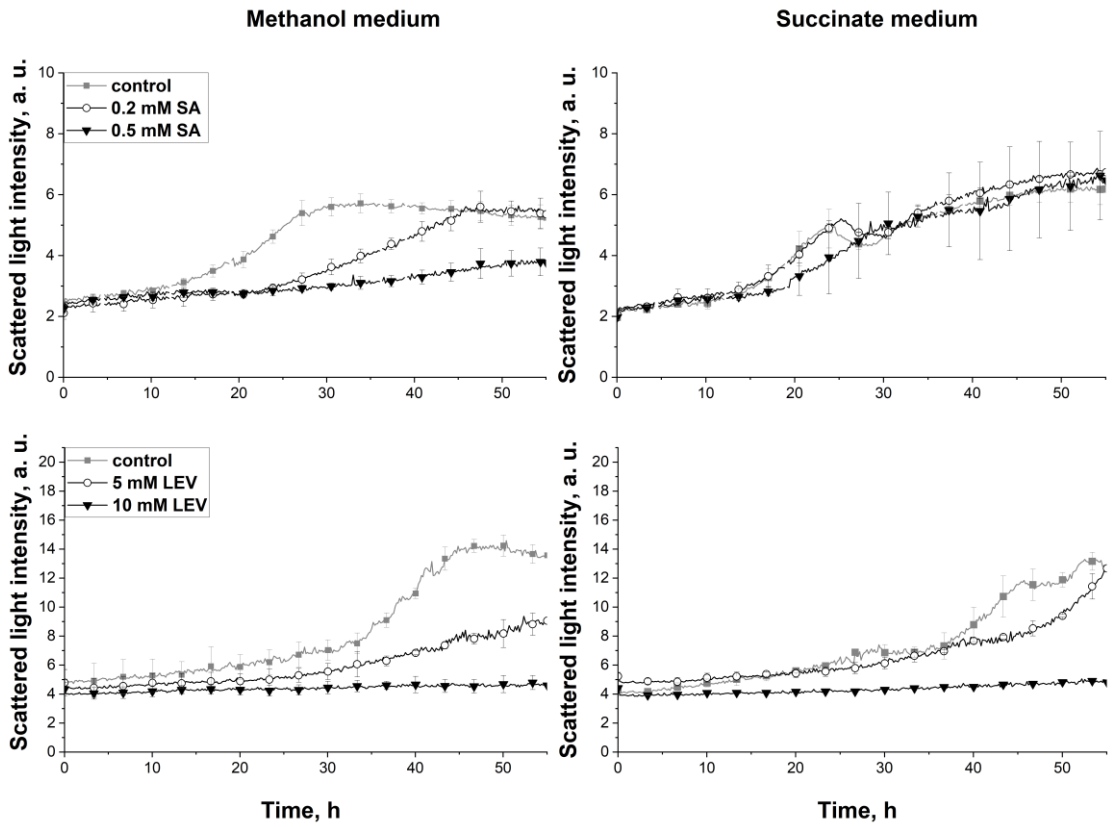

Fig. 30 Growth curves of *M. extorquens* DSM 1338 in methanol and succinate media under addition of of LEV or SA. *M. extorquens* DSM 1338 grown in the same media without LEV and SA addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

As shown in Fig. 30, a concentration-dependent growth inhibition was detected in methanol medium when SA was added: the control cultures reached the maximum cell density already after 30 h of growth, while a prolonged lag-phase was observed for the cultures grown in the presence of 0.2 mM of SA, where the maximum cell density was reached after 45 h. The inhibitory effect of 0.5 mM SA addition was even stronger, since these cell cultures did not achieve the growth comparable with that of the control cultures even after 50 h.

On the contrary, no growth inhibitory effect could be observed upon addition of 0.2 mM SA in succinate medium, while 0.5 mM SA caused a slight decrease of *M. extorquens* growth. Although no inhibition in succinate medium was expected, the observed effect was not so remarkable as in methanol medium.

For LEV, a dose-dependent inhibition of *M. extorquens* growth was detected in methanol medium. A prolonged lag-phase was observed in the presence of 5 mM LEV, while addition of 10 mM LEV completely prevented the growth of *M. extorquens* in methanol medium. The inhibitory action of LEV could be also demonstrated in the succinate medium and the growth inhibition was comparable with the effect in methanol medium: 5 mM LEV caused a slight growth decrease of *M. extorquens*, while no growth was observed upon addition of 10 mM LEV.

In spite of these unexpected findings, it was decided to further investigate the effect of LEV and SA. Since for mutant selection experiments it was necessary to find a vitamin B_{12} precursor analogue being able to interrupt vitamin B_{12} synthesis in *M. extorquens*, the addition of vitamin B_{12} should restore the normal growth of *M. extorquens* in methanol medium in this case. To investigate whether this theory works for the tested cobalamin precursor analogues, the growth behavior of *M. extorquens* in methanol medium upon simultaneous addition of each compound and vitamin B_{12} was examined. Since the strongest growth inhibition was detected for 0.5 M of SA and 10 mM of LEV, growth experiments with *M. extorquens* in methanol medium in the presence of 0.5 mM of SA and 10 mM of LEV and upon supplementation of various vitamin B_{12} concentrations were performed.

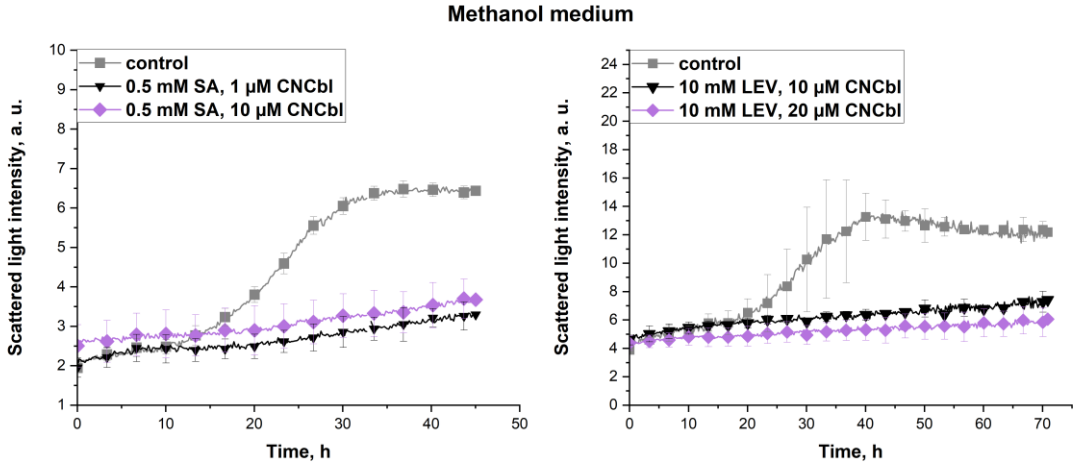

Fig. 31 Growth curves of *M. extorquens* DSM 1338 in methanol medium under addition of various vitamin B_{12} concentrations in the presence of SA or LEV. *M. extorquens* DSM 1338 grown in the same medium without cobalamin and precursor analogues addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

For both tested compounds, all tested vitamin B_{12} concentrations had no effect on the growth of *M. extorquens* in methanol medium.

In the experiment with SA, the control cultures achieved the maximum cell density after 35 h, while it was not the case for the cells grown in the presence of 0.5 mM of SA and 1 µM and 10 µM vitamin B_{12} addition even after 45 h and the experiment was stopped.

As Fig. 31 shows, there were also no significant differences between the growth curves obtained after addition of 10 µM and 20 µM vitamin B_{12} in the presence of 10 mM LEV: in both cases no culture growth in methanol medium was detected, while the control cultures demonstrated normal growth.

Due to the inability of vitamin B_{12} to restore the normal growth of *M. extorquens* in methanol medium when LEV or SA are added and to the presence of inhibitory effect of the compounds on the growth of *M. extorquens* in succinate medium, LEV and SA were excluded from the further investigations as inappropriate selection agents.

2,2-Difluorosuccinic acid

Other than in case of the above-described vitamin B_{12} derivatives or precursor analogues, DFS has a completely different chemical structure (Fig. 32).

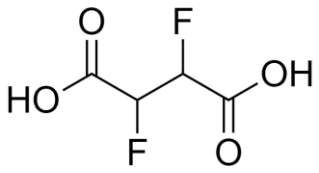

Fig. 32 Structure of DFS

Nevertheless, earlier investigations on the toxicity of DFS have shown that when 10 mg/L DFS was added to methanol medium, *M. extorquens* growth was completely inhibited, while the growth on succinate as a carbon source was not affected by the addition of DFS (Pöschel et al. 2022). As an active EMCP is required for the utilization of methanol in *M. extorquens*, while it is not essential during the growth on succinate, it was proposed that the DFS mode of action can cause an inhibition of the EMCP. Since EMCP carries the vitamin B_{12}-dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase, it was decided to test, whether vitamin B_{12} addition can restore the activity of EMCP and enable normal growth of *M. extorquens*. For this purpose, the growth behavior of the strain was examined in methanol medium upon addition of 10 mg/L DFS with simultaneous vitamin B_{12} supplementation (Fig. 33).

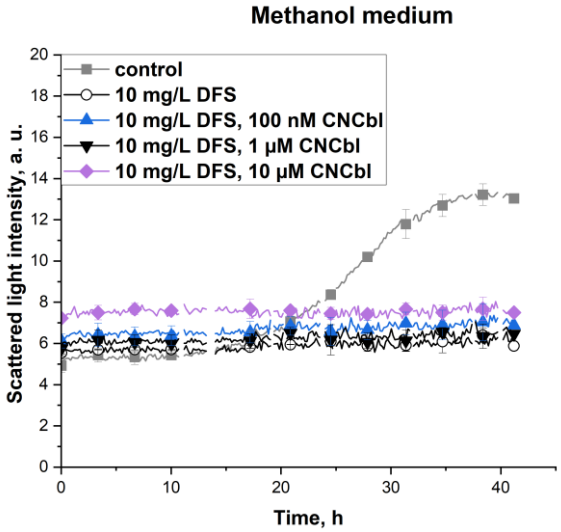

Fig. 33 Growth curves of *M. extorquens* DSM 1338 in methanol medium under addition of various vitamin B_{12} concentrations in the presence of 10 mg/L DFS. *M. extorquens* DSM 1338 grown in methanol

medium without DFS addition was used as control. The cultivations were performed in BioLector microbioreactor, the data points represent the mean values and standard deviations of three biological replicates

The results of the performed cultivations show that the applied concentration of DFS completely inhibited the growth of *M. extorquens* in methanol medium. Three concentrations of vitamin B_{12} were tested for their ability to restore the growth of the strain starting from the low concentration of 100 nM and increasing the added CNCbl concentration up to 10 µM. As Fig. 33 shows, no growth of *M. extorquens* in methanol medium could be observed for the tested vitamin B_{12} concentrations upon DFS addition. Since the tested concentration of 10 µM CNCbl is very high and still does not provide the growth of *M. extorquens* under tested conditions, it was assumed, that the inhibitory effect of DFS is not related to vitamin B_{12} in *M. extorquens,* which made this compound inappropriate for the mutant selection.
Due to the lack of an appropriate screening agent, the proposed strategy was not further followed and experiments on the selection of mutants with enhanced vitamin B_{12} synthesis were not performed.

3.4 Identification of new vitamin B_{12} producers via the search for homologues of *bluB/cobT2* fusion gene from *P. freudenreichii*

P. freudenreichii is a well-known vitamin B_{12}-synthesizing strain and production of high amounts of vitamin B_{12} by *P. freudenreichii* DSM 20270 was shown in this work (Fig. 22). The capability of *P. freudenreichii* to produce such high amounts of nearly exclusively the active form of vitamin B_{12} was demonstrated to be the result of the fusion protein BluB/CobT2 (Deptula et al. 2015). Therefore, to identify new vitamin B_{12}-producing bacterial strains, it was decided to search for microorganisms with a *bluB/cobT2* fusion gene and to investigate them for their vitamin B_{12} production capabilities.

3.4.1 Identification of strains containing a *bluB/cobT2* fusion gene

The first step of this research was to look for microorganisms carrying the fusion protein BluB/CobT2 encoded by the *bluB/cobT2* fusion gene. For this purpose, the protein sequence of the BluB/CobT2 fusion protein (GenBank: CBL56167.1, protein sequence in Appendix 3) from *P. freudenreichii* was used for the BLAST search in the NCBI non-redundant protein database. From the resulting list of 1000 queries sequences showing an identity of more than 50% to the BluB/CobT sequence from *P. freudenreichii* were chosen. From the resulting list of 338 sequences, several non-pathogenic organisms (risk group 1) were randomly selected and the protein sequences from *C. indicus, Y. lutea, Terrabacter* sp., *Blastococcus* sp., *R. antarctica* (protein sequences in Appendix 4) were compared to that of *P. freudenreichii*.

The BluB/CobT2 sequences from the selected strains showed between 52.56% and 56.12% identity to BluB/CobT2 query sequence from *P. freudenreichii.* The BluB/CobT2 fusion protein from *P. freudenreichii* is 626 amino acids long, while the gene products from the selected candidates were shorter reaching the length between 563 and 602 amino acids. To identify which regions are responsible for the differences, the sequences were aligned using ClustalW online tool (full alignment in Appendix 5). Besides some differences in the C- and N-terminal parts of the candidate protein sequences (full alignment in Appendix 5), the alignment has shown that the spacer region located in BluB/CobT from *P. freudenreichii* between amino acid residuals between 258 and 293 is shorter in all selected candidate strains (Fig. 34).

```
P. freudenreichii CBL56167.1                241 AWSHKLPLDQVVMTDRWPDNGPEPPVSALAGMAPAEPVESLITRPIPDDPSSVLWSDVHA  300
Terrabacter sp. 28 KJK13062.1               219 GWSRRLPLEQVVMRERWTERD------------------------------APTSHLRA  247
Blastococcus sp. DSM 44272 WP_089338383.1   220 GWSRRLPLRDVVLADRWPDEAG-A--------------------------PPPPPSRLRA  251
R. antarctica WP_245703061.1                232 AWSRRLPVEDVLLHDHWPHADQPARP------------------------ASHLKVTA  265
Y. lutea WP_141927336.1                     224 AWSRKAPLDDVVLQERWPDDSAPPA--------------------------PPTSHLRG  256
C. indicus WP_115921595.1                   224 AWSRKAPLDDVVLQERWPHDSAPPA--------------------------PPTSHLRG  256
```

Fig. 34 Alignment of the sequences from *P. freudenreichii* and selected candidates, a part corresponding to the region between amino acid residuals 241 and 300 from *P. freudenreichii* containing the spacer region and the aligned sequence regions from the selected candidates are shown. Identical residues are marked in grey

Overall, various bacterial species belonging to five different genera were among the selected candidates (Fig. 35). Only *R. antarctica* belonged to the order Propionibacteriales which also includes *P. freudenreichii*, while other selected strains belonged to the orders Geodermatophilales and Micrococcales.

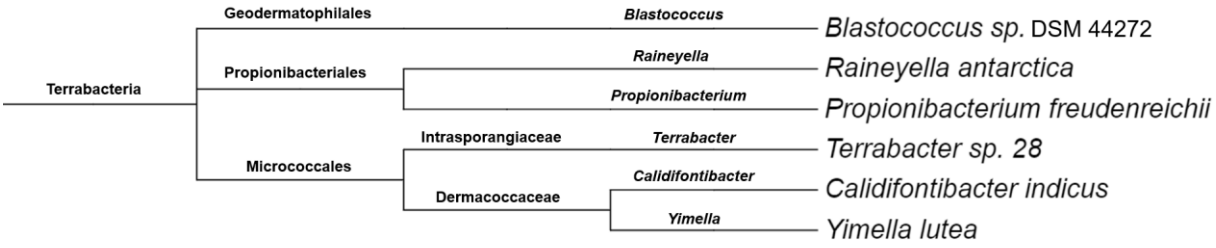

Fig. 35 Phylogenetic tree of the candidate species selected for the analysis of vitamin B_{12} production

In the next step, strains available at DSMZ at the time of the study were ordered and 6 microorganisms were taken for the further investigation: *Terrabacter* sp. DSM 102553, *Terrabacter* sp. DSM 102554, *C. indicus* DSM 22967, *R. antarctica* DSM 100494, *Y. lutea* DSM19828 and *Blastococcus* sp. DSM 44272.

3.4.2 Determination of vitamin B_{12} and pseudovitamin B_{12} concentrations produced by the selected strains

The developed LC-MS/MS method was applied to investigate the ability of the identified strains to produce cobalamins. For this purpose, the extracts obtained from the cells after four days of cultivation

in the complex media recommended by DSMZ were analyzed and the obtained results were compared with the chromatograms of the reference compounds.

Since it was important to distinguish between the active vitamin B_{12} and pseudocobalamin, the cell extracts were monitored for the presence of both forms. The analysis was performed in the MRM mode, since it allows the most sensitive determination of the target compounds. Moreover, due to the defined parent ion and fragment ion masses, a better differentiation between the active cobalamin and pseudocobalamin is possible.

The spectrum of the cyanocobalamin reference compound demonstrated a characteristic peak with the retention time of 1.77 min and parent ion with m/z 678.40 and fragment signals with m/z 146.95 [DMBI + H] $^+$, m/z 359.10 [DMBI + sugar + PO_3 + H] $^+$ and m/z 456.75. The peak identified in the extract of pseudocobalamin-producing *L. reuteri* demonstrated the retention time of 1.4 min and a parent ion with m/z 672.75 and fragment signals with m/z 136.05 [adenine + H] $^+$ and m/z 348.05 [adenine + sugar + PO_3 + H] $^+$ (Fig. 36).

LC-MS/MS analysis of the cell extracts of *Terrabacter* sp. DSM 102554, *R. antarctica* DSM 100494 and *Blastococcus* sp. DSM 44272 revealed no production of the active cobalamin (chromatograms in Appendix 6). Since the focus of this work was synthesis of the active form of vitamin B_{12}, additional analysis of these strains was not performed and they were excluded from the further investigations.

On the contrary, cobalamin production was detected for *Terrabacter* sp. DSM 102553, *C. indicus* DSM 22967 and *Y. lutea* DSM 19828 after cultivation in PP, TSYE and GYM-medium, respectively. Moreover, the active form was prevalent in the cell extracts of these strains since the identified product demonstrated the same retention time and parent and fragment ion masses as those detected in the cyanocobalamin standard solution (Fig. 36).

