Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Establishing 3-hydroxypropionic acid production pathway in *Bacillus methanolicus* by heterologous expression of malonyl coenzyme A reductase and malonic semialdehyde reductase

Master's thesis in MBIOT5
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Abstract

3-Hydroxypropionic acid is considered among the top ten biobased chemicals with opportunities from renewable carbohydrates. It is currently used as a precursor for multiple chemical compounds to produce consumer goods. It is necessary to establish a new method of 3-hydroxypropionic acid production as the chemical synthesis of the compound use potentially toxic catalyzers which follow harsh production conditions. *Bacillus methanolicus* MGA3, a thermotolerant methylotroph, has a natural pathway for producing the precursor malonyl-CoA from methanol. The genes involved in methanol conversion in *Bacillus methanolicus* MGA3 has a high methanol conversion rate. This study ascertained that *Bacillus methanolicus* MGA3 was tolerant toward 3-hydroxypropionic acid and possessed no degradation pathways for the compound. A production host of 3-hydroxypropionic acid is dependent on malonyl coenzyme A reductase (MCR) (and malonic semialdehyde reductase (MSR)) for being capable of 3-hydroxypropionic acid production from malonyl-CoA. Recombinant strains of *Bacillus methanolicus* MGA3 expressing malonyl coenzyme A reductase (MCR) and malonic semialdehyde reductase (MSR) were created, and catalytic activity up to 0.57 U mg^{-1} protein was observed. The introduction of additional genetic expressions is conducted to optimize the inherent catalytic activity and outcompete competing pathways. The reduction of malonyl-CoA to 3-hydroxypropionic acid uses NADPH as a cofactor which cytoplasmic soluble transhydrogenase (SthA) regulates. Recombinant strains introduced with cytoplasmic soluble transhydrogenase (SthA) activities were also assembled to evaluate the plausibility of optimized production of 3-hydoxypropionic acid in *Bacillus methanolicus* MGA3. This work has shown the potential of 3-hydroxypropionic acid production in *Bacillus methanolicus* MGA3.

Sammendrag

3-Hydroxypropionsyre regnes å være en av de mest sentrale biobaserte kjemikaliene som kan produseres fra fornybare karbohydrater. Syren brukes som en forløper for flere ulike kjemiske forbindelser i produksjon av forbruksvarer. Det er nødvendig å etablere en ny metode for å produsere 3-hydroxypropionsyre siden kjemisk syntese bruker potensielle giftige katalysatorer som kan være skadelig for mennesker. *Bacillus methanolicus* MGA3 er en termotolerant metylotrof som produserer malonyl-CoA naturlig fra metanol. Genene som er involvert i prosesseringen av metanol i *Bacillus methanolicus* MGA3 har en høy omgjøringsrate. Dette studiet har vist at *Bacillus methanolicus* MGA3 tåler å være i miljøer med 3-hydroxypropionsyre og at den mikrobielle organismen ikke kan degradere forbindelsen. Det er nødvendig at en produksjonsvert av 3-hydroxypropionsyre har malonyl coenzym A reduktase (MCR) (og malonat semialdehyd reduktase (MSR)) for å produsere 3-hydroxypropionsyre fra malonyl-CoA. I dette studiet har det blitt satt sammen rekombinante stammer av *Bacillus methanolicus* MGA3 som uttrykker malonyl coenzym A reduktase (MCR) og malonat semialdehyd reduktase (MSR), med enzym aktivitet opp til 0.57 U mg^{-1}. Flere gener kan bli introdusert for å optimalisere produksjonen av 3-hydroxypropionsyre. Slike gener kontrollerer aktiviteten av enzymer som bidrar til å forbedre katalytisk aktivitet og utkonkurrerer sideliggende synteseveier. Membran løselig transhydrogenase (SthA) regulerer NADPH som brukes når malonyl-CoA reduseres til 3-hydroxypropionsyre. Rekombinante stammer av *Bacillus methanolicus* MGA3 har blitt satt sammen med membran løselig transhydrogenase (SthA). Dette ble gjort med bakgrunn i å evaluere muligheten for utbedret produksjon av 3-hydroxypropionsyre i *Bacillus methanolicus* MGA3.

Acknowledgement

I want to thank Trygve Brautaset for letting me be part of his cell factory group at NTNU. A special thanks to my supervisor Marta Katarzyna Irla and my co-supervisor May Laura Kilano Khider, who has been remarkable in guiding me through a whole year of laboratory work and leading me in the right direction in writing my thesis. I also want to thank Luciana Fernandes de Brito for serving her knowledge with HPLC, in addition, to always being available to answer questions. When the problems occurred regarding the HPLC, Kåre Andre Kristiansen made persistent attempts with LC-MS and suggested methods to try for HPLC, thank you so much for taking your time helping me on my project. I also want to thank Helga Ertesvåg for giving me genomic DNA of wild type *Azotobacter vinelandii*. The mood within the cell factory group and in the laboratory in general has been remarkable, making me look forward to each day this year. Thank you for being such good colleagues and friends.