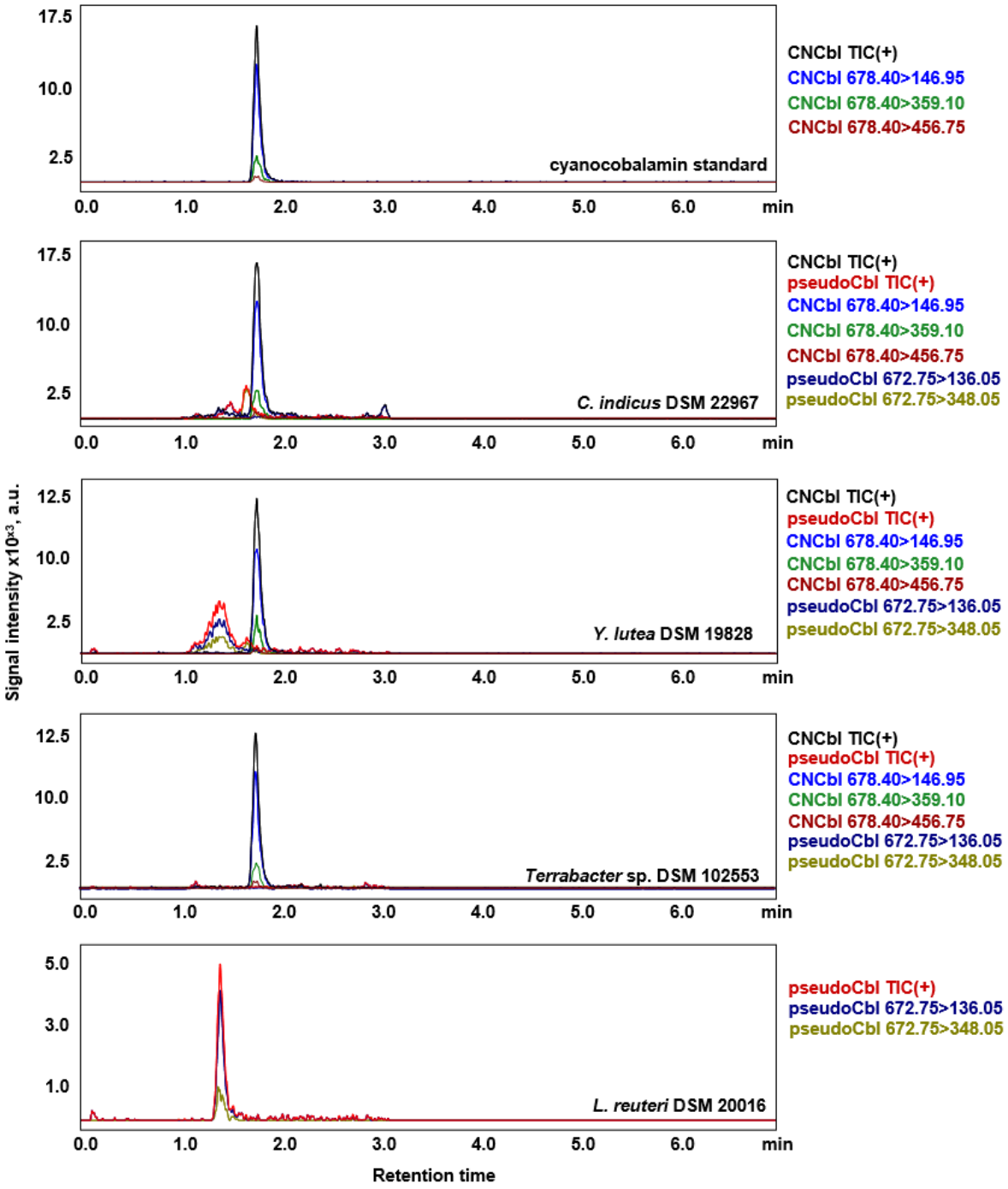

Fig. 36 Identification of the active and inactive cobalamin forms in the cell extracts of the selected producing strains. Shown are representative LC-MS/MS chromatograms of the cyanocobalamin reference compound, cell extracts of the selected strains and of the pseudocobalamin produced by *L. reuteri* used instead of the commercially unavailable pseudocobalamin standard

Active vitamin B_{12} was exclusively produced by *Terrabacter* sp. DSM 102553, since no other products were detected in the chromatogram of this strain. In contrast to *Terrabacter* sp. DSM 102553, minor

peaks corresponding to products other than active vitamin B_{12} were also detected in the cell extracts of *Y. lutea* and *C. indicus*. Due to the characteristic retention time and fragmentation pattern, the second minor peak in the chromatogram of *Y. lutea* was identified as pseudovitamin B_{12}. Additionally, an unidentified peak was detected in the chromatogram of *C. indicus* since its retention time and ion masses did not correspond to those of cobalamin and pseudovitamin B_{12}.

3.4.3 Investigations on the growth of the selected strains in complex media

To get a first impression of the growth behaviour of the vitamin B_{12}-producing strains, their growth curves in respective complex media recommended by DSMZ were recorded. For this purpose, *Y. lutea, C. indicus* and *Terrabacter* sp. DSM 102553 were cultivated in a Biolector® microbioreactor and the biomass accumulation was monitored online via scattered light measurement over 100 h (Fig. 37).

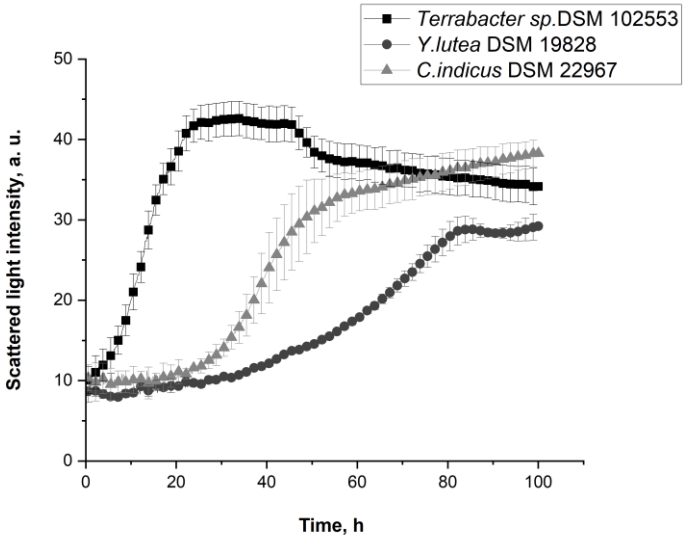

Fig. 37 Comparison of *Y. lutea, C. indicus* and *Terrabacter* sp. DSM 102553 growth curves in the GYM, PP and TSYE medium. The data points represent the mean values and standard deviations of three biological replicates

As Fig. 37 shows, remarkable differences in the growth behaviour of the investigated strains could be observed. The cultures of *C. indicus* demonstrated a lag-phase of 30 h and exponential growth for further 20 h, after that a linear biomass accumulation continued. The growth of *Y. lutea* under the tested conditions was characterized by a prolonged lag-phase if compared to *C. indicus* and *Terrabacter* sp. DSM 102553. Moreover, the maximum biomass monitored as scattered light intensity of 28 a. u. achieved after 80 h of cultivation was significantly lower than those observed for the cultures of *C. indicus*

and *Terrabacter* sp. DSM 102553. *Terrabacter* sp. DSM 102553 demonstrated the shortest lag-phase combined with the ability to achieve the highest cell density under these conditions reaching the maximum scattered light intensity of 42 a. u. after 24 h of cultivation.

As all the results show, in comparison to the other candidate strains, active vitamin B_{12} was the only form produced by *Terrabacter* sp. DSM 102553. Moreover, *Terrabacter* sp. DSM 102553 demonstrated the fastest biomass accumulation in the preliminary experiments. For these reasons, this strain was selected for the further investigations.

3.4.4 Investigation on the growth and active vitamin B_{12} production capability of *Terrabacter* sp. DSM 102553 in different media

Although production of the active vitamin B_{12} was detected in the PP medium, one aim of this work was to identify the optimal medium providing production of the highest vitamin B_{12} amounts with *Terrabacter* sp. DSM 102553. Moreover, since peptones are one of the most expensive components of microbial media, it was also important to find a low-cost medium suitable for cobalamin synthesis. For this purpose, identification of a cheap mineral medium enabling growth of *Terrabacter* sp. DSM 102553 was required in the first step. To achieve this, various defined mineral media were tested for their ability to provide the growth of *Terrabacter* sp. DSM 102553 (Appendix 7).

M9 medium with glucose as C-source was identified as appropriate for *Terrabacter* sp. DSM 102553 growth and further growth experiments were performed in this medium. Moreover, in order to improve cell density and thereby volumetric vitamin B_{12} productivity of *Terrabacter* sp. DSM 102553, two-fold concentrated PP medium was also selected and the growth of *Terrabacter* sp. DSM 102553 was investigated in the selected M9 medium, standard PP medium and two-fold concentrated PP medium. For this purpose, precultures grown in the standard PP medium were inoculated in PP, 2xPP and M9 medium and *Terrabacter* sp. DSM 102553 was cultivated in a Biolector® microbioreactor, the biomass accumulation was monitored online via scattered light measurement. (Fig. 38).

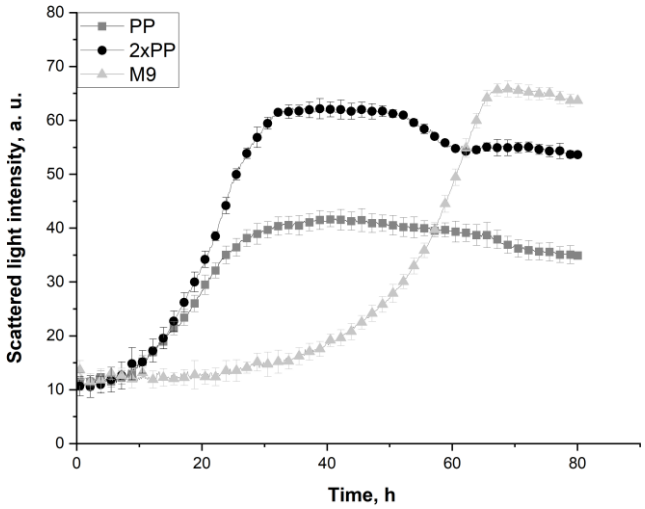

Fig. 38 Growth curves of *Terrabacter* sp. DSM 102553 in PP, 2xPP and M9 medium. The data points represent the mean values and standard deviations of three biological replicates

As shown in Fig. 38, the maximal cell density of 62 a. u. reached in 2xPP medium after 30 h of cultivation was about 1.5 times higher in comparison to 41 a. u. achieved in PP. Although a prolonged lag phase of 40 h was observed in M9 medium, the maximum cell density in M9 medium was comparable with that reached in 2xPP. Due to these results, further investigations were performed in order to improve the growth of *Terrabacter* sp. DSM 102553 in M9 medium and to make the lag-phase shorter.

In the first step, the impact of the preculture medium on the duration of lag-phase was assessed. Therefore, M9 medium was inoculated with the *Terrabacter* sp. DSM 102553 precultures grown in the standard PP medium or in M9 medium but no great differences in the lag-phase duration were detected (Appendix 8).

In the next step, the effect of each single component of the M9 medium on the growth of *Terrabacter* sp. DSM 102553 was evaluated. To investigate this, the concentrations of glucose, mineral salts, biotin, thiamine, trace elements, $MgSO_4$ and $CaCl_2$ were altered and media with the two-fold increased or reduced amount of each single component were tested. The standard M9 medium was used as reference and the growth of *Terrabacter* sp. DSM 102553 under the standard and altered conditions was investigated in the Biolector® microbioreactor.

The performed growth experiment has shown that biotin and thiamine have no influence on the growth of the strain (Appendix 9) which is why they were excluded from the medium composition in the further vitamin B_{12} quantification experiments.

On the contrary, the reduction of glucose concentration in the M9 medium by 50% resulted in a decreased biomass accumulation which was expressed in the decline of the scattered light intensity from 84 a. u. to 40 a. u., while the increase of glucose amount did not lead to a higher biomass formation (Fig. 39).

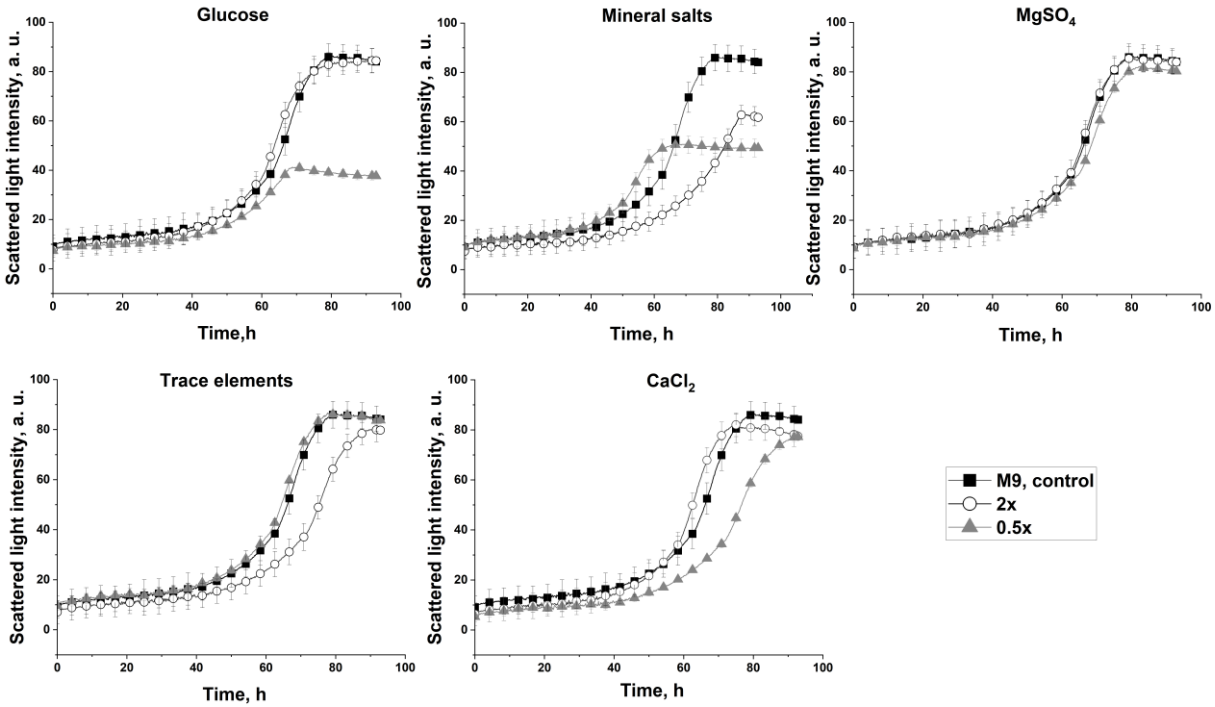

Fig. 39 Influence of the M9 mineral medium components on the growth of *Terrabacter* sp. DSM 102553. The data points represent the mean values and standard deviations of three biological replicates

In case of mineral salts, both increased and decreased amounts led to a lower accumulation of *Terrabacter* sp. DSM 102553 biomass, while higher mineral salts concentration resulted also in a longer lag-phase. A slight decrease in *Terrabacter* sp. DSM 102553 biomass formation was observed when $MgSO_4$ concentration was reduced by half, while an increased concentration had no effect on the strain growth. Opposite effects were observed for trace elements and $CaCl_2$, where reduction of biomass accumulation was detected in response to the two-fold increase and decrease of the components, respectively. Nevertheless, no remarkable biomass accumulation increase could be detected when the concentrations of $CaCl_2$ or trace elements were changed.

According to these results, it was decided not to introduce any further changes in the medium composition and to use the standard M9 medium without biotin and thiamine and the precultures grown

in the standard PP medium for the vitamin B_{12} quantification experiments which were performed in the next step.

The analysis of vitamin B_{12} production was performed in PP, 2xPP and M9 medium. For this purpose, 50 mL cultures were cultivated for four days in shaking flasks and 25 mL of the culture broths were applied for the vitamin B_{12} analysis. The obtained cell extracts were analyzed for the active vitamin B_{12} with the previously developed LC-MS/MS method. Due to the significant differences between the OD_{600} values, which have already been observed in the preliminary experiments (Fig. 38), the remaining 25 mL of the cultures were used for the dry cell weight (DCW) determination and the amount of cobalamin was expressed per g DCW for the relative comparison.

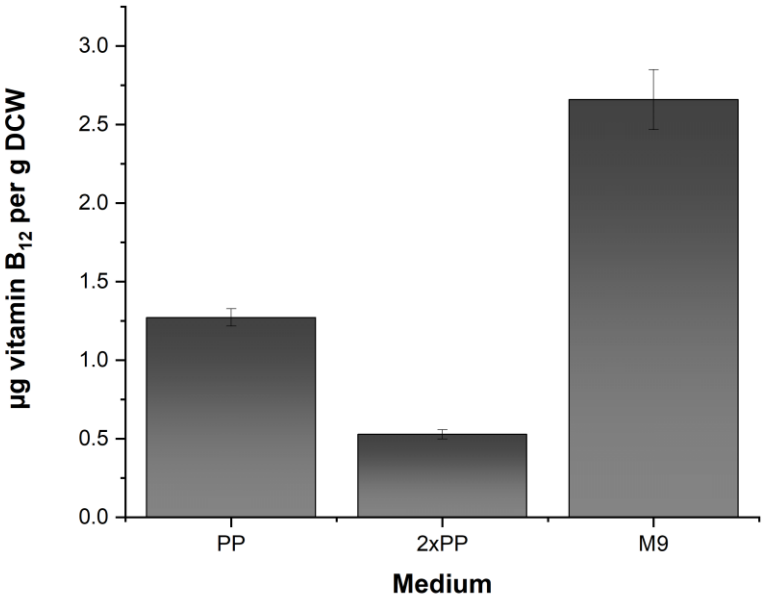

Fig. 40 Vitamin B_{12} content in the cells of *Terrabacter* sp. DSM 102553 cultured in PP, 2xPP and M9 medium. The data points represent the mean values and standard deviations of three biological replicates

Although higher biomass accumulation was observed in 2xPP in comparison to PP medium, 1.27 ± 0.05 µg vitamin B_{12} per g DCW detected in the latter medium were remarkably higher than the value measured in cells cultivated in 2xPP (Fig. 40). Among the three media, the highest vitamin B_{12} production was observed in M9 medium. The amounts of 2.65 µg of vitamin B_{12} per g dry cell weight DCW obtained with this medium were two-fold and five-fold higher than those produced in PP or 2xPP medium,

respectively. In comparison to PP medium, faster growth and higher cell density, but lower vitamin B_{12} concentrations were observed in 2xPP medium.

3.5 Identification of new vitamin B_{12} producers via the search for methylotrophs containing the EMCP

In contrast to *P. freudenreichii,* methylotrophs do not belong to bacterial species used for the industrial production of vitamin B_{12}. Nevertheless, there are several reports presenting data on cobalamin production in methylotrophic bacteria (Danilova et al. 2004; Ivanova et al. 2006; Trotsenko et al. 2001). Many methylotrophic microorganisms, for example *M. extorquens*, operate the EMCP upon growth on methanol as sole carbon source. Since this cycle contains the vitamin B_{12}-dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase, the ability of such methylotrophs to grow on methanol as sole C-source without vitamin B_{12} supplementation can indicate their capability of cobalamin production. Moreover, production of the active form of vitamin B_{12} by *M. extorquens* DSM 1338 upon growth on methanol has already been confirmed in this work (Fig. 24). For these reasons, the main aim of this part of the work was to identify microbial species like *M. extorquens* which have an essential function of vitamin B_{12} in the primary metabolism and to investigate them for their ability to produce vitamin B_{12}.

3.5.1 Identification of strains with probably essential EMCP

To identify microorganisms with a high vitamin B_{12} demand and potential for high vitamin B_{12} synthesis, it was necessary to find bacterial strains with essential function of the cobalamin-dependent mutases in the EMCP. In order to find microorganisms that possess an essential EMCP, a search for bacterial genomes carrying the Ccr-encoding gene and lacking the Icl-encoding gene was performed. The *M. extorquens* AM1 Ccr protein sequence (GenBank: ACS38140.1, protein sequence in Appendix 10) and the Icl of *E. coli* (Uniprot P0A9G6, Appendix 11) were used as query sequences.

Among all at the time of the search available bacterial genomes, 65 strains could be identified, which contained a Ccr-encoding gene and lacked an Icl-encoding gene. Analysis of literature data revealed that three out of the 65 strains, *X. autotrophicus* Py2, *Hyphomicrobium* sp. MC1 and *P. dioxanivorans* CB1190 should be able to grow on methanol as sole carbon source (van Ginkel and Bont 1986; Harder et al. 1973; Grostern and Alvarez-Cohen 2013), while *R. pomeroyi* DSS-3 was reported to be capable of ethanol utilization (González et al. 2003).

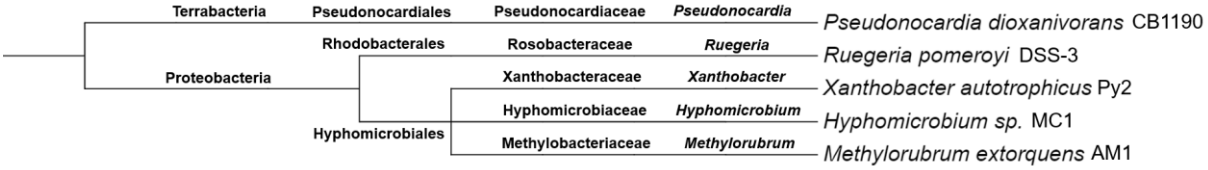

Fig. 41 Phylogenetic tree of the identified candidate species which probably contain an essential EMCP and were selected for the analysis of vitamin B_{12} production

As Fig. 41 shows, the identified bacterial species belong to four different genera. Among them, *X. autotrophicus* Py2 and *Hyphomicrobium* sp. MC1 belong to the order Hyphomicrobiales which also includes *M. extorquens*, while *P. dioxanivorans* CB1190 and *R. pomeroyi* DSS-3 belong to the orders Pseudonocardiales and Rhodobacterales, respectively.

As the EMCP should be only essential during growth with C1 or C2 carbon sources, further investigations focused on these five bacteria. For this reason, strains available at DSMZ at the time of the study were ordered and further investigations were performed with *X. autotrophicus* DSM 1618, *X. autotrophicus* DSM 432, *Hyphomicrobium* sp. DSM 3646, *P. dioxanivorans* DSM 44775 and *R. pomeroyi* DSM 15171.

3.5.2 Verification of active vitamin B_{12} production in the cells of selected strains

To investigate the ability of the selected strains to produce vitamin B_{12}, the cells were cultured for five days on agar plates with minimal medium, to which ethanol was added as a C-source for *R. pomeroyi* DSM 15171, while methanol was used as carbon source for all other analyzed strains. In contrast to previously reported data (González et al. 2003), we could not detect growth of *R. pomeroyi* DSM15171 in the respective medium, while *X. autotrophicus* DSM 1618, *X. autotrophicus* DSM 432, *Hyphomicrobium* sp. DSM 3646 and *P. dioxanivorans* DSM44775 were able to grow on agar plates with solid minimal medium containing 0.5% (v/v) methanol. For this reason, *R. pomeroyi* DSM15171 was excluded from the further investigation and only the cell extracts of *X. autotrophicus* DSM 1618, *X. autotrophicus* DSM 432, *Hyphomicrobium* sp. DSM 3646 and *P. dioxanivorans* DSM44775 were subsequently analyzed with the developed LC-MS/MS method for the production of cobalamins.

Since the aim of this investigation was to identify strains capable of active vitamin B_{12} production and exclude those producing the inactive form, the obtained cell extracts were analyzed for the presence of both forms. For this purpose, the results obtained for the extracts of the selected candidates were compared with the cyanocobalamin reference compound and with the cell extract of pseudocobalamin-producing *L. reuteri* DSM 20016.

Analysis of the cell extracts obtained after the cultivation in the minimal methanol medium demonstrated a peak at 1.77 min showing the parent ion signal with m/z of 678.40 and fragment ion signals with m/z 146.95 and m/z 359.10 present in the chromatograms of the samples from all strains (Fig. 42). These values correspond to the retention time and fragmentation pattern of cyanocobalamin and confirm the ability of the identified strains for the synthesis of active vitamin B_{12}. On the contrary, pseudovitamin B_{12} production could be excluded, since the characteristic peak with the retention time of 1.4 min and a parent ion with m/z 672.75 and fragment signals with m/z 136.05 [adenine + H] $^+$ and m/z 348.05 [adenine + sugar + PO_3 + H] $^+$ identified in the extract of *L. reuteri* was not detected in the cell extracts of the selected candidates. Moreover, no other unidentified peaks were present in the chromatograms of the analyzed candidate strains, which showed that the active vitamin B_{12} was the only cobalamin form produced by all selected bacteria.

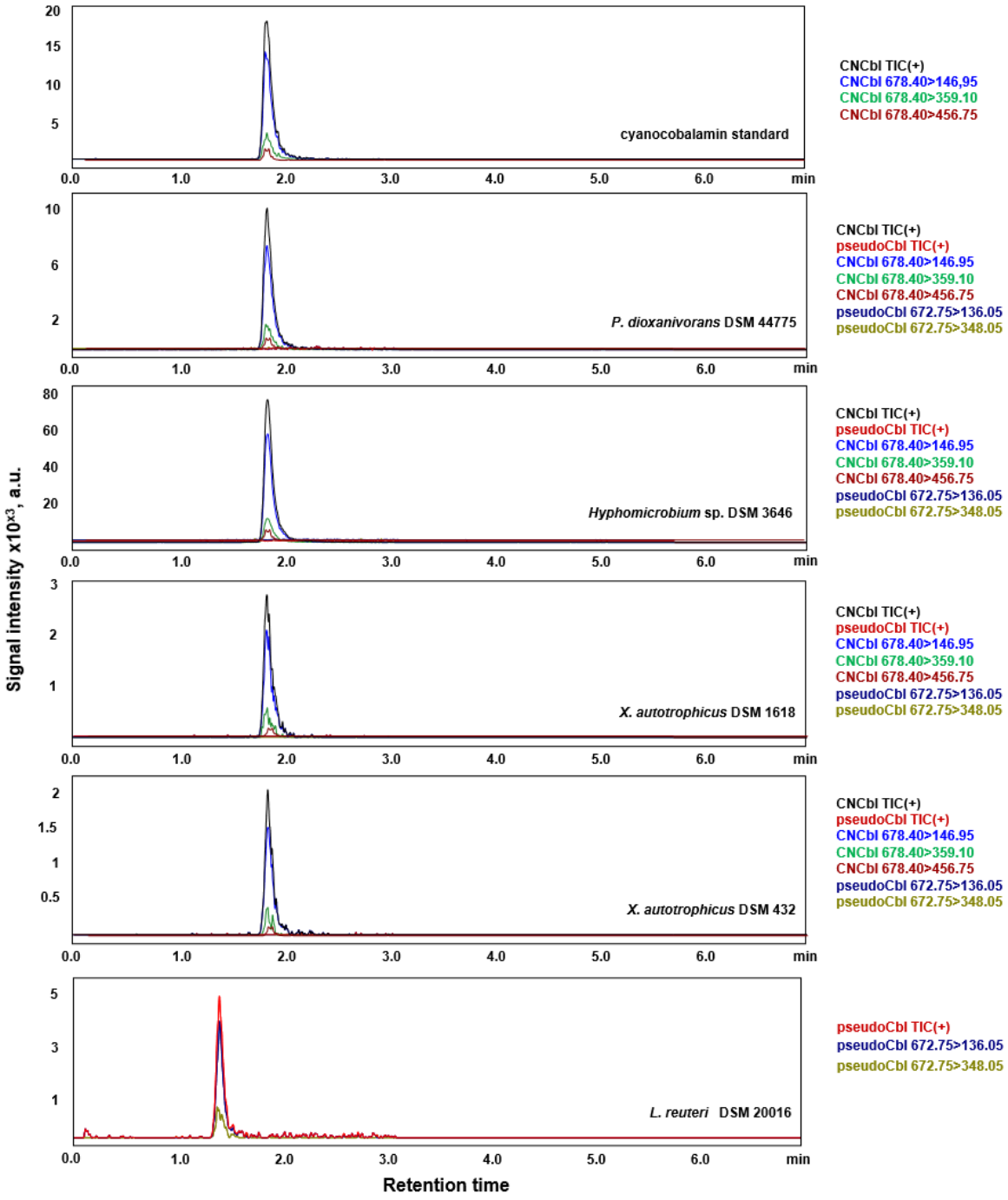

Fig. 42 Analysis of the cell extracts of the selected candidate strains for active and inactive cobalamin production. Shown are representative LC-MS/MS chromatograms of the cyanocobalamin reference compound, cell extracts of the selected strains and of the pseudocobalamin produced by *L. reuteri* used instead of the commercially unavailable pseudocobalamin standard

Due to these results, *X. autotrophicus* DSM 1618, *X. autotrophicus* DSM 432, *Hyphomicrobium* sp. DSM 3646 and *P. dioxanivorans* DSM44775 were selected for further investigation and quantitative vitamin B_{12} analysis was performed in the next step.

3.5.3 Quantitative comparison of vitamin B_{12} production capability of the selected strains

In order to identify the most efficient vitamin B_{12}-producing strain, the cells were grown on agar plates with minimal medium containing 0.5% (v/v) methanol, the obtained biomass was used for the preparation of the cell extracts, which were then used for the LC-MS/MS analysis and the cyanocobalamin peak areas obtained from the cell extracts were compared.

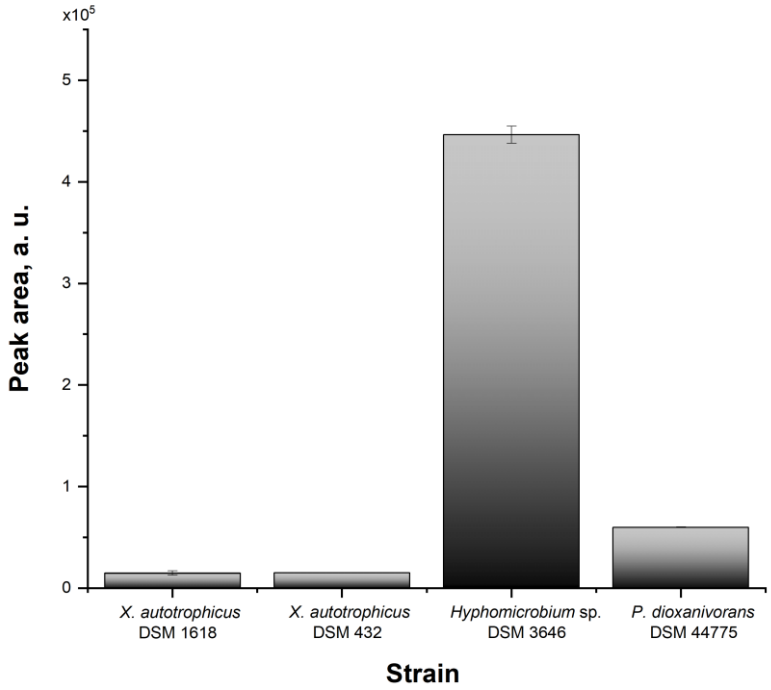

Fig. 43 Quantitative comparison of vitamin B_{12} produced by the investigated strains. Shown are the peak areas of the TICs of the cyanocobalamin detected in the respective cell extracts. Data are shown as mean values of technical replicates ± standard deviation (n = 3)

As Fig. 43 shows, although all bacteria were capable of vitamin B_{12} production, the peak areas varied greatly between the samples from the four strains. The extracts of the studied *X. autotrophicus* strains DSM 1618 and DSM 432 demonstrated comparable amounts of cyanocobalamin showing peak areas of 14784.67 ± 2107.42 and 15096.33 ± 336.52 a. u., respectively, which were remarkably lower than those detected for the other strains. The peak area corresponding to the cell extract of *Hyphomicrobium*

sp. DSM 3646 was 7.5 times higher than the peak area of *P. dioxanivorans* DSM 44775 cell extract and nearly 30 times higher than those determined for the cell extracts of *X. autotrophicus* DSM 1618 and *X. autotrophicus* DSM 432.

In addition to the highest vitamin B_{12} peak areas, only *Hyphomicrobium* sp. DSM 3646 was able to grow in liquid medium, while no growth in liquid medium was observed for *P. dioxanivorans* DSM44775, *X. autotrophicus* DSM1618 and *X. autotrophicus* DSM432. For these reasons, the latter microbial strains were excluded from the further study and only *Hyphomicrobium* sp. DSM 3646 was selected for the detailed investigations.

3.5.4 Quantitative comparison of vitamin B_{12} production by *M. extorquens* AM1 and the newly identified *Hyphomicrobium* sp. DSM 3646

Since the initial strain *M. extorquens* DSM 1338 has already demonstrated a high capability for cobalamin production (Fig. 24), while *Hyphomicrobium* sp. DSM 3646 showed good results in comparison to other newly identified strains (Fig. 43), it was decided to compare the strains for their ability to synthesize vitamin B_{12} after 6 days of growth in minimal methanol medium in order to find the best producing strain (Fig. 44).

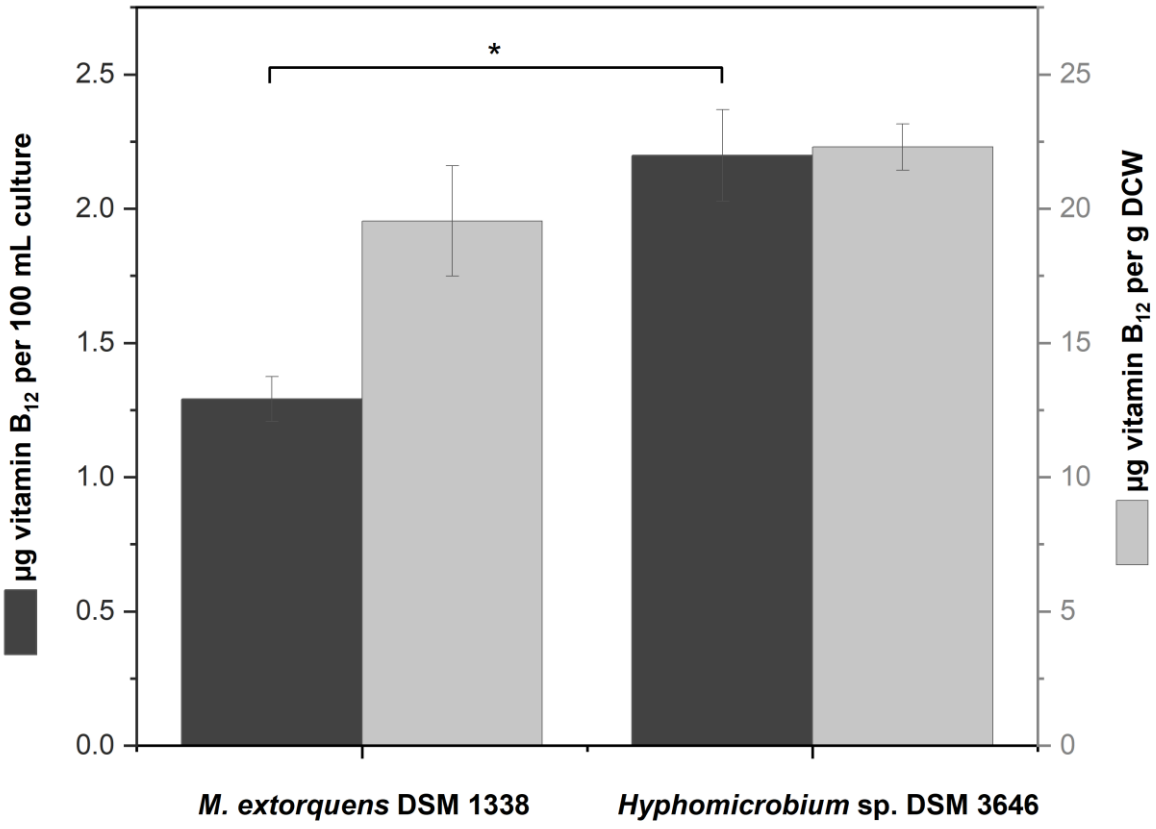

Fig. 44 Quantitative comparison of vitamin B_{12} amounts produced with *Hyphomicrobium* sp. DSM 3646 and *M. extorquens* DSM 1338 in the minimal medium with 0.5% methanol. Demonstrated are vitamin B_{12} amounts expressed in µg per 100 mL culture (dark grey) and per g DCW (light grey). The data are represented as the mean values and standard deviations of three biological replicates. Two-sample t-test was used for statistical comparison between the strains, differences considered as significant at $p < 0.05$ are denoted by an asterisk

The OD_{600} value of 2.97 ± 0.06 measured in the culture of *Hyphomicrobium* sp. DSM 3646 was significantly higher than 2.06 ± 0.06 reached by *M. extorquens*. Therefore, the amount of cobalamin was calculated per g DCW for the relative comparison. The amount of 22.30 ± 0.87 µg vitamin B_{12} per g DCW detected in the culture of *Hyphomicrobium* sp. DSM 3646 was slightly higher than 19.54 ± 2.05 µg vitamin B_{12} per g DCW reached by *M. extorquens* DSM 1338. Nevertheless, these differences were not significant.

On the contrary, the subsequently performed t-test showed that the vitamin B_{12} amounts varied significantly between the producing strains when the cobalamin concentrations were calculated per 100

mL culture. The yield of vitamin B_{12} achieved by *Hyphomicrobium* sp. DSM 3646 was 2.2 ± 0.17 µg per 100 mL culture, which is 1.7 times higher than 1.29 ± 0.08 µg per 100 mL culture produced by *M. extorquens*. Due to the higher volumetric yield of vitamin B_{12}, which is at least partly caused by higher biomass formation, *Hyphomicrobium* sp. DSM 3646 was chosen for further investigation.

3.5.5 Optimization of *Hyphomicrobium* sp. DSM 3646 cultivation medium

In order to improve the biomass accumulation and thereby volumetric vitamin B_{12} productivity, it was decided to evaluate whether alterations in component concentrations of the minimal medium can have an effect on the growth of *Hyphomicrobium* sp. DSM 3646. For this purpose, the concentration of methanol, buffer, $CaCl_2$, trace elements and mineral salts was changed and media with the two-fold increased or reduced amount of each single component were tested. The standard minimal medium was used as reference and the growth of *Hyphomicrobium* sp. DSM3646 under the standard and altered conditions was investigated in the Biolector® microbioreactor.

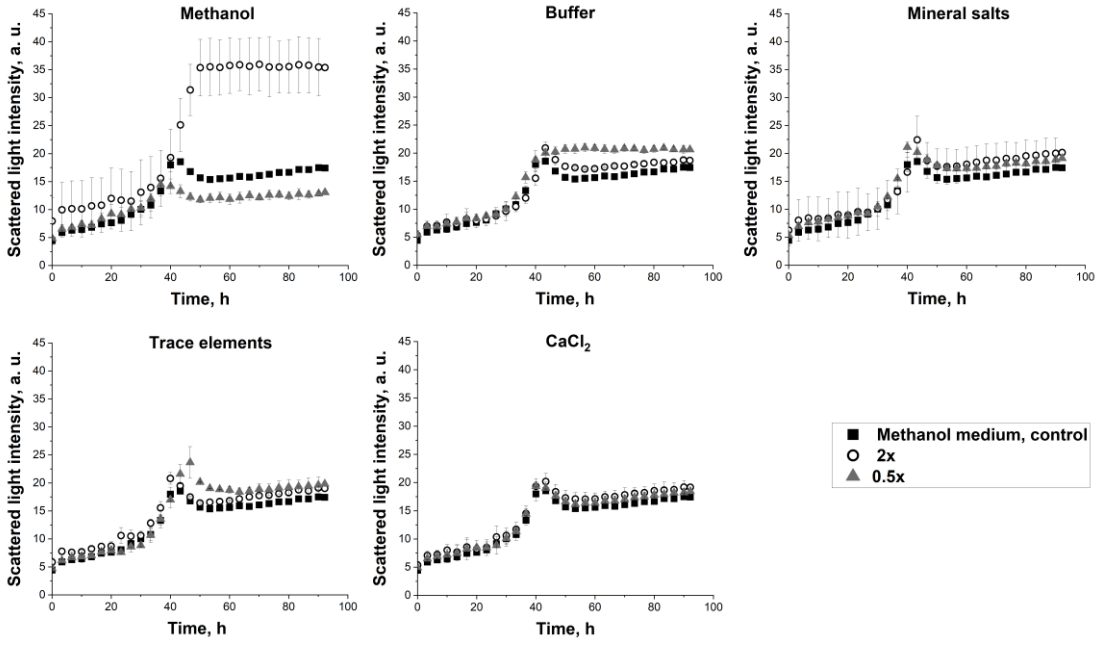

Fig. 45 Influence of the methanol mineral medium components on the growth of *Hyphomicrobium* sp. DSM 3646. The data points represent the mean values and standard deviations of three biological replicates

The effect of different media components on the growth of the organism is presented in Fig. 45. Among all investigated components, methanol was identified as the factor having the greatest influence on the

growth of *Hyphomicrobium* sp. DSM 3646. The results show that both increase and decrease of methanol concentration had a significant effect on biomass formation. The values observed in the medium with 1% methanol reached above 35 a. u. after 50 h, while 0.25% of methanol in the medium resulted in a scattered light intensity of 12 a. u., which was significantly lower than 15 a. u. achieved in the standard medium with 0.5% methanol used as a control. On the contrary, no effect on the growth of *Hyphomicrobium* sp. DSM 3646 was observed when the concentration of $CaCl_2$ was altered, while only slight variations in biomass accumulation were detected upon the change in the concentration of the buffer, trace elements and mineral salts. Nevertheless, since these effects were not comparable with the methanol impact on the growth of *Hyphomicrobium* sp. DSM 3646, only the latter was selected for a more detailed investigation.