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Table of content

Abbreviations

3-HP – 3-hydroxypropionic acid

ACC – Acetyl-CoA carboxylase

AOD – Alcohol oxidase

BSA – Bovine serum albumin

CRISPRi – Clustered Regulatory Interspaced Short Palindromic Repeats interference

DHAP – Dihydroxyacetone phosphate

F6P – Fructose 6-phosphate

FAD – Flavin adenine dinucleotide

FBF – Fructose-1,6-biphosphate

FBPA – Fructose-1,6-biphosphatase

GABA– γ-aminobutyric acid

GAP – Glyceraldehyde-3phosphate

GapN – Glyceraldehyde-3-phosphate dehydrogenase

GFP – Green fluorescent protein

H6P – Hexulose-6-phosphate

HCC – 3-hydroxipropionic acid/4-hydroxypropionic acid cycle

HPS – 3-hexulose 6-phosphate synthase

LC-MS – Liquid chromatography-mass spectrometry

MCR – Malonyl coenzyme A reductase

MCR-C – C-terminal part of MCR

MCR-N – N-terminal part of MCR

MDH – methanol dehydrogenase

MSR – Malonic semialdehyde reductase

NAD(P) – Nicotinamide adenine dinucleotide phosphate

NAD^+ – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate hydrogen

PHI – 6-phospho-3-hexuloisomerase

PKF – Phosphofructokinase

PntAB – membrane bound nucleotide transhydrogenase

PQQ – Pyrroloquinoline

Ru5P – Ribulose 5-phosphate

RuMP – Ribulose monophosphate

sfGFP – Superfolder green fluorescent protein

SthA – Cytoplasmic soluble nucleotide transhydrogenase

XuMP – Xylose monophosphate

1 Introduction

Organic synthesis methods were developed in the 20th century to use oil to produce synthetic polymers, which today have a central role in the industry, such as food, pharmaceutics, and other consumer goods [1]. White biotechnology, also known as industrial biotechnology, is devoted to replacing petroleum-based synthetics with processes that use isolated enzymes, cell extracts, or whole organisms as cell factories (See "Cell factories") [1-3]. White biotechnology attracts widespread interest due to the possibility of producing chemicals, drugs, bio-colorants, solvents, bioplastics, vitamins, food additives, and biofuel from renewable sources [4-6]. From a Forward-looking perspective taking advantage of such natural processes can lead to independence from fossil resources and, at the same time, decrease the environmental impact [1]. However, the challenge is to produce high yields while maintaining production costs lower than production from fossil resources [7].

1.1 Cell factories

Cell factories are engineered organisms used for enhanced production of native chemicals or heterologous products. Organisms used in such processes are created by plausible genetic or environmental manipulations or systematic modifications that remove or overexpress genes to regulate the metabolic flux in the engineered organisms [8].

Escherichia coli (*E. coli*) is a primary choice for metabolic engineering and industrial production due to being one of the best characterized microorganisms, possessing an exceptional genetic toolbox, and being relatively easy to cultivate [9, 10]. *E. coli* is a workhorse in the field of biotechnological production. However, there are some disadvantages of its use predominantly for protein production, such as glycosylated proteins, proteins that are difficult to assemble, and proteins that hold several disulfide bonds [11, 12]. In addition, *E. coli* does not grow at temperatures above 45 °C nor in acidic or alkaline environments ($4.5 < pH > 9$) [9], which increases the chances of contamination by other microorganisms and phages that thrive under similar growth conditions [13-15].

Corynebacterium glutamicum (*C. glutamicum*) is also used in biotechnological production, although the genetic toolbox is not comparable with the one of *E. coli* [16]. *C. glutamicum* has been used for industrial production of amino acids [17], such as the flavor enhancer L-glutamate and the food additive L-lysine [16], and is a promising production host for production of other value-added chemicals [16, 18, 19]. A notable obstacle for *C. glutamicum* is the low efficiency in plasmid insertion during genomic integration and subsequent gene editing, owing to its deficient homologous recombination and the difficulties in penetrating its cell wall [20, 21].