The impact of methanol concentrations of 1%, 2%, 3% or 4% on the growth of *Hyphomicrobium* sp. DSM 3646 was tested and compared with the growth of the strain in the standard minimal medium containing 0.5% methanol (control) to determine methanol concentration providing optimal growth. The cultivations were performed in the Biolector® microbioreactor, the biomass accumulation was monitored online via scattered light measurement (Fig. 46).

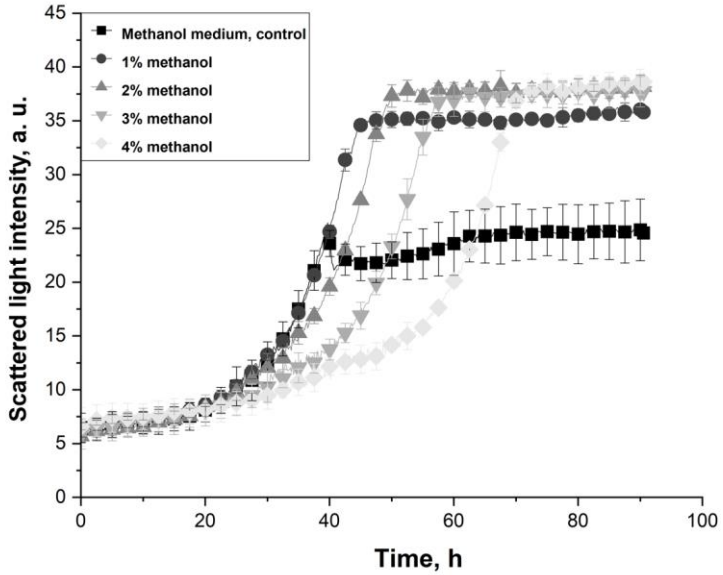

Fig. 46 Comparison of the growth behaviour of *Hyphomicrobium* sp. DSM 3646 in the media with increased methanol concentrations of 1-4% and the standard medium with 0.5% methanol (control). The data points represent the mean values and standard deviations of three biological replicates

Each tested altered methanol concentration resulted in the changes of the *Hyphomicrobium* sp. DSM 3646 growth. All increased methanol concentrations led to a maximal scattered light intensity which was 1.6 times higher than that in the reference medium. The highest scattered light intensity was determined in the media with methanol concentrations of 2% and higher, while no further signal increase was detected in the media containing 3% and 4% of methanol. Nevertheless, elongation of the lag phases was observed upon methanol concentration higher than in the control medium. *Hyphomicrobium* sp. DSM 3646 could reach the maximum scattered light intensity after 40 h in the control medium, while it took 45 h, 50 h, 58 h and 70 h in the media with 1%, 2%, 3% and 4% of methanol, respectively.

Overall, the most beneficial growth properties were obtained in the medium with 2% of methanol, where efficient biomass formation was combined with only slight growth inhibition at the beginning. Therefore, this methanol concentration was chosen for the investigation in the following experiment.

To avoid the inhibitory effect of methanol observed at the beginning of the *Hyphomicrobium* sp. DSM 3646 growth in this medium, the cultures were inoculated in the medium containing 1% of methanol, while further 1% of methanol was added in the mid-exponential phase of growth. To investigate, whether the increase of other medium components can provide a positive effect on the *Hyphomicrobium* sp. DSM 3646 growth under these growth conditions, they were then investigated in the Biolector® microbioreactor in combination with the doubled amounts of the buffer, mineral salts, trace elements and $CaCl_2$ (Fig. 47A).

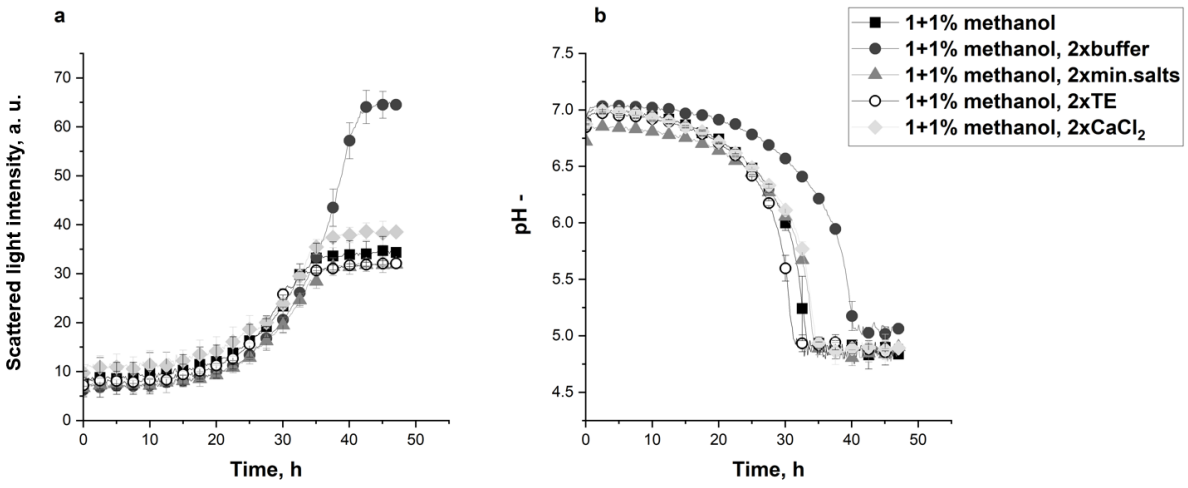

Fig. 47 Investigation of the growth (A) of *Hyphomicrobium* sp. DSM 3646 and the corresponding pH changes (B) in the medium with 2% methanol combined with the two-fold increased concentration of each other medium component. The data points represent the mean values and standard deviations of three biological replicates

Stepwise methanol addition enabled to shorten the lag-phase and to achieve the maximum scattered light intensity after 35 h, which was comparable with the medium with standard methanol concentration (Fig. 46). Among all tested components, the increased amount of the buffer had the greatest influence on the biomass formation leading to the significant increase of the scattered light intensity over 64 a. u. This value was 1.7 times higher than in the medium with the standard buffer concentration and 2% methanol and 2.8 times higher than the signal reached in the medium with standard buffer concentration and 0.5% methanol.

Due to the effect observed upon buffer concentration increase, it was proposed that pH was the growth-limiting factor in the media with the standard buffer concentration and pH measurement in all tested media was performed. As. Fig. 47B shows, the pH value changes correlated with the growth of the cultures. In the media with the standard buffer amounts the pH value decreased to 5 after 35 h, while the cells grown in these media achieved the stationary growth phase at this time point. In case of the medium with the increased buffer concentration, the same trend was observed 5 h later, when acidic pH and the stationary growth phase were achieved after 40 h of cultivation.

Since a positive effect of the increased methanol concentration in combination with the buffer amount increase was observed, it was decided to investigate whether further addition of these components can positively influence the growth of *Hyphomicrobium* sp. DSM 3646. The experiment was conducted in the Biolector® microbioreactor and the growth of the organism was monitored with 2% and 3% methanol and with the two-fold and three-fold buffer concentration.

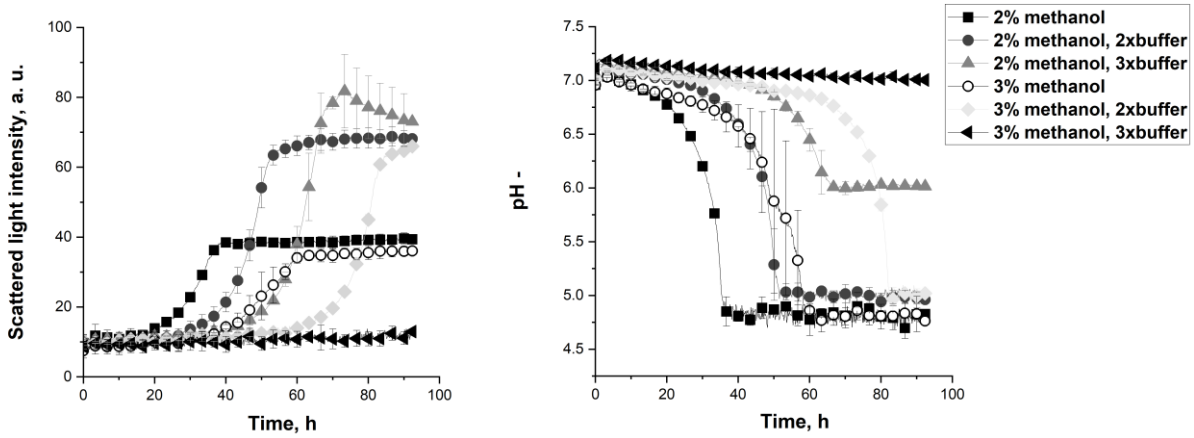

Fig. 48 Investigation of the growth (A) of *Hyphomicrobium* sp. DSM 3646 and the corresponding pH changes (B) in the media with 2% and 3% methanol combined with the standard, two-fold or three-fold

increased concentration of the buffer. The data points represent the mean values and standard deviations of three biological replicates

As Fig. 48A shows, three-fold concentration of the buffer in the medium with 2% methanol resulted in additionally increased *Hyphomicrobium* sp. DSM 3646 biomass accumulation which was expressed in the maximum scattered light intensity of 80 a. u., which was remarkably higher than 68 a. u. achieved in 2% methanol medium with the doubled buffer amount. Nevertheless, immediately after the maximum scattered light intensity was reached in the 2% methanol medium with 3xbuffer, a rapid signal decrease was observed. Moreover, the growth of the strain in this medium was characterized by a prolonged lag-phase in comparison to 2% methanol medium with the standard and two-fold buffer concentration. In case of 3% methanol medium, the two-fold buffer increase resulted in the biomass accumulation comparable with that in 2% methanol medium with the increased buffer amount, although further lag-phase prolongation was observed. Combination of 3% methanol with 3xbuffer had a negative effect on the growth of *Hyphomicrobium* sp. DSM 3646 since no changes in the scattered light intensity were detected even after 90 h of cultivation.

Similar to the results of the previous experiment, pH changes correlated with the growth behaviour of the cultures (Fig. 48B). Practically no changes in the pH value were observed for the medium with 3% methanol and three-fold buffer concentration. Acidic pH below 5 was reached after 35 h, 52 h, 60 h and 82 h in the standard, 2% methanol and 2xbuffer, 3% methanol, 3% methanol 2xbuffer medium, respectively, which corresponded to the beginning of the stationary phase of the respective cultures. In the medium with 2% methanol and 3xbuffer after the cultures reached the stationary phase the pH achieved the value of 6 and no further decrease was observed.

Due to the obtained results, the medium with the two-fold buffer amount supplemented with 2% of methanol was selected as optimal for the growth of *Hyphomicrobium* sp. DSM 3646 and taken for the further investigation of vitamin B_{12} production.

3.5.6 Investigation of vitamin B_{12} production capability of *Hyphomicrobium* sp. DSM 3646 in the standard and optimized minimal medium

In order to identify optimal conditions for the production of the highest vitamin B_{12} amounts with *Hyphomicrobium* sp. DSM 3646, the ability of the strain to produce cobalamin was compared in the

standard, 2% methanol and 2% methanol medium with the doubled buffer concentration. For this purpose, 50 mL cultures were cultivated for 6 days in shaking flasks, subsequently 25 mL of the cultures were used for vitamin B_{12} quantification, while remaining 25 mL were used to determine the DCW. As it has already been shown in the previous experiments, that there are great differences in the biomass formation between the investigated media, the amount of cobalamin was expressed per 100 mL culture and also per g DCW for the relative comparison. The analysis of the active vitamin B_{12} production was performed with the previously developed LC-MS/MS method.

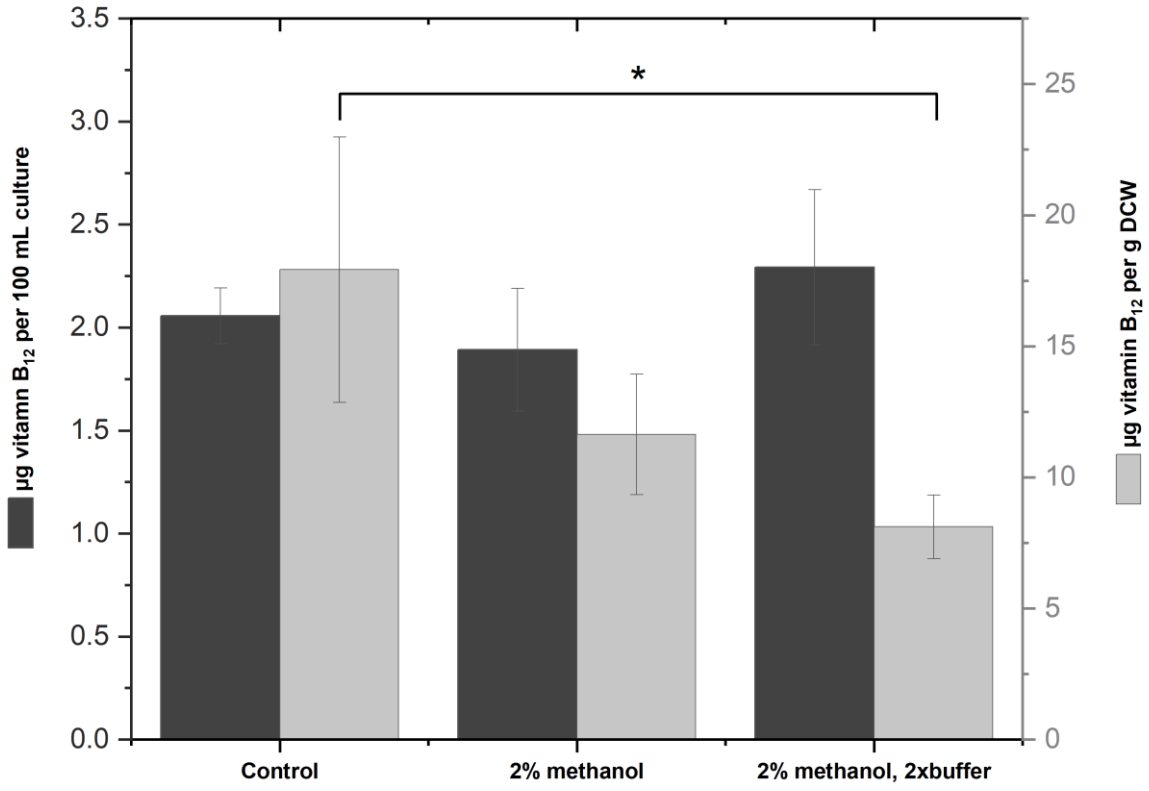

Fig. 49 Quantitative comparison of vitamin B_{12} amounts produced with *Hyphomicrobium* sp. DSM 3646 in the standard minimal medium with 0.5% methanol (control), in the medium with 2% methanol and in the medium with 2% methanol and two-fold increased buffer concentration. Demonstrated are vitamin B_{12} amounts expressed in µg per 100 mL culture (dark grey) and per g DCW (light grey). The data are represented as the mean values and standard deviations of three biological replicates. Two-sample t-test was used for statistical comparison between the media, differences considered as significant at $p < 0.05$ are denoted by an asterisk

As Fig. 49 shows, higher biomass accumulation did not increase the yield of vitamin B_{12} in the optimized media in comparison to the standard medium. The data obtained when vitamin B_{12} concentration is calculated per 100 mL culture show that the amounts of cobalamin in the control medium were slightly higher than those reached in the 2% methanol medium, and minor increase of cobalamin content in comparison to control medium was detected in the 2% methanol medium with the doubled buffer concentration. However, the subsequently performed unpaired t-test showed that the observed differences between the tested media were not significant.

On the contrary, significant differences were revealed between the media when the vitamin B_{12} amounts were calculated per g dry cell weight DCW for the purpose of relative comparison. In this case it was again shown that the optimization of the cultivation medium leading to the increased biomass formation had no positive effect on cobalamin synthesis. Surprisingly, the highest concentration of 17.9 ± 5.05 µg per g DCW was obtained in the standard medium, which was 2.2 times higher than 8.11 ± 1.21 µg per g DCW of vitamin B_{12} achieved in the 2% methanol medium with the increased buffer concentration, where the highest biomass formation was observed.

4. Discussion

4.1 Development of vitamin B_{12} analytics

4.1.1 LC-MS/MS method development and optimization

Although there are many different techniques described for the analysis of vitamin B_{12} (Kumar et al. 2010), LC-MS analysis provides opportunities for a fast and sensitive analytical procedure. For this reason, the first objective of this study was to establish and optimize a sensitive LC-MS/MS method for vitamin B_{12} analysis. According to the obtained results (Fig. 10), the developed LC-method can be successfully applied for the detection and separation of CNCbl and MetCbl and AdoCbl, which are naturally produced by the microorganisms. Nevertheless, since vitamin B_{12} was extracted in the cyano-form in this work, further steps were focused on method optimization for CNCbl.

The number of theoretical plates is an important parameter which is often used for the evaluation of the HPLC column performance. Among the two tested columns, Luna Omega 3 µm PS C18 100 Å was found to perform better, since the number of theoretical plates corresponding to the peak obtained with this column was remarkably higher than with Luna Omega 1.6 µm Polar C18 100 Å. The higher signal intensity observed for the peak produced with the first column underlined the finding that Luna Omega 3 µm PS C18 100 Å provides a better opportunity for the vitamin B_{12} analysis. The difference between the two tested columns lies in the column stationary phase particles: Luna Omega 1.6 µm Polar C18 100 Å is characterized by a polar modified particle surface, while the surface of Luna Omega 3 µm PS C18 100 Å contains a positive charge (Fig. 8).

According to the manufacturer (Phenomenex®), the latter column allows better analysis and a greater separation between the compounds with varying functional groups. Since the vitamin B_{12} molecule has a complex chemical structure carrying different functional groups (Fig. 1), the obtained results are very plausible. Moreover, the mentioned stationary phase feature of the Luna Omega 3 µm PS C18 100 Å column makes it also the most appropriate for the separation of the active vitamin B_{12} and pseudovitamin B_{12}.

Overall, the best differentiation between these two vitamin B_{12} forms was achieved with the selected column, the flow rate of 0.4 mL/min and mass spectrometer parameters optimized for the double charged ions. Due to the differences in the lower ligand structure (Fig. 1), identification of the different

characteristic fragmentation patterns corresponding to the cyanocobalamin standard and pseudovitamin B_{12}-producing *L. reuteri* cell extract was achieved. Pseudocobalamin is unavailable as a commercially produced standard. Nevertheless, Santos et al. 2007 and Crofts et al. 2013 reported, that pseudovitamin B_{12} is the corrinoid exclusively produced by *L. reuteri*, which enabled the use of this strain as a reference for the control of pseudocobalamin production. The spectrum of the cyanocobalamin reference compound demonstrated a parent ion with m/z 678.40 and fragment signals with m/z 146.95 and m/z 359.10, which were identified as $[DMBI + H]^+$ and $[DMBI + sugar + PO_3 + H]^+$, respectively, while a parent ion with m/z 672.75 and fragment signals with m/z 136.05 $[adenine + H]^+$ and m/z 348.05 $[adenine + sugar + PO_3 + H]^+$ were detected in the spectrum of the pseudovitamin B_{12} (Fig. 13). These results are in line with the data reported in previous studies (Chamlagain et al. 2018; Bernhardt et al. 2019). Moreover, the fragment with m/z 456.75 which was detected in the spectrum of the cyanocobalamin in this study, was also reported as an unidentified fragment by Bernhardt et al. 2019, which supports the obtained findings.

4.1.2 Factors influencing vitamin B_{12} signal intensity

In the performed investigation, the condition of the ion source, water storage, concentration of the formic acid in the mobile phase and the sample storage time were detected as the factors having a great influence on the detected vitamin B_{12} signal in the LC-MS/MS analysis performed with the used analytic system under the described conditions.

Since in the applied analysis technique the m/z signal is measured as the outcome and the analyte ionization occurs in the ion source, the results showing that the measured signal intensity and peak area increased 1.5 times after the ion source was cleaned were not surprising. The low signal detected in the initial measurement can be easily explained with the ion source contamination which can result in the lower ionization efficiency of the target analyte due to the reduction of ion transmission.

Although it is known that water quality can influence the results of the LC-MS measurement (Regnault et al. 2004), only minor changes in the vitamin B_{12} signal were expected after the fresh water was introduced into the system. On the contrary, the influence was comparable with that observed for the ion source condition. This can be possibly explained with the water quality degradation effect characterized by the increased amounts of organics in the high purity water after storage period (Gabler et al. 1983). This assumption can be underlined by the findings of Regnault et al. 2004, who showed

that a good quality of water is very important when ESI-MS measurements are performed. This report has demonstrated that MS detection methods are sensitive to organic impurities in water since a higher total organic content leads to a higher baseline intensity which especially complicates the detection of very low concentrated analytes. For these reasons, the accumulation of the organic impurities in the water with the storage time can be the reason for the comparably low signal obtained in the initial measurement. According to the Merck technical bulletin (Merck KGaA 2017), the storage of solvents in original packaging amber glass bottles can maintain their purity. Since the mobile phase used for the initial measurement was stored in a clear Schott glass bottle for a longer period of time while the use of a mobile phase mixture prepared with the water from the original packaging amber glass bottle directly on the day of the measurement positively influenced the signal, we suppose that the latter procedure is important for the LC-MS analysis of vitamin B_{12} under the conditions described in this study.

0.1% formic acid in the mobile phase is commonly used as a proton source in the LC-MS applications since the trifluoroacetic acid widely used when UV detection is applied can cause strong ion suppression in mass spectrometry (Merck KGaA 2017). This concentration was found to be optimal for the LC-MS analysis of vitamin B_{12} in this study. 0.1% formic acid in the mobile phase was also used in earlier reports on the LC-MS-based vitamin B_{12} investigations (Chamlagain et al. 2016; Chamlagain et al. 2018; Tanioka et al. 2014; Bernhardt et al. 2019).

The standard sample storage time was also identified to have an influence on the detected signal. Although CNCbl is more stable than the very light sensitive adenosyl- and methyl- forms, nevertheless, CNCbl degradation upon light exposure has already been described (Juzeniene and Nizauskaite 2013). Since CNCbl samples used in the measurement were stored in the amber glass vials for three months, such slow CNCbl degradation can explain the signal decrease detected between the fresh and old samples (Fig. 15). However, the signal decrease observed between two consecutive days (Fig. 16) was probably caused by a technical problem because further measurements performed in triplicates on three sequential days did not demonstrate remarkable deviations (Table 13). Nevertheless, the chosen strategy to set up the standard rows from the stocks directly on the day of the measurement is probably the best way to avoid undesired signal fluctuations.

4.1.3 Method validation

One of the main challenges in vitamin B_{12} analysis is the fact that it is present in the food sources or produced by bacteria only in small amounts. For example, the contents between 0.4 – 158.5 µg vitamin B_{12} per 100 g were mentioned for different food sources of animal origin (Watanabe 2007). *P. freudenreichii* often used for food fortification, was reported to produce between 120 ng vitamin B_{12} per mL culture and approximately 0.9 µg vitamin B_{12} per mL culture in different studies (Deptula et al. 2017; Chamlagain et al. 2016). For this reason, development of a very sensitive vitamin B_{12} method was very important for this work. The demonstrated LOD of 5 nM and LOQ of 15 nM make the method appropriate for the sensitive analysis even of the samples containing very low amounts of vitamin B_{12}.

The method also performed well in terms of linearity in the working concentration range and showed a good selectivity in the experiment with the blank and spiked acetate buffer and *E.coli* DSM 18039 cell extracts. Although an unidentified peak was observed in the chromatogram of the cell extract of *E.coli* DSM 18039 (Fig. 19C), the fragment signals with m/z 456.75 and m/z 359.10 characteristic for cyanocobalamin were not detected. This allows to exclude that the observed peak corresponds to CNCbl and is rather evidence for a substance with the same precursor ion mass as cyanocobalamin but another chemical structure.

The developed method demonstrated a high accuracy in the experiments with various commercially available CNCbl supplements. In the experiments on the robustness of the method, the expected influence of the flow rate and column oven temperature on the retention time can be easily explained by the change of the pressure in the system when these parameters are altered. On the contrary, formic acid concentration had a stronger effect on the peak area and higher peak areas were obtained for the lower formic acid concentration in the mobile phase. This finding correlates with the results from the experiments on the factors influencing the vitamin B_{12} signal (Fig. 15). Nevertheless, since the changes in the retention time and peak area were not remarkable, the method demonstrated a high robustness to the described changes introduced into the system.

The method developed in this study provides a good approach for the reliable analysis of vitamin B_{12}. Although there have been several LC-MS/MS methods for vitamin B_{12} determination reported, they are mostly dealing with the qualitative analysis of B_{12} in the samples (Chamlagain et al. 2018; Bernhardt et al. 2019). On the contrary, HPLC-based methods or traditional MBA are often applied in food analytics for the quantitative analysis of vitamin B_{12} (Heudi et al. 2006; Chamlagain et al. 2018; Chamlagain et al.

2015). In comparison to these methods, the proposed analytical procedure enables a much more sensitive analysis of vitamin B_{12} demonstrating very low LOD and LOQ of 5 nM and 15 nM of cyanocobalamin, respectively.

Moreover, due to such high sensitivity the developed method does not require large amounts of the sample material: while earlier reported LCMS/MS-based methods applied 15-30 g of sample material for the subsequent vitamin B_{12} analysis (Luo et al. 2006; Szterk et al. 2012), only few milligrams of cell material were sufficient for the successful B_{12} analysis with the developed method.

Finally, the developed method is faster than other previously described LC-MS/MS-based methods. In comparison to 12-40 min required for vitamin B_{12} analysis with the methods reported by Lu et al. 2008; Schwertner et al. 2012; Szterk et al. 2012; Lee et al. 2015, LC-MS/MS-method described in this work is an attractive tool for a high sample throughput analysis.

4.2 Comparison of previously described vitamin B_{12}-synthesizing strains

Earlier studies describing vitamin B_{12} production are usually dealing with vitamin B_{12} amounts produced with one specific strain rather than comparing amounts obtained from different bacteria. Moreover, analysis of the literature data reported earlier for the strains known as vitamin B_{12} producers from the literature and selected for this research has shown that the vitamin B_{12} amounts described for the same species vary greatly between different studies depending on the cultivation conditions and B_{12} extraction protocol. For example, amounts between 3.1 – 13.9 µg/L (Piwowarek et al. 2018) and 20 – 124. 8 µg/L (Deptula et al. 2017) of vitamin have been described for *P. freudenreichii*. Various investigations on vitamin B_{12} synthesis in *B. megaterium* reported between 0.26 µg/L (Biedendieck et al. 2010) and 204.46 µg/L (Mohammed et al. 2014) for different wild-type strains. Vitamin B_{12} concentrations between 177.49 ± 2.98 mg/L (Li et al. 2008) and 198.27 ± 4.60 mg/L (Xia et al. 2015) have been achieved in the large-scale production experiments with *P. denitrificans* after 162 and 180 h of fermentation, respectively. Although the differences between the amounts reported for *S. meliloti* in different studies were not so great as for above mentioned species, they still varied between 108 mg/L (Cai et al. 2018) and 140 mg/L (Dong et al. 2016) of vitamin B_{12}. Finally, production of 41 - 54 ng of vitamin B_{12} per g wet biomass was reported for different *M. extorquens* type strains (Ivanova et al. 2006). Due to the various concentrations reported from the literature before, a comparison of the ability capability of various bacterial strains to produce vitamin B_{12} under comparable conditions was performed in this study.

To the best of our knowledge, this is the first report comparing the amounts of vitamin B_{12} produced by the different bacterial strains previously described in the literature. Since the variations in the vitamin B_{12} amounts reported in the earlier studies for the same species can be explained by the differences in the cultivation conditions and duration, they were kept constant in this study. The only exception was made for *B. megaterium* and *P. freudenreichii*, where a special cultivation procedure was applied.

A two-step cultivation widely applied for vitamin B_{12} production with *P. freudenreichii* (Chamlagain et al. 2016; Chamlagain et al. 2018; Deptula et al. 2017) was also performed in this study. The first oxygen-free stage was necessary since *Propionibacteria* are microaerophilic microorganisms and produce the vitamin B_{12} precursor cobamide under anaerobic conditions (Martens et al. 2002). Nevertheless, oxygen is needed for the formation of DMBI and its incorporation in the vitamin B_{12} molecule (Martens et al. 2002; Deptula et al. 2015), which is why the second aerobic stage was included.

B. megaterium also uses the anaerobic way of vitamin B_{12} biosynthesis (Martens et al. 2002; Fang et al. 2017) but, on the contrary, is an aerobic microorganism. For this reason, *B. megaterium* was grown aerobically to accumulate the biomass during the first cultivation stage, which was then followed by the second anaerobic stage necessary for the vitamin B_{12} production.

Among the tested strains, the highest contents of vitamin B_{12} were detected in the cultures of *M. extorquens* DSM 1338 and *P. freudenreichii* DSM 20270. These results were not surprising, since vitamin B_{12} is important for the activity of enzymes of the EMCP (Fig. 6), which is operated by *M. extorquens* upon growth on methanol (Smejkalová et al. 2010), while *P. freudenreichii* is well known for the ability to synthesize high cobalamin amounts and is also used for the industrial vitamin B_{12} production (Martens et al. 2002; Fang et al. 2018).

Interestingly, the amounts of vitamin B_{12} obtained from *P. denitrificans*, the other strain used for the industrial vitamin B_{12} production were relatively low in comparison to cobalamin levels detected for *M. extorquens* DSM 1338 and *P. freudenreichii* DSM in this investigation and to the amounts reported for *P. denitrificans* in the earlier studies (Li et al. 2008; Xia et al. 2015). The low vitamin B_{12} levels observed in this study can be explained by the applied cultivation conditions, while the earlier reported high concentrations were obtained in the optimized large-scale production processes. The other reason for the high levels of 300 mg/L of vitamin B_{12} known for the industrial processes (Martens et al. 2002) is the use of a genetically engineered highly effective vitamin B_{12} - producing *P. denitrificans* strain, while a wild-type strain was used in this study.

4.3 Identification of mutants with an enhanced vitamin B_{12} production

4.3.1 *M. extorquens* as appropriate organism for the search for mutants with the enhanced vitamin B_{12} production

M. extorquens meets all criteria important for the developed strategy for the mutant selection: it carries vitamin B_{12}-dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase from the EMCP (Fig. 6) which is active in *M. extorquens* upon growth on methanol (Erb et al. 2007; Smejkalová et al. 2010; Peyraud et al. 2009). Secondly, production of vitamin B_{12} was demonstrated in *M. extorquens* earlier (Ivanova et al. 2006), although the type of the produced corrinoid compound was not defined in that study, which was performed in this investigation. The parent ion and fragment ion signals detected in the chromatogram of *M. extorquens* cell extract (Fig. 24) were identical with those detected in the spectrum of the cyanocobalamin standard, which confirmed production of the active vitamin B_{12} by *M. extorquens* DSM 1338 and made it appropriate for the selection of mutants according to the developed strategy.

4.3.2 Influence of the possible selection agents on the growth of *M. extorquens* on methanol

All possible selection agents tested in this study can be divided into two groups: vitamin B_{12}-b-monocarboxylic acid and vitamin B_{12}-c-lactam had no influence on the growth of *M. extorquens*, while vitamin B_{12}-dodecylamine, LEV, ALA, SA and DFS provided inhibitory effect on the strain growth, which, nevertheless, could not be compensated by vitamin B_{12} addition.

Due to the similarities in the structure, vitamin B_{12}-b-monocarboxylic acid and vitamin B_{12}-c-lactam were expected to act as vitamin B_{12} antagonists replacing it as cofactor of ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase according to the developed screening strategy. The absence of the expected effect can be due to different reasons. Firstly, since vitamin B_{12} production occurs in the cells of *M. extorquens*, it should be verified whether the added agents can enter the cell or whether no antagonistic effect was observed because they are not taken up by *M. extorquens* (Fig. 50A). Investigations on the transport of vitamin B_{12} analogues through the cell membrane and measurement of their extracellular concentration can give insights into this question. Secondly, if the analogues are transported into the cells, it should be also examined whether they are able to bind the vitamin B_{12} -

dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase or no interaction with the enzymes takes place (Fig. 50B).

Earlier report on the antagonistic activity of vitamin B_{12}-monoacid has shown that the sensitivity against this analogue varied depending on the microorganism: a strong activity against *E. coli* was observed in contrast to only weak growth inhibitory effect towards *O. malhamensis* (Ford 1959). We propose that if vitamin B_{12}-b-monoacid is taken up by the cells in our experiments and can bind the target enzymes, the insensitivity of *M. extorquens* against this agent can be due to the following mechanisms: either the microorganism is able to utilize vitamin B_{12}-b-monoacid as the cofactor for ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase (Fig. 50C) or it is able to convert the compound to the vitamin B_{12} molecule (Fig. 50D).

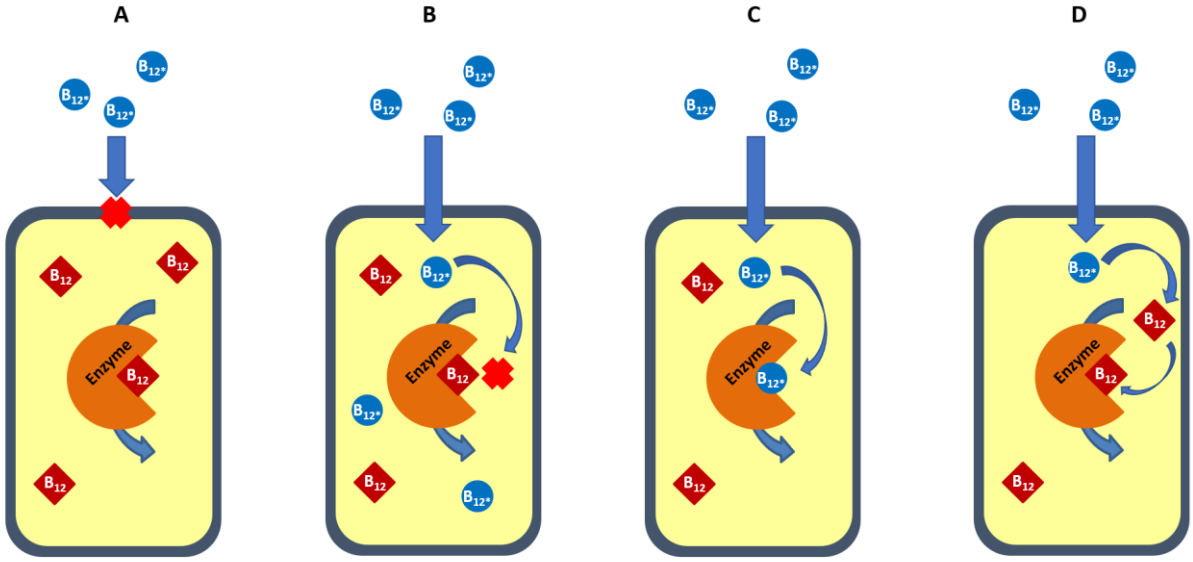

Fig. 50 Possible mechanisms explaining the absence of the inhibitory effect upon addition of vitamin B_{12}-b-monocarboxylic acid and vitamin B_{12}-c-lactam. (A) the agent is not able to bypass the cell membrane and enter the cell, the transport of the agent through the cell membrane occurs but it is not able to bind the vitamin B_{12}-dependent enzyme (B), is used by the cell as a coenzyme (C) or is converted to vitamin B_{12} (D)

Although Stabler et al. 1991 demonstrated that cobalamin-c-lactam can act as vitamin B_{12} inhibitor in the experiments with rat tissues, no effect was detected in this study. Nevertheless, this contradiction can be easily explained: being mammals, rats possess a typical mammalian vitamin B_{12}-dependent enzyme, methionine synthase, which was affected in the experiments carried out by Stabler et al. 1991,

while ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase were the target enzymes in this study. Moreover, cobalamin-c-lactam was able to act as cofactor for the methionine synthase reaction in the cell-free experiments with purified apo methionine synthetase, which suggests that the observed effect was due to the interruption of the cellular vitamin B_{12} uptake (Matthews 1998), rather than to the direct competitive inhibition between cobalamin-c-lactam and vitamin B_{12}. Since the cellular vitamin B_{12} uptake mechanisms in the mammals and bacterial cells differ greatly, this can explain that no inhibitory effect of cobalamin-c-lactam was observed in the experiments with *M. extorquens*.

In comparison to vitamin B_{12}-b-monocarboxylic acid and vitamin B_{12}-c-lactam, an inhibitory effect was observed for vitamin B_{12}-dodecylamine, LEV, SA and DFS, which suggests that these agents can enter and affect *M. extorquens* cells.

Inhibition of the activity of vitamin B_{12}-dependent enzymes by cyanocobalamin-dodecylamine in *Caenorhabditis elegans* was demonstrated by Bito et al. 2014. Kinetic studies performed in that study showed that the affinity of dodecylamine derivative for both *C. elegans* cobalamin-dependent enzymes methylmalonyl-CoA mutase and methionine synthase was greater than that of cobalamin. The ability of dodecylamine to serve as a competitive inhibitor of vitamin B_{12} also for *M. extorquens* enzymes was expected in this work. Nevertheless, the fact that the addition of high vitamin B_{12} concentrations did not compensate cyanocobalamin-dodecylamine inhibition provided evidence, that another mechanism caused the inhibitory effect observed for *M. extorquens* and made this agent inappropriate for the screening experiments.

Other than the above-mentioned agents, LEV and SA were expected to act as antagonists of vitamin B_{12} biosynthesis in *M. extorquens*. LEV and SA have already been reported to act as inhibitors of ALA - dehydratase (Fig. 51), the enzyme catalysing the condensation of two molecules of ALA to one molecule of porphobilinogen, a precursor of vitamin B_{12} biosynthesis in *Clostridium tetanomorphum* (Brumm and Friedmann 1981).