Saccharomyces cerevisiae (*S. cerevisiae*) is commonly used to produce numerous consumer goods such as beer, bread, wine, bioethanol, nutraceuticals, and pharmaceuticals [22]. There are several reasons for its widespread use; a well-developed collection of genetic tools, industrial robustness, fermentation capacity, and resistance to stress [23, 24]. However, a few issues have been reported while using *S. cerevisiae* as a production host, including oxidative stress and prolonged fermentation processes at low temperatures [25]. Additionally, *S. cerevisiae* does not tolerate temperatures above 40 °C [26]. The common feature of all these cell factories is that they feed on sugars that are not future-oriented compared to *Bacillus methanolicus* (*B. methanolicus*) MGA3 that uses methanol as a carbon and energy source [9, 16, 22, 27].

1.2 The production host *Bacillus methanolicus* MGA3

There is ongoing interest in finding suitable feedstock for biotechnological production that does not originate from food crops, thus avoiding competition with the food industry. *B. methanolicus* MGA3 is a facultative methylotroph which utilizes methanol, a one-carbon (C_1) compound as a carbon and energy source, next to multi-carbon sugar and sugar alcohol glucose and mannitol, respectively [27, 28]. *B. methanolicus* was isolated from freshwater marsh soil in 1990 and has since emerged as a promising cell factory for the synthesis of recombinant proteins and value-added compounds with methanol as feedstock [29-35]. Its optimal growth at a relatively high temperature (50 °C) leads to decreased likelihood of contamination and reduction in cooling costs during production [36-38]. The presence of methanol in the cultivation broth is an additional factor that can prevent contamination as it is toxic to most organisms [38].

The genome of *B. methanolicus* MGA3 was first sequenced in 2012 and completed in 2014 which has given insight on its metabolic landscape [39, 40]. Continuous research has also made transcriptomic [41, 42], proteomic [43], and metabolomic -data available for this strain [36, 44, 45]. *B. methanolicus* has an extensive toolbox which includes rolling circle- and theta-replicating plasmids for controlled gene overexpression [46, 47], reporter proteins such as superfolder green fluorescent protein (sfGFP) and mCherry [48, 49], and Clustered Regulatory Interspaced Short Palindromic Repeats interference (CRISPRi) for gene silencing [50].

GFP is not a thermostable enzyme and for that reason it is not suited as a reporter protein for *B. methanolicus* MGA3. sfGFP was generated by Frenzel et al. (2018) and proven to be functional as a probe in thermophilic organisms for cell tracking and monitoring of biological processes [48]. CRISPRi is a genetic perturbation technique which sequence-specific repressions or activations. CRISPRi is likely to contribute to an expansion in strain engineering in *B. methanolicus* for simplified selective genetic silencing [51].

Wild type (WT) *B. methanolicus* MGA3 is capable of producing significant titers of L-glutamate and the classical mutant strains of L-lysine [31]. Both L-lysine, one of the nine essential amino acids, and L-glutamate [16], are precursors to several other amino acids and additional compounds [52]. *B. methanolicus* MGA3 has also been engineered to produce cadaverine which is used for polyamide production [32], γ-aminobutyric acid (GABA) which is used as a precursor for bioplastics [33], the flavor and fragrance agent (R)-acetoin [33, 53] and 5-aminovalinate (5AVA) [54]. 5AVA can be used as a precursor for producing several polyamides, plasticizers, and intermediates for bioplastic preparation [35, 55-60]. It has also been confirmed that recombinant strains of *B. methanolicus* MGA3 has the potential of being a production host of C_{30} terpenoids (4,4'-dipolycopene and 4,4'-dihyponeurosporene) which is used in the pharmaceutical industry [61]. The engineered strains of *B. methanolicus* MGA3 cultivated in seaweed extract and mannitol had slightly higher titers of terpenoids than in methanol cultivation [61]. An overview of all compounds produced by WT and recombinant strains of *B. methanolicus* MGA3 with methanol as feedstock is shown in Table 1.1.

Table 1.1: Chemicals produced by *B. methanolicus* MGA3.

Compound	Titer [g L^{-1}]	Fermentation method	Reference
L-glutamate	59	Fed-batch	Brautaset et al. (2010) [31]
L-lysine	65		Brautaset et al. (2010) [31]
Cadaverine	11.3		Nærdal et al. (2015) [32]
γ-aminobutyric acid	9.0		Irla et al. (2017) [33]
(R)-acetoin	0.42 ± 0.01	Flask	Drejer et al. (2020) [34]
5-aminovalerate	0.02 ± 0.002		Fernandes et al. (2021) [54]
C_{30} terpenoids	0.0240 ± 0.001		Hakvåg et al. (2020) [61]