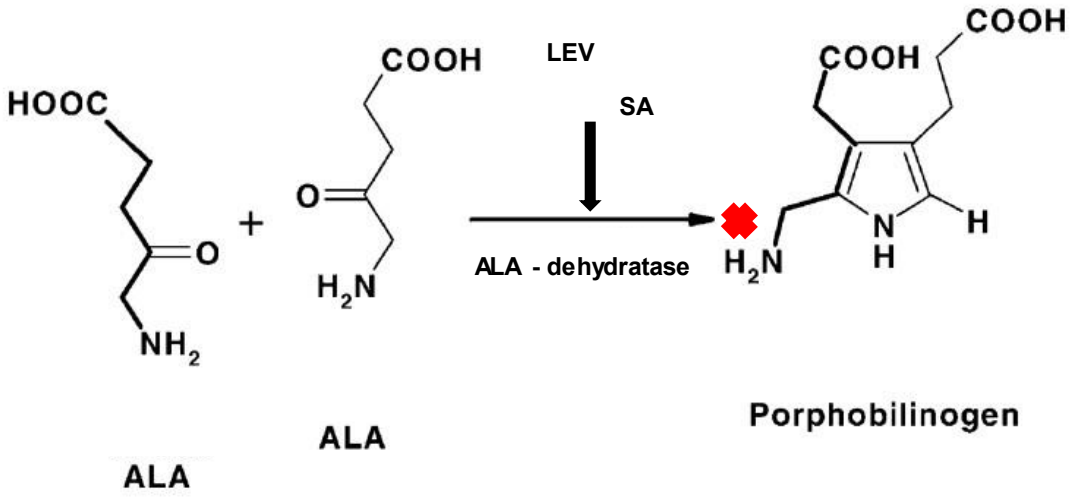

Fig. 51 Possible mechanism of the ALA - dehydratase inactivation: being structurally similar to ALA, the actual substrate of ALA - dehydratase, LEV and SA are used by the enzyme preventing the formation of porphobilinogen, thus, blocking further vitamin B_{12} biosynthesis (modified from Lee et al. 2003)

In this case, addition of vitamin B_{12} was expected to compensate the interrupted biosynthesis and restore the growth of *M. extorquens.* Since this was not observed in the performed experiments, the ability of *M. extorquens* to transport the extracellularly added vitamin B_{12} into the cell should be investigated. These experiments can clarify whether vitamin B_{12} could not compensate LEV and SA inhibition because it was not taken up by the cells or because the inhibitory action of LEV and SA is not connected to vitamin B_{12} biosynthesis. However, as the inhibitory effect of LEV was also observed upon growth in the succinate medium, when the vitamin B_{12} - dependent ethylmalonyl-CoA mutase and methylmalonyl-CoA mutase from the EMCP are down-regulated (Smejkalová et al. 2010), a vitamin B_{12}-independent mechanism is most likely to be the reason of the inhibition.

In comparison to SA and LEV, the DFS inhibitory effect was observed for *M. extorquens* cells growing on methanol but not on succinate which indicates that the target is located in one of the pathways important for C1 utilisation (Pöschel et al. 2022). Moreover, the addition of glyoxylate to methanol medium also performed by Pöschel et al. 2022 partially compensated the DFS inhibition which shows that the EMCP contains the target. Nevertheless, vitamin B_{12} addition to *M. extorquens* cultures grown on methanol performed in this study could not compensate the DFS inhibitory effect. For this reason, two probable mechanisms of inhibition are possible.

In the first case, EMCP can be affected by DFS, which makes the pathway unfunctional and prevents the growth of *M. extorquens* on methanol. Due to the structural similarity of DFS to the dicarboxylic acids that constitute the EMCP intermediates, DFS can act as competitive inhibitor (Pöschel et al. 2022) causing the inactivation of vitamin B_{12}-independent enzymes while methylmalonyl-CoA mutase and ethylmalonyl-CoA mutase remain unaffected.

Alternatively, conversion of DFS in the EMCP to a toxic compound which causes the cell death is possible. In both proposed mechanisms vitamin B_{12} biosynthesis is not affected, which makes DFS inappropriate as a selection agent for the proposed approach.

As the described results show, substances having structural similarities to vitamin B_{12} or potential to act as inhibitors of its biosynthesis were tested in the study, which offered various opportunities to identify a selection agent. Since among different proposed and examined mechanisms of vitamin B_{12} inhibition no appropriate selection agent could be identified, it was decided to stop further investigations and the strategy was not further followed.

4.4 New vitamin B_{12} producers identified via the search for homologues of *P. freudenreichii* fusion gene

4.4.1 *bluB/cobT2* fusion gene homologues in the identified candidate strains

The BluB/CobT2 protein belongs to the fusion proteins with a proline-rich spacer region. Proline is a common amino acid residue widespread in the natural non-helical linkers having a rigid structure and serving to separate the domains (George and Heringa 2002). Interestingly, the linker spacer regions were shorter in all identified homologues in comparison to the *bluB/cobT2* fusion gene from *P. freudenreichii.* The shorter region separating the BluB and CobT domains in organisms other than *Propionibacteria* has already been reported in the earlier study (Deptula et al. 2015).

Among the identified homologues, the protein sequence from *R. antarctica* demonstrated the highest identity to the initial query sequence, which was not surprising since similar to *P. freudenreichii* this strain belongs to the order Propionibacteriales.

4.4.2 Vitamin B_{12} and pseudovitamin B_{12} synthesis by the selected strains

According to the obtained results, the selected strategy proved to be successful for the identification of new producing strains. Production of vitamin B_{12} was detected in 3 from 6 selected strains, which was confirmed by the identification of the characteristic precursor and fragment ion masses present also in the spectrum of the cyanocobalamin standard (Fig. 36).

Besides vitamin B_{12}, microorganisms are known to produce also other corrinoid compounds (Crofts 2013), which was also confirmed in this work. For example, production of pseudovitamin B_{12} previously reported for a wide range of other microorganisms (Watanabe et al. 1999; Santos et al. 2007; Bernhardt et al. 2019; Hashimoto et al. 2012; Tanioka et al. 2009) was detected for *Y. lutea.*

Production of an unknown compound was also observed for *C. indicus*. Although the parent ion mass of the compound was similar to that of the pseudocobalamin, the detected retention time did not correspond to the retention time of pseudovitamin B_{12}. Moreover, since the characteristic fragment ion masses were also not detected, pseudovitamin B_{12} synthesis can be excluded for *C. indicus* and production of a compound that has the same mass as pseudocobalamin, but another chemical structure most likely takes place in this case.

Since active vitamin B_{12} was the exclusively produced corrinoid only in case of *Terrabacter* sp. DSM 102553, this strain was assumed to be the most promising for the vitamin B_{12} production among the identified candidates.

4.4.3 Growth comparison of vitamin B_{12}-producing strains in complex media

Growth differences between the investigated strains seemed to be remarkable already from the beginning, when only *Terrabacter* sp. DSM 102553 demonstrated visible cell growth compared to the prolonged lag-phase observed for the cultures of both other candidates. This difference was especially noticeable for *Y. lutea* DSM 19828 which showed the longest lag-phase among all investigated candidates. Since the same media were applied for the precultures and main cultivation experiment, which was started at the same OD_{600} for all strains, these differences cannot be explained by the adaptation of the cells to the new conditions but are rather due to the characteristic features of each specific strain. Such ability to accumulate higher biomass during shorter time period underlined the earlier finding that *Terrabacter* sp. DSM 102553 is the most appropriate strain for the vitamin B_{12} production among the identified candidates under the tested conditions. Nevertheless, selection of an optimal medium and optimization of the cultivation conditions might be the possible ways to improve the vitamin B_{12} contents produced by *Y. lutea* and *C. indicus*.

4.4.4 Comparison of the growth and active vitamin B_{12} production capability of *Terrabacter* sp. DSM 102553 in different media

The increase of medium components concentration in 2xPP in comparison to PP medium, resulted in a short lag-phase and the ability to achieve remarkably higher optical densities. The prolonged lag-phase observed for the M9 medium was firstly proposed to be the result of the cell adaptation to the new conditions since the precultures were grown in the PP medium and the preculture history is known to affect the length of the lag phase (Bertrand 2019). Nevertheless, further experiments have shown that precultures grown in M9 medium did not provide any positive effect on the lag-phase duration (Appendix 8). Possibly sequential transfers of *Terrabacter* sp. DSM 102553 from M9 medium to fresh medium can help to reduce the long lag time since such effect has already been demonstrated for the cells of *E. coli* BL21(DE3) (Kim et al. 2021).

The analysis of vitamin B_{12} production in *Terrabacter* sp. DSM 102553 was performed after 100 h of cultivation as higher amounts of produced cobalamin at later growth stages have already been reported for *P. freudenreichii* (Deptula et al. 2017; Bernhardt et al. 2019). In contrast to comparable OD_{600} values in M9 and 2xPP media, the amounts of vitamin B_{12} obtained in M9 medium were much higher than those achieved in PP and 2xPP (Fig. 40). These remarkable differences show that under the investigated conditions the production of vitamin B_{12} was not linked to biomass formation in *Terrabacter* sp. DSM 102553. Moreover, high vitamin B_{12} concentrations measured for the M9 medium samples demonstrate that this medium is the most favourable for vitamin B_{12} production. M9 is a low-cost medium which opens new opportunities for a cost-effective biotechnological vitamin B_{12} production with *Terrabacter* sp. DSM 102553.

It is unquestionable that the vitamin B_{12} contents reported in this study cannot compete with the values of 25 - 204 mg per g wet cell mass reported earlier for *P. freudenreichii* (Chamlagain et al. 2016). Nevertheless, a two-step cultivation including an anaerobic stage is essential for vitamin B_{12} production with *Propionibacterium* strains (Chamlagain et al. 2015; Chamlagain et al. 2016; Chamlagain et al. 2018; Deptula et al. 2017), while the cultivation of *Terrabacter* sp. DSM 102553 occurs under aerobic conditions. The omission of the anaerobic cultivation step can be an attractive advantage since it makes the synthesis procedure more easy-to-handle and time effective. Moreover, the vitamin B_{12} levels achieved in the first preliminary experiments can be optimized. Future optimization steps can involve further medium optimization, for example via supplementation of DMBI (Chamlagain et al. 2016) or cobalt (Mohammed et al. 2014) as well as identification of mutants with higher productivity.

The identified *Terrabacter* sp. DSM 102553 is a new promising strain and its ability to synthesize active cobalamin in the low-cost medium demonstrated in this work opens new opportunities for biotechnological vitamin B_{12} synthesis in a fairly simple aerobic manner, increasing the cost-effectiveness of biotechnological vitamin B_{12} production.

4.5 Newly identified strains with probably essential EMCP as promising vitamin B_{12} producers

4.5.1 Strains carrying probably functional EMCP

Although this issue has not been investigated so far, it was assumed in this study, that organisms with an essential need for vitamin B_{12} might possess a high capability for its synthesis. The reduction of pyruvate to propionate, a key reaction of *P. freudenreichii* metabolism, occurs in the Wood–Werkman cycle, which includes coenzyme B_{12}-dependent methylmalonyl-CoA mutase (Thierry et al. 2011). The vitamin B_{12}-producing plant symbiont *S. meliloti* (Burton and Lochhead 1952) requires a cobalamin-dependent ribonucleotide reductase to establish the symbiosis with its plant host (Taga and Walker 2010). Similar to these microorganisms, methylotrophs with cobalamin-dependent enzymes in the EMCP seemed to be promising as a new source for vitamin B_{12}.

Since the search for microorganisms lacking the isocitrate lyase - coding gene was performed, it was assumed that the identified candidates do not carry the glyoxylate way and use EMCP for glyoxylate regeneration. EMCP is essential for growth on C1 and C2 carbon substrates, therefore, the ability of the selected strains to utilize methanol or ethanol provided a further prove for the functional EMCP.

4.5.2 Production of active vitamin B_{12} in the cells of selected strains

The obtained findings show that investigation of microorganisms with essential functions of cobalamin in their primary metabolism is a successful strategy for the identification of new vitamin B_{12} producers. Since in this case it was also necessary to exclude the production of pseudocobalamin, all candidates were investigated for the synthesis of the active and inactive vitamin B_{12} form. Interestingly, in comparison to the producing strains identified via *bluB/cobT2* homology (Fig. 36), active vitamin B_{12} was the form exclusively synthesized by all EMCP - candidates. These results are very promising and provide a good opportunity for the identification of further new vitamin B_{12}-producing microorganisms.

4.5.3 *Hyphomicrobium* sp. DSM 3646 as the most efficient producer among the investigated strains

The peak areas corresponding to cobalamin measured for the cultures of *Hyphomicrobium* sp. DSM 3646 exceeded those of other identified strains, which demonstrated that *Hyphomicrobium* sp. DSM 3646 is the most efficient producing strain. The ability of the strain to accumulate high biomass in the

liquid medium and direct comparison of vitamin B_{12} production capability of the newly identified *Hyphomicrobium* sp. DSM 3646 with *M. extorquens* AM1 also underlined the finding that the latter is the most appropriate strain for vitamin B_{12} production.

To best of our knowledge, the essential role of vitamin B_{12} in *Hyphomicrobium* sp. DSM 3646 metabolism and the vitamin B_{12} production capability of the strain have not been investigated before, although the positive effect of vitamin B_{12} addition on the growth of other *Hyphomicrobium* strain has already been reported (Matzen and Hirsch 1982).

4.5.4 Optimization of *Hyphomicrobium* sp. DSM 3646 cultivation medium

The methanol concentration was identified to be the limiting factor for *Hyphomicrobium* sp. DSM 3646 growth. Generally, standard medium used in this study contained methanol concentrations higher than those described for *Hyphomicrobium* sp. in earlier research (Harder et al. 1973). Moreover, performed *Hyphomicrobium* sp. DSM 3646 growth experiments identified conditions enabling higher biomass formation compared to the standard medium and demonstrated that increased methanol concentrations had a positive influence on biomass accumulation (Fig. 45, Fig. 46). These results were not expected, since high methanol amounts are known to have an inhibitory effect on the growth of *M. extorquens*, which could be overcome only after evolutionary experiments in other studies (Cui et al. 2018; Belkhelfa et al. 2019). No evolutionary experiments were performed in this investigation and only a prolonged lag-phase was observed for the media with the increased methanol concentrations, which can indicate that *Hyphomicrobium* sp. DSM 3646 is a more robust strain possessing a higher ability to adapt to harsh environmental conditions than *M. extorquens.*

Acidification was detected as another factor limiting growth of *Hyphomicrobium* sp. DSM3646 in the media with the increased methanol concentration. Nevertheless, this inhibitory effect could be easily overcome via the addition of higher buffer concentrations (Fig. 47). pH control achieved via addition of alkali agents could be another possible way to avoid strong medium acidification.

4.5.5 Production of vitamin B_{12} by *Hyphomicrobium* sp. DSM 3646 in the standard and optimized minimal medium

Although an increased methanol concentration positively influenced biomass formation, the analysis of vitamin B_{12} synthesis revealed that the highest cobalamin amounts were produced in the standard

medium (Fig. 49). This implies that vitamin B_{12} production does not directly correlate with biomass formation for *Hyphomicrobium* sp. DSM 3646. This finding is in line with the results previously obtained for another producer newly identified in this study, *Terrabacter* sp. DSM 102553, and makes the standard medium more advantageous for vitamin B_{12} production with *Hyphomicrobium* sp. DSM 3646. Interestingly, high biomass accumulation observed in the medium with the increased methanol and buffer concentration was also accompanied by production of lower vitamin B_{12} amounts in comparison to standard medium. This observation underlined the finding that the standard medium is the most appropriate for vitamin B_{12} production. As in the case of *Terrabacter* sp. DSM 102553, the achieved cobalamin levels are lower than the concentrations reported previously for various *P. freudenreichii* strains (Chamlagain et al. 2016; Chamlagain et al. 2018). However, it is worth mentioning, that the values described for *P. freudenreichii* were obtained after optimization of the production process, while the current study describes the first attempts on vitamin B_{12} production with *Hyphomicrobium* sp. DSM 3646. Moreover, as already mentioned above, the completely aerobic cultivation process for vitamin B_{12} production with *Hyphomicrobium* sp. DSM 3646 is advantageous in comparison to the two-step cultivation procedure necessary for vitamin B_{12} synthesis with *P. freudenreichii* (Chamlagain et al. 2016; Chamlagain et al. 2018; Deptula et al. 2017).

Production of active vitamin B_{12} with *Hyphomicrobium* sp. DSM 3646 was demonstrated in this work, which makes it attractive for possible applications in the food fortification field. The use of low-cost mineral medium with low methanol concentrations for vitamin B_{12} production with *Hyphomicrobium* sp. DSM 3646 can contribute to the development of an economically viable process. The described cultivation process necessary for vitamin B_{12} production with *Hyphomicrobium* sp. DSM 3646 is also very fast and easy-to-handle. Finally, among all producers described for the first time in this work, *Hyphomicrobium* sp. DSM 3646 demonstrated the highest vitamin B_{12} production capability, which makes it the most promising candidate for biotechnological vitamin B_{12} production.

5. Conclusions and Outlook

Due to the growing demand for vitamin B_{12} and, therefore, new vitamin B_{12} sources, the main goal of this work was a detailed characterization and comparison of vitamin B_{12} production capability of synthesizing species earlier described in literature and strains newly identified in this work.

This was achieved with the help of a fast, sensitive and robust LC-MS/MS method developed in this study, which enabled quantification of very low cobalamin amounts and differentiation between the active and inactive vitamin B_{12} forms.

Among the previously described vitamin B_{12}-synthesyzing strains, *P. freudenreichii* and *M. extorquens* were identified as the most efficient producers, which allowed to develop new strategies for the search for the mutants with enhanced vitamin B_{12} production and identification of new candidate strains.

M. extorquens was identified as an appropriate strain to be applied for the proposed strategy for the search for the mutants with enhanced vitamin B_{12} production and different substances were tested as possible agents to be applied for the selection of *M. extorquens* mutants. Although various cobalamin derivatives and analogues of the precursors of vitamin B_{12} synthesis were used in the experiments, no possible selection agent matching the proposed searching strategy could be identified. Investigations on the transport of possible selection agents into the cells of *M. extorquens* and their affinity for cobalamin-dependent EMCP enzymes can be performed to provide better information on the mechanisms of interaction between these compounds and vitamin B_{12}-dependent methylmalonyl-CoA mutase and ethylmalonyl-CoA mutase. Moreover, further vitamin B_{12} derivatives such as vitamin B_{12} amides or other vitamin B_{12} monoacids can be tested as possible selection agents.

On the contrary, the proposed strategy for the identification of new vitamin B_{12}-producing microorganisms via the search for the BluB/CobT2 fusion protein homologues resulted in the description of bacteria with the ability to produce active vitamin B_{12} under aerobic conditions. The analysis identified *Terrabacter* sp. DSM 102553 as a promising strain which demonstrated a fast growth in the tested media and produced exclusively the active form of vitamin B_{12}. Moreover, M9 medium was identified as a cheap mineral medium appropriate for the synthesis of higher vitamin B_{12} concentrations than those achieved in the peptone-based media with *Terrabacter* sp. DSM 102553.

The second suggested strategy for the identification of the microorganisms with the active EMCP as possible new vitamin B_{12} producing microorganisms was also successful and enabled identification of microorganisms capable of active vitamin B_{12} production. Analysis performed with the LC-MS/MS

method developed in this study revealed production of the active B_{12} form in the cells of all selected strains with *Hyphomicrobium* sp. DSM 3646 being the most promising strain. Further investigation on the medium composition optimization resulted in biomass yields significantly higher than in the initial medium. Nevertheless, the highest vitamin B_{12} content was obtained after the cultivation of *Hyphomicrobium* sp. DSM 3646 in the standard minimal methanol medium.

The obtained results propose that the identified strains might be successfully used for biotechnological vitamin B_{12} synthesis, while the developed analytical method is a reliable tool for the analysis of low vitamin B_{12} amounts in food sources. Further studies can focus on optimization of the cultivation conditions and vitamin B_{12} amounts produced with newly identified *Terrabacter* sp. DSM 102553 and *Hyphomicrobium* sp. DSM 3646 in controlled bioreactor experiments. After optimization of *Terrabacter* sp. DSM 102553 and *Hyphomicrobium* sp. DSM 3646 productivity against the commonly used strains the economic benefits of vitamin B_{12} production processes with these strains can be evaluated in future studies. Moreover, although *Terrabacter* sp. DSM 102553 and *Hyphomicrobium* sp. DSM 3646 are not included in the GRAS (FDA) or QPS (EFSA) list, further investigations on their safety can be made to check the possibility to include these strains into the mentioned lists and possibly use them for food fortification.

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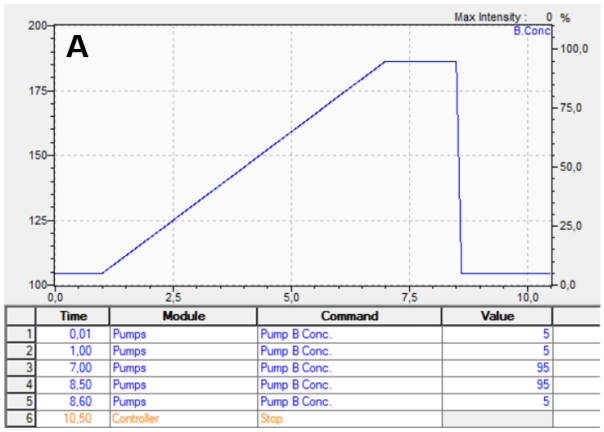
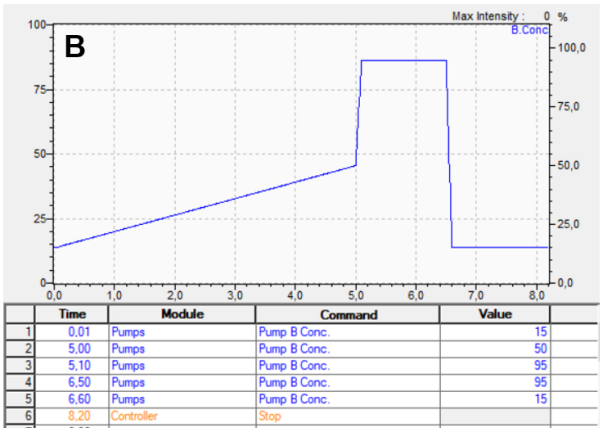
7. Appendix

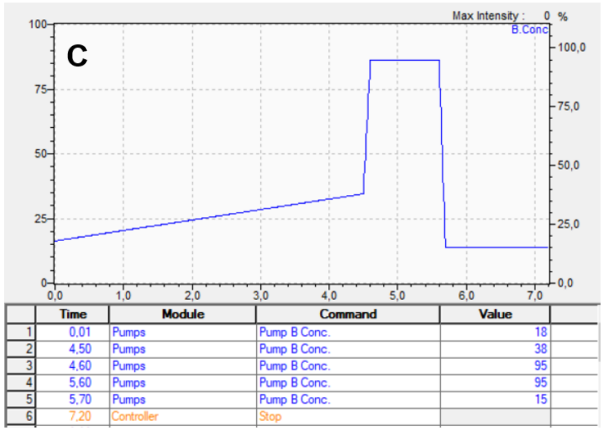
1. Optimization steps performed during the development of LC-program for the analysis of CNCbl, MetCbl, AdoCbl



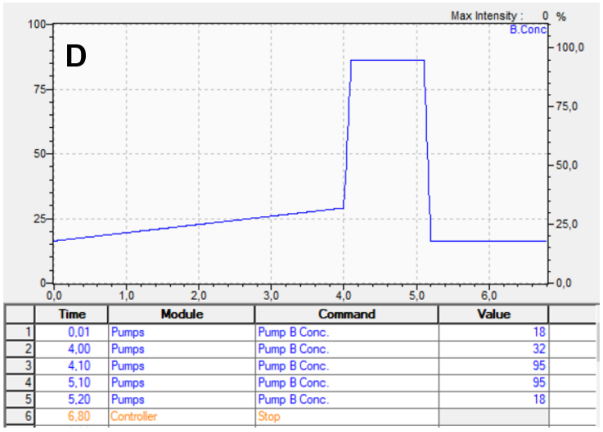
	Time	Module	Command	Value
1	0,01	Pumps	Pump B Conc.	5
2	1,00	Pumps	Pump B Conc.	5
3	7,00	Pumps	Pump B Conc.	95
4	8,50	Pumps	Pump B Conc.	95
5	8,60	Pumps	Pump B Conc.	5
6	10,50	Controller	Stop	



	Time	Module	Command	Value
1	0,01	Pumps	Pump B Conc.	15
2	5,00	Pumps	Pump B Conc.	50
3	5,10	Pumps	Pump B Conc.	95
4	6,50	Pumps	Pump B Conc.	95
5	6,60	Pumps	Pump B Conc.	15
6	8,20	Controller	Stop	

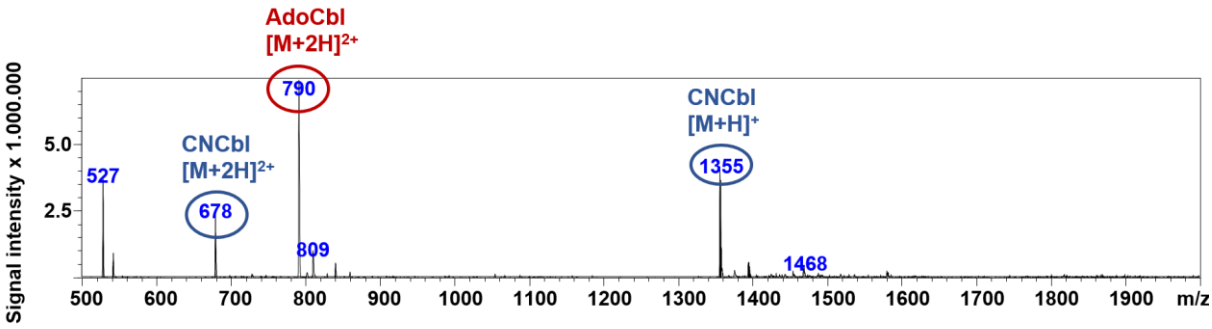


	Time	Module	Command	Value
1	0,01	Pumps	Pump B Conc.	18
2	4,50	Pumps	Pump B Conc.	38
3	4,60	Pumps	Pump B Conc.	95
4	5,60	Pumps	Pump B Conc.	95
5	5,70	Pumps	Pump B Conc.	15
6	7,20	Controller	Stop	



	Time	Module	Command	Value
1	0,01	Pumps	Pump B Conc.	18
2	4,00	Pumps	Pump B Conc.	32
3	4,10	Pumps	Pump B Conc.	95
4	5,10	Pumps	Pump B Conc.	95
5	5,20	Pumps	Pump B Conc.	18
6	6,80	Controller	Stop	

2. Mass spectrum of the peak detected at 4.3 min during the first step of the development of LC-method for the analysis of CNCbl, MetCbl, AdoCbl. Masses corresponding to the single and double charged CNCbl ion are marked in blue, mass corresponding to the single charged AdoCbl ion is marked in red



3. Sequence of BluB/CobT2 fusion protein from *P. freudenreichii* subsp. *shermanii* CBL56167.1

>CBL56167.1 Phosphoribosyltransferase/nitroreductase (fusion gene)
(Nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase)
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1]

MSDEARDPETTNPADEPLPGDHLFERPVPNVGDDSSVNERDRDVSGWAFDQATQAALDRVIGARRDIRRF
RPDPVSDELVREVLNAGHGGPSVGQSQPWRFIIVKDRRTRERAALMSDRERMRQSRQLTAERSQRLLDLQ
LEGIREAPLGIVVACDRRAPAAGVLGRNTFHDADMWSCAAAIENMWLTARALGLGMGWVTLMQPDELAGL
LNLPEGVTTLGWLCLGWPNERPPYPGLERRAWSHKLPLDQVVMTDRWPDNGPEPPVSALAGMAPAEPVES
LITRPIPDDPSSVLWSDVHAPSPQQVVDARDKGEKLLTPPGSLGKLDQALDRLVAASGDQVTGGTLVLVG
ADHPLNAHKVSAFDQSVSRQVMEAALEGRAVGVVTARSAGLDVMVVDAGIDGGPVAGCELARPEDVRGDL
VNTPAMTTADVRRLVTRGRELGARAAERGVVCLGEIGIGNTTIASALACVFTGITPEQAAGIGAGSDAKM
VEHKAEVLRAIFARTDITALRADPALALAEVGGPEFAVLAGVILGAVEAGSTVVLDGLAGSVPALAVVEV
NPAVQSYLIAGQVSREFAHGAVLTRLGLEPLVSLRLRAGEGVGASLATQMLFSGLAVRRQSGRTEE

4. Protein sequences of from the candidate strains identified via BLAST search for the BluB/CobT2 fusion protein homologues from *P. freudenreichii* subsp. *shermanii*

>KJK13062.1 phosphoribosyltransferase [Terrabacter sp. 28]
MSYRRPVPTIGDATSAAERAQAPDAWAMPDDLASLERVVGARRDIRRYRPEPVPDDVLTAVLTAGHRGPS
VGHSQPWRFVVVTEAQTRDTAALMADRCRLRQAAGMAEESARGLLDLRLEGIREAPVGVVVACDRRTPAA
GVLGRATFPDADLWSCAAAIENMWLTARAHGLGLGWVTLFEPDELKELLGLPDGVETLGWLCIGWPDERP
PEPGLERAGWSRRLPLEQVVMRERWTERDAPTSHLRAPDQAHVVGARDRSDDLLTVPGSLGVLDSVLDRV
TALPAVDGGGTLVVAAADHAVTAYGVTAFDPSVTADVARATREGTSMGAVAAAAAGLDVELIDAGIGCSR
GDLVNEDALDETTYAGLLALGRERGRALAPGGPVALGEVGVGNTTVAAAVAAALLDLSADAVVGRGSSAD
SAMVDRKRDVVTRALERVGPGARADEPLDPLEAVRRLGGGELAVLTGVVLGAAEAGGVVVLDGLATSVCA
LAAVRAEPAVAAHLVAGQRSREKAHAAVLHELGLEPLLDLRIRAGEGVGAALATGLVKDALALRRGVART
STV

>WP_089338383.1 5,6-dimethylbenzimidazole synthase [Blastococcus sp. DSM 44272]
MTQPGHPWPRPVPLVGDATAASERAADPTAWRLPPGTRAGVYEAIGARRDVRRFRPDPVPDEVLERVLGA
AHAAPSVGHSQPWRFLVVRDPGIRDRAAVLTDRERLRQAERLEADAARRLLDLQLEGVREAPLGIVVCCD
RRAPAAGVLGRATFPDADLWSCAAAIQNLWLAARAEGLGLGWVTLFRPEDLAELLRLPEGVVTLGWLCLG
WPDERPPAPGLERAGWSRRLPLRDVVLADRWPDEAGAPPPPPSRLRAPDQPAVVGARDEADRLLAVPGSL
GVLDRAVDRAVALGRSAATGGVLVLAAADHPLAAHQVSPYSQSVTGDVLRAAVAGTSLGATAARAVGLEL
RIVDAGVAGDPVPGVTATRPSGYRGDLVNAPAMTLADTRRLVASGRRLGREAGAAGIVALGEVGVANTTV
AAALACGMLGTDADAVIGLGSGADAAMLDRKRGVVSAAVDRARRNHPALGEDPLTALAEVGGPELAVLAG
VAWGAAQARAVVVVDGFAVSLAALVAVQLEPAVQACLVAGQRSRERGHALVLEHLGCEPLLDVRMRAGEG
VGAALATGLLLDGLQLRRGTAQVDR

>WP_245703061.1 5,6-dimethylbenzimidazole synthase [Raineyella antarctica]
MSDDTRERPGAPMTWPRPVPTVGDTTSARTRRDDPAGWGFPEADVDALTRIIDARRDIRRYRPDPVPAAL
VERVVTAGHHGPSVGHSQPWRFIVVGDPGIRDRAAAMADRGRVRQAAGMTEDRAARLLDLKLEGLREAPL
GIVVACDRRVPADGVLGRATFPDADMWSCAAAIENMWLTSRALGLGMGWVTLFEPAELAGLLGLPEGVET
LGWLCLGWPDERPPSPGLERAAWSRRLPVEDVLLHDHWPHADQPARPASHLKVTAPAADRMVEATDGSDE

LLSPPESLGLLDRVLNRITAIGGAALDSGILVLAGADHPVAAYGVSAFPSSAGRDVVEAAAAGRSVGVAA
ATSAGLAHLVIDAGIDGGPVPGVRHVLPSGPRGDLAGSDALGRADVDRMLEAGREIGREVARHGLVAIGE
VGIGNTTVAAALTCALTGLEPGQAVGLGSGADAAMLARKQRVVEAALARWRAGTPAEAPTESVSGHNGVS
ELLAVLGGGEIAVLTGVVLGAVEAGSPVVLDGLAGSLPGLCAARIEPAVQAYLVAGQVSRERAHRVVLDE
LGLEPLLALRMRAGEGVGACLAASMILQGLSVRRLAARTSDA

>WP_141927336.1 5,6-dimethylbenzimidazole synthase [Yimella lutea]
MSEQSAPQRPVPLVGDTTSAQQRRDDPAGWAMLPEVVDALAAVVGGRRDIRRFRPDPVPDELLRQVLEAA
HSAPSVGHSQPWRFIVVRSQTTRDRAAHLADRARLEQASELTSERAARLLDLKLEGLREAPVGVVVTCDR
RTPAAGVLGRATFPDADLWSCACAVQNLWLTARALGLGVGWVTLFDQAELADLLHLPDGVVTLGWLCVGW
PDERPPAPGLERAAWSRKAPLDDVVLQERWPDDSAPPAPPTSHLRGPEPGRRVDGTDDTDRLLSPPEALG
ALDRALHLARAAAGPDLDTGQLVLVGADHPVTAHAVSAYDRSVTRDVLTSAVGGISLGAAHARAAGLDVV
VVDAGVGEAHVEGAVDVRPHDPRGDLVTTDAMSAADVRRLMDAGRRLGAGVPGIAPGLVALGEVGVGNTT
VAAALTCALTGTPAADVVGLGSGADADIVARKTEVVAAAIDRLGHTDDVHRMLAAVGGPEFAVLTGVVLG
AARAGRPVVLDGLATSVAALAAVGIEPSVQAYLIAGQRSREKAHGLVLRRLGLEPLLQLRLRAGEGVGAC
LAAGMVLQGMAARRMTVRTSLRTAQRNA

>WP_115921595.1 5,6-dimethylbenzimidazole synthase [Calidifontibacter
indicus]
MSEQSAPQRPVPLVGDPTSAQQRRDDPAGWAMPPEVVDALAAVVGGRRDIRRFRPDPVPDELLRQVLEAA
HSAPSVGHSQPWRFIVVRSQTTRDRAAHLADRARLEQASELTSERAARLLDLKLEGLREAPVGVVVTCDR
RTPAAGVLGRATFPDADLWSCACAVQNLWLTARALGLGVGWVTLFDQAELADLLHLPDGVVTLGWLCVGW
PDERPPAPGLERAAWSRKAPLDDVVLQERWPHDSAPPAPPTSHLRGPAPGRRVDGTDDTDRLLSPPEALG
ALDRALNLVRAAAGPDLDTGTLVLVGADHPVTAHAVSAYDRSVTRDVLTSAVGGGSLGAAHARAAGLDVV
VVDAGVGEAHVEGAVDVRPHDPRGDLVTTDAMSAADVRRLMDAGRRLGAGLPGLVALGEVGVGNTTVAAA
LTCALTGAAAADVVGLGSGADADIVARKTEVVAAAIDRLGDTDDVHRMLAAVGGPEFAVLTGVVLGAAGA
GRPVVLDGLATSVAALAAVGIEPSVQAYLIAGQRSREKAHGLVLRRLGLEPLLQLRLRAGEGVGACLAAG
MVLQGMAARRTTVRTSARTAQRNA

5. CLUSTAL 2.1 multiple sequence alignment of the BluB/CobT2 fusion protein from *P. freudenreichii* and the corresponding sequences from *C. indicus, Y. lutea, Terrabacter* sp., *Blastococcus* sp., *B. colisei, R. antarctica*

```
KJK13062.1          ----------------------MSYRRPVPTIGDATSAAERAQAPDAWAMP-DDLASLERV
WP_089338383.1      ----------------MTQPGHPWPRPVPLVGDATAASERAADPTAWRLPPGTRAGVYEA
WP_245703061.1      ---------MSDDTRERPGAPMTWPRPVPTVGDTTSARTRRDDPAGWGFPEADVDALTRI
WP_141927336.1      -----------------MSEQSAPQRPVPLVGDTTSAQQRRDDPAGWAMLPEVVDALAAV
WP_115921595.1      -----------------MSEQSAPQRPVPLVGDPTSAQQRRDDPAGWAMPPEVVDALAAV
CBL56167.1          MSDEARDPETTNPADEPLPGDHLFERPVPNVGDDSSVNERDRDVSGWAFDQATQAALDRV
                                         **** :** ::.  *     .* :      .:

KJK13062.1          VGARRDIRRYRPEPVPDDVLTAVLTAGHRGPSVGHSQPWRFVVVTEAQTRDTAALMADRC
WP_089338383.1      IGARRDVRRFRPDPVPDEVLERVLGAAHAAPSVGHSQPWRFLVVRDPGIRDRAAVLTDRE
WP_245703061.1      IDARRDIRRYRPDPVPAALVERVVTAGHHGPSVGHSQPWRFIVVGDPGIRDRAAAMADRG
WP_141927336.1      VGGRRDIRRFRPDPVPDELLRQVLEAAHSAPSVGHSQPWRFIVVRSQTTRDRAAHLADRA
WP_115921595.1      VGGRRDIRRFRPDPVPDELLRQVLEAAHSAPSVGHSQPWRFIVVRSQTTRDRAAHLADRA
CBL56167.1          IGARRDIRRFRPDPVSDELVREVLNAGHGGPSVGQSQPWRFIIVKDRRTRERAALMSDRE
                    :..***:**:**:**.  ::  *: *.* .****:******::* .   *: ** ::**

KJK13062.1          RLRQAAGMAEESARGLLDLRLEGIREAPVGVVVACDRRTPAAGVLGRATFPDADLWSCAA
WP_089338383.1      RLRQAERLEADAARRLLDLQLEGVREAPLGIVVCCDRRAPAAGVLGRATFPDADLWSCAA
WP_245703061.1      RVRQAAGMTEDRAARLLDLKLEGLREAPLGIVVACDRRVPADGVLGRATFPDADMWSCAA
WP_141927336.1      RLEQASELTSERAARLLDLKLEGLREAPVGVVVTCDRRTPAAGVLGRATFPDADLWSCAC
WP_115921595.1      RLEQASELTSERAARLLDLKLEGLREAPVGVVVTCDRRTPAAGVLGRATFPDADLWSCAC
CBL56167.1          RMRQSRQLTAERSQRLLDLQLEGIREAPLGIVVACDRRAPAAGVLGRNTFHDADMWSCAA
                    *:.*:  :  : :  ****:***:****:*:** ****.** ***** ** ***:****.

KJK13062.1          AIENMWLTARAHGLGLGWVTLFEPDELKELLGLPDGVETLGWLCIGWPDERPPEPGLERA
WP_089338383.1      AIQNLWLAARAEGLGLGWVTLFRPEDLAELLRLPEGVVTLGWLCLGWPDERPPAPGLERA
WP_245703061.1      AIENMWLTSRALGLGMGWVTLFEPAELAGLLGLPEGVETLGWLCLGWPDERPPSPGLERA
WP_141927336.1      AVQNLWLTARALGLGVGWVTLFDQAELADLLHLPDGVVTLGWLCVGWPDERPPAPGLERA
WP_115921595.1      AVQNLWLTARALGLGVGWVTLFDQAELADLLHLPDGVVTLGWLCVGWPDERPPAPGLERA
CBL56167.1          AIENMWLTARALGLGMGWVTLMQPDELAGLLNLPEGVTTLGWLCLGWPNERPPYPGLERR
                    *::*:**::** ***:*****:   :*  ** **:** ******:***:**** *****
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KJK13062.1          GWSRRLPLEQVVMRERWTERD------------------------------APTSHLRA
WP_089338383.1      GWSRRLPLRDVVLADRWPDEAG-A--------------------------PPPPPSRLRA
WP_245703061.1      AWSRRLPVEDVLLHDHWPHADQPA-------------------------RPASHLKVTA
WP_141927336.1      AWSRKAPLDDVVLQERWPDDSAP---------------------------PAPPTSHLRG
WP_115921595.1      AWSRKAPLDDVVLQERWPHDSAP---------------------------PAPPTSHLRG
CBL56167.1          AWSHKLPLDQVVMTDRWPDNGPEPPVSALAGMAPAEPVESLITRPIPDDPSSVLWSDVHA
                    .**:: *: :*:: ::*..                                  : .

KJK13062.1          PDQAHVVGARDRSDDLLTVPGSLGVLDSVLDRVTALP-AVDGGGTLVVAAADHAVTAYGV
WP_089338383.1      PDQPAVVGARDEADRLLAVPGSLGVLDRAVDRAVALGRSAATGGVLVLAAADHPLAAHQV
WP_245703061.1      PAADRMVEATDGSDELLSPPESLGLLDRVLNRITAIGGAALDSGILVLAGADHPVAAYGV
WP_141927336.1      PEPGRRVDGTDDTDRLLSPPEALGALDRALHLARAAAGPDLDTGQLVLVGADHPVTAHAV
WP_115921595.1      PAPGRRVDGTDDTDRLLSPPEALGALDRALNLVRAAAGPDLDTGTLVLVGADHPVTAHAV
CBL56167.1          PSPQQVVDARDKGEKLLTPPGSLGKLDQALDRLVAASGDQVTGGTLVLVGADHPLNAHKV
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KJK13062.1          TAFDPSVTADVARATREGTSMGAVAAAAAGLDVELIDAGIG-------------CSRGDL
WP_089338383.1      SPYSQSVTGDVLRAAVAGTSLGATAARAVGLELRIVDAGVAGDPVPGVTATRPSGYRGDL
WP_245703061.1      SAFPSSAGRDVVEAAAAGRSVGVAAATSAGLAHLVIDAGIDGGPVPGVRHVLPSGPRGDL
WP_141927336.1      SAYDRSVTRDVLTSAVGGISLGAAHARAAGLDVVVVDAGVGEAHVEGAVDVRPHDPRGDL
WP_115921595.1      SAYDRSVTRDVLTSAVGGGSLGAAHARAAGLDVVVVDAGVGEAHVEGAVDVRPHDPRGDL
CBL56167.1          SAFDQSVSRQVMEAALEGRAVGVVTARSAGLDVMVVDAGIDGGPVAGCELARPEDVRGDL
                    :.:  *.  :*  ::  * ::*.. * :.**   ::***:              ****

KJK13062.1          VNEDALDETTYAGLLALGRERGRALAP--GGPVALGEVGVGNTTVAAAVAAALLDLSADA
WP_089338383.1      VNAPAMTLADTRRLVASGRRLGREAGA--AGIVALGEVGVANTTVAAALACGMLGTDADA
WP_245703061.1      AGSDALGRADVDRMLEAGREIGREVAR--HGLVAIGEVGIGNTTVAAALTCALTGLEPGQ
WP_141927336.1      VTTDAMSAADVRRLMDAGRRLGAGVPGIAPGLVALGEVGVGNTTVAAALTCALTGTPAAD
WP_115921595.1      VTTDAMSAADVRRLMDAGRRLGAGLP----GLVALGEVGVGNTTVAAALTCALTGAAAAD
CBL56167.1          VNTPAMTTADVRRLVTRGRELGARAAER--GVVCLGEIGIGNTTIASALACVFTGITPEQ
                    .   *:  :    ::  **. *        * *.:**:*:.***:*:*::. : .  .

KJK13062.1          VVGRGSSADSAMVDRKRDVVTRALERVGPGARADEPLDPLE-------AVRRLGGGELAV
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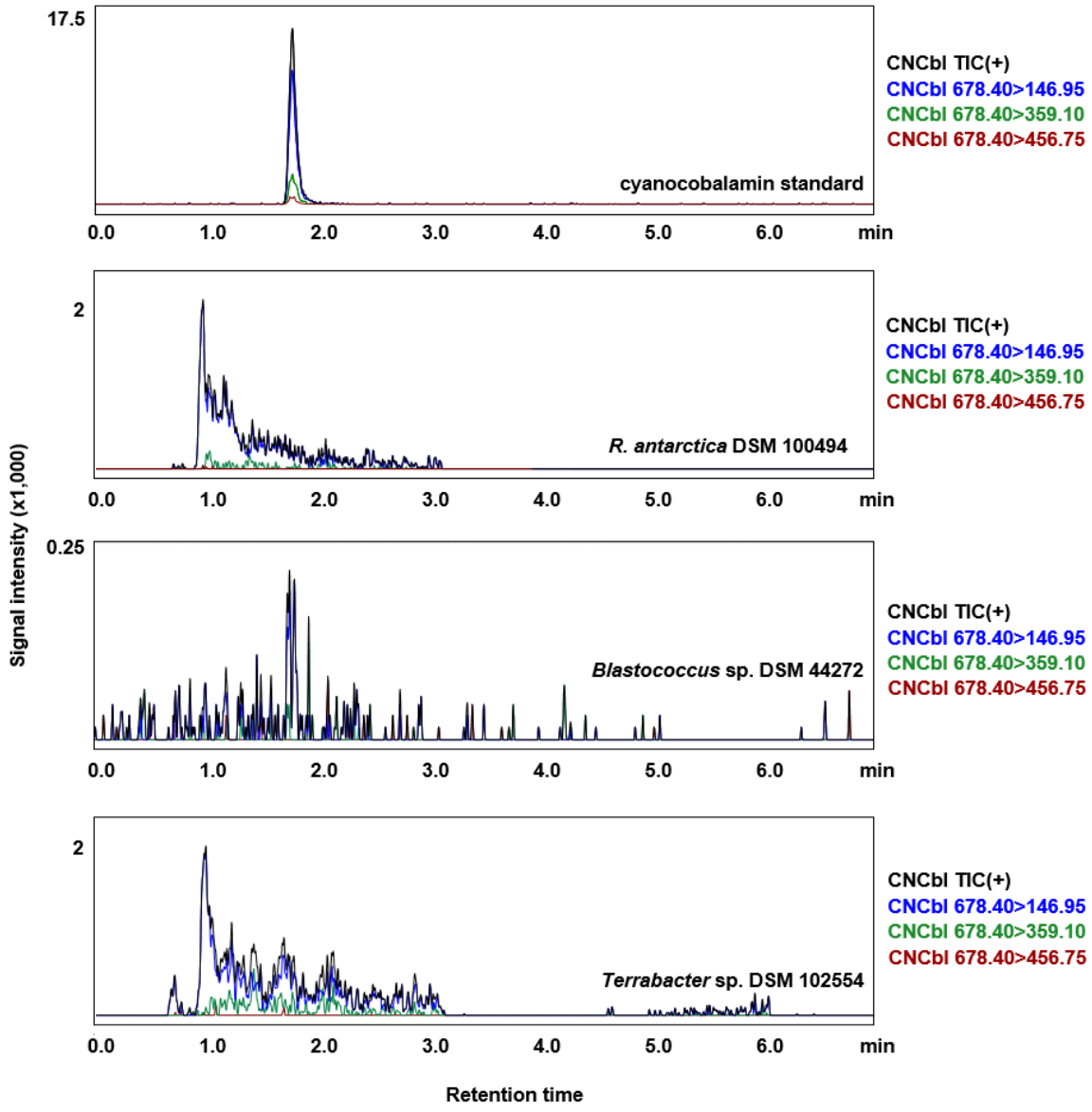
```
WP_089338383.1      VIGLGSGADAAMLDRKRGVVSAAVDRARRNHPALG-EDPLT-------ALAEVGGPELAV
WP_245703061.1      AVGLGSGADAAMLARKQRVVEAALARWRAGTPAEAPTESVSGHNGVSELLAVLGGGEIAV
WP_141927336.1      VVGLGSGADADIVARKTEVVAAAIDRLGHTDDVHR-------------MLAAVGGPEFAV
WP_115921595.1      VVGLGSGADADIVARKTEVVAAAIDRLGDTDDVHR-------------MLAAVGGPEFAV
CBL56167.1          AAGIGAGSDAKMVEHKAEVLRAIFARTDITALRADP----------ALALAEVGGPEFAV
                    . * *:.:*: :: :*  *:   . *                     :  :** *:**

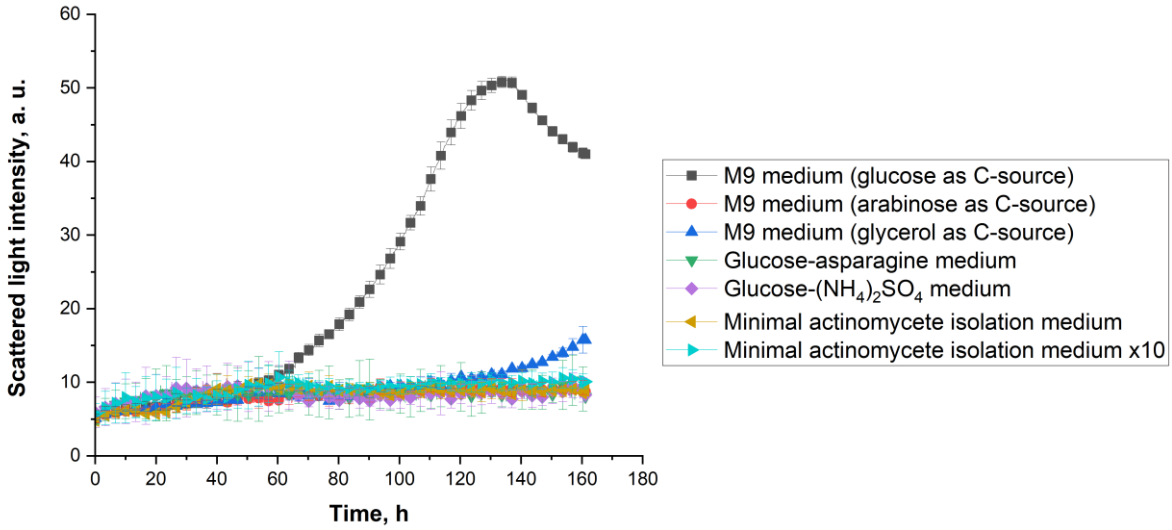
KJK13062.1          LTGVVLGAAEAGGVVVLDGLATSVCALAAVRAEPAVAAHLVAGQRSREKAHAAVLHELGL
WP_089338383.1      LAGVAWGAAQARAVVVVDGFAVSLAALVAVQLEPAVQACLVAGQRSRERGHALVLEHLGC
WP_245703061.1      LTGVVLGAVEAGSPVVLDGLAGSLPGLCAARIEPAVQAYLVAGQVSRERAHRVVLDELGL
WP_141927336.1      LTGVVLGAARAGRPVVLDGLATSVAALAAVGIEPSVQAYLIAGQRSREKAHGLVLRRLGL
WP_115921595.1      LTGVVLGAAGAGRPVVLDGLATSVAALAAVGIEPSVQAYLIAGQRSREKAHGLVLRRLGL
CBL56167.1          LAGVILGAVEAGSTVVLDGLAGSVPALAVVEVNPAVQSYLIAGQVSREFAHGAVLTRLGL
                    *:**  **. *   **:**:* *: .* ..  :*:* : *:*** *** .*  ** .**

KJK13062.1          EPLLDLRIRAGEGVGAALATGLVKDALALRRGVARTSTV------
WP_089338383.1      EPLLDVRMRAGEGVGAALATGLLLDGLQLRRGTAQVDR-------
WP_245703061.1      EPLLALRMRAGEGVGACLAASMILQGLSVRRLAARTSDA------
WP_141927336.1      EPLLQLRLRAGEGVGACLAAGMVLQGMAARRMTVRTSLRTAQRNA
WP_115921595.1      EPLLQLRLRAGEGVGACLAAGMVLQGMAARRTTVRTSARTAQRNA
CBL56167.1          EPLVSLRLRAGEGVGASLATQMLFSGLAVRRQSGRTEE-------
                    ***: :*:********.**: :: ..:  **   :..
```

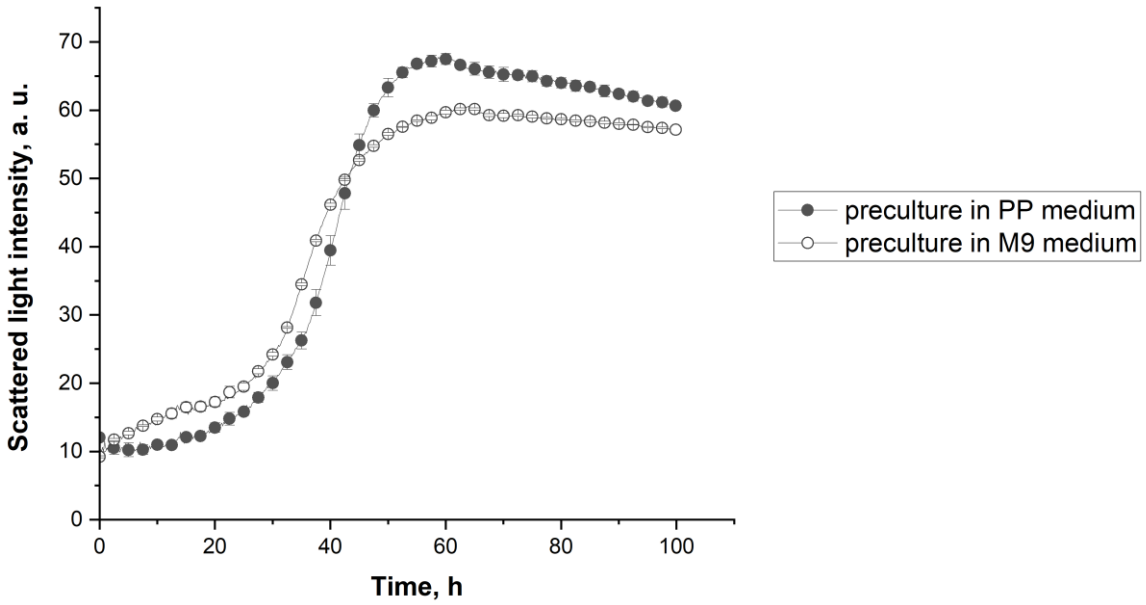
6. LC-MS/MS chromatograms of the cyanocobalamin reference compound and the cell extracts of *R. antarctica* DSM 100494, *Blastococcus* sp. DSM 44272 and *Terrabacter* sp. DSM 102554. Shown are representative LC-MS/MS chromatograms of the cyanocobalamin reference compound and of the cell extracts of the selected strains



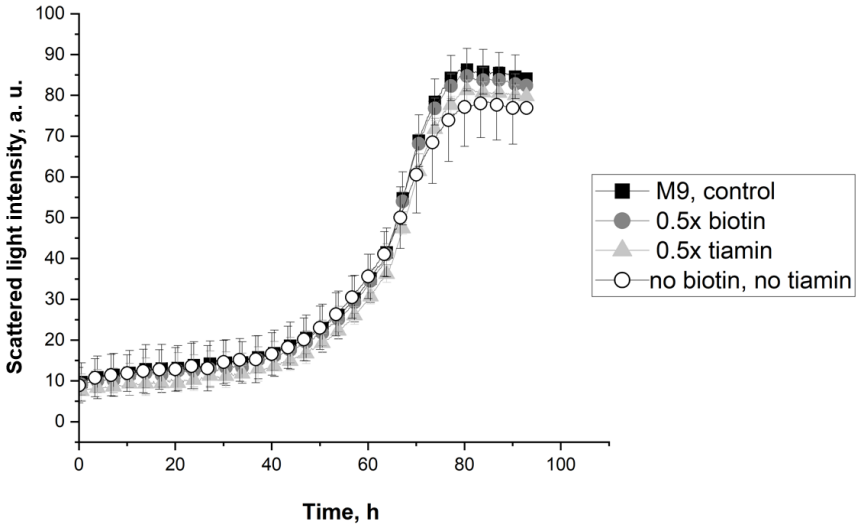
7. Identification of the mineral defined medium appropriate for the growth of *Terrabacter* sp. DSM 102553. The cultivations were performed in the Biolector® microbioreactor and the growth was monitored online by scattered light intensity. The data points represent the mean values and standard deviations of three biological replicates



8. Impact of the pre-cultural medium on the lag-phase duration of the main culture grown in M9 medium. 3-day-old precultures grown in PP or M9 medium were used for inoculation, M9 medium containing 0.24 mM $CaCl_2$ was used for the cultivation of the main cultures. The cultivations were performed in the Biolector® microbioreactor and the growth was monitored online by scattered light intensity. The data points represent the mean values and standard deviations of three biological replicates



9. Influence of biotin and thiamine on the growth of *Terrabacter* sp. DSM 102553. The cultivations were performed in the Biolector® microbioreactor and the growth was monitored online by scattered light intensity. The data points represent the mean values and standard deviations of three biological replicates



10. Sequence of crotonyl-CoA carboxylase/reductase from *M. extorquens* AM1 ACS38140.1

>ACS38140.1 crotonyl-CoA carboxylase/reductase [Methylorubrum extorquens AM1]

MAASAAPAWTGQTAEAKDLYELGEIPPLGHVPAKMYAWAIRRERHGPPEQSHQLEVLPVWEIGDDEVLVY
VMAAGVNYNGVWAGLGEPISPFDVHKGEYHIAGSDASGIVWKVGAKVKRWKVGDEVIVHCNQDDGDDEEC
NGGDPMFSPTQRIWGYETGDGSFAQFCRVQSRQLMARPKHLTWEEAACYTLTLATAYRMLFGHAPHTVRP
GQNVLIWGASGGLGVFGVQLCAASGANAIAVISDESKRDYVMSLGAKGVINRKDFDCWGQLPTVNSPEYN
TWLKEARKFGKAIWDITGKGNDVDIVFEHPGEATFPVSTLVAKRGGMIVFCAGTTGFNITFDARYVWMRQ
KRIQGSHFAHLKQASAANQFVMDRRVDPCMSEVFPWDKIPAAHTKMWKNQHPPGNMAVLVNSTRAGLRTV
EDVIEAGPLKAM

11. Sequence of isocitrate lyase from *E. coli* P0A9G6

>sp|P0A9G6|ACEA_ECOLI Isocitrate lyase OS=Escherichia coli (strain K12)
OX=83333 GN=aceA PE=1 SV=1
MKTRTQQIEELQKEWTQPRWEGITRPYSAEDVVKLRGSVNPECTLAQLGAAKMWRLLHGESKKGYINSLGALTGG
QALQQAKAGIEAVYLSGWQVAADANLAASMYPDQSLYPANSVPAVVERINNTFRRADQIQWSAGIEPGDPRYVDY
FLPIVADAEAGFGGVLNAFELMKAMIEAGAAAVHFEDQLASVKKCGHMGGKVLVPTQEAIQKLVAARLAADVTGV
PTLLVARTDADAADLITSDCDPYDSEFITGERTSEGFFRTHAGIEQAISRGLAYAPYADLVWCETSTPDLELARR
FAQAIHAKYPGKLLAYNCSPSFNWQKNLDDKTIASFQQQLSDMGYKFQFITLAGIHSMWFNMFDLANAYAQGEGM
KHYVEKVQQPEFAAAKDGYTFVSHQQEVGTGYFDKVTTIIQGGTSSVTALTGSTEESQF