1.3 Methanol as feedstock

Methylotrophic organisms can use methanol for growth and production of various compounds such as proteins and amino acids [27]. Assimilation of methanol in most methylotrophic bacteria begins with its oxidation to formaldehyde (FA). The oxidation of methanol can be catalyzed by pyrroloquinoline (PQQ)-dependent methanol dehydrogenase (MDHs), nicotinamide adenine dinucleotide (NAD^+)-dependent MDHs, or the flavin adenine dinucleotide (FAD)-containing alcohol oxidase (AODs) depending on the organism's electron acceptors. The first two oxidoreductases are mainly found in methylotrophic procaryotes, and the FAD-AODs is found in methylotrophic yeast. FA is highly toxic, and it needs to be rapidly assimilated to prevent its negative effect on cell metabolism. The assimilation of FA can proceed through three different pathways; the ribulose monophosphate (RuMP) pathway and the serine pathway mainly found in procaryotes, and the xylose monophosphate (XuMP) pathway found primarily in yeast [62, 63].

Methanol is assimilated through the RuMP cycle in *B. methanolicus* MGA3. The RuMP cycle generates the three-carbon compound pyruvate, which can enter the tricarboxylic acid (TCA) cycle and be a precursor to a variety of amino acids [31]. The Genome of *B. methanolicus* MGA3 comprises a circular chromosome and two plasmids; pBM19 and pBM69 [40, 41]. The organism is dependent on the pBM19 plasmid for its methylotrophy. The pBM19 plasmid contains the methanol dehydrogenase gene (*mdh)*. *mdh* encodes the nicotinamide adenine dinucleotide phosphate (NAD(P))-dependent MDHs that catalyze the oxidation of methanol to three FAs [41, 64]. In addition, the pBM19 plasmid encodes different enzymes of the RuMP cycle with five genes; *glpX, fba, tkt, pfk* and *rpe* [41, 64].

One FA molecule is assimilated and in combination with ribulose 5-monophosphate (Ru5P), leads to the formation of hexulose-6-phosphate (H6P) catalyzed by 3-hexulose 6-phosphate synthase (HPS) [65, 66]. Next, fructose 6-phosphate (F6P) is formed in a reaction catalyzed by 6-phospho-3-hexuloisomerase (PHI). Phosphofructokinase (PFK) catalyze the conversion of F6P to fructose-1,6-biphosphate (FBP). Fructose-1,6-biphosphatase (FBPA) cleaves FBP which results in formation of glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). Through several glycolytic reactions pyruvate is formed from DHAP. GAP enters the regeneration of the pathway. Pyruvate formed in the RuMP cycle can further be oxidized by pyruvate dehydrogenase leading to acetyl-CoA formation [45, 65].

1.4 3-Hydroxypropionic acid production through the malonyl-CoA pathway

Bozell and Petersen revised a list of top 10 biobased chemical opportunities from renewable carbohydrates based on a 2004 publication by the US Department of Energy, with one of them being 3-hydroxypropionic acid (3-HP) [67, 68]. The compounds were chosen according to specific criteria such as the possibility of conversion technology, economic value, industrial viability, size of markets, and the ability to serve as a precursor for additional value-added compounds.

3-HP is a three-carbon platform chemical that can be synthesized from numerous precursors such as glycerol [69], lactate [70], β-alanine [71], and malonyl-CoA [72]. 3-HP possesses both carboxylic- and hydroxyl groups and works as a precursor for multiple chemical compounds such as 1,3-propanediol, acrylic acid, methyl acrylate, and acrylamide. These compounds are used as raw materials for production of consumer goods and health products [68, 73]. 3-HP can be produced in a well-established traditional chemical synthesis process. Nevertheless, the harsh production conditions and use of potentially toxic catalyzers in chemical synthesis of 3-HP does not meet the sustainable development goals. Therefore, it is found necessary to find new methods of 3-HP production. The malonyl-CoA pathway is one out of three alternatives for production of 3-HP. Malonyl-CoA is reduced to the intermediate malonic semialdehyde and then to 3-HP. The metabolic pathway from malonyl-CoA to 3-HP can be catalyzed by the bifunctional malonyl coenzyme A reductase (MCR) or by two enzymes: MCR and malonic semialdehyde reductase (MSR). Biosynthesis has attracted wide attention in finding an improved production method of 3-HP [74-76]. In some bacteria, 3-hydroxypropionic acid dehydrogenase (HpdH) and (methyl)malonic semialdehyde dehydrogenase (MmsA) or 3-hydroxyisobutyrate dehydrogenase (HbdH-4) have catalytic activity of 3-HP degradation [77].

The genes encoding these enzymes are upregulated by supplementation of growth medium with 3-HP and this process is regulated by the transcriptional regulator protein LysR [77].. An overview of a proposed 3-HP biosynthesis pathway that can be engineered into *B. methanolicus* MGA3 is shown in Figure 1.1.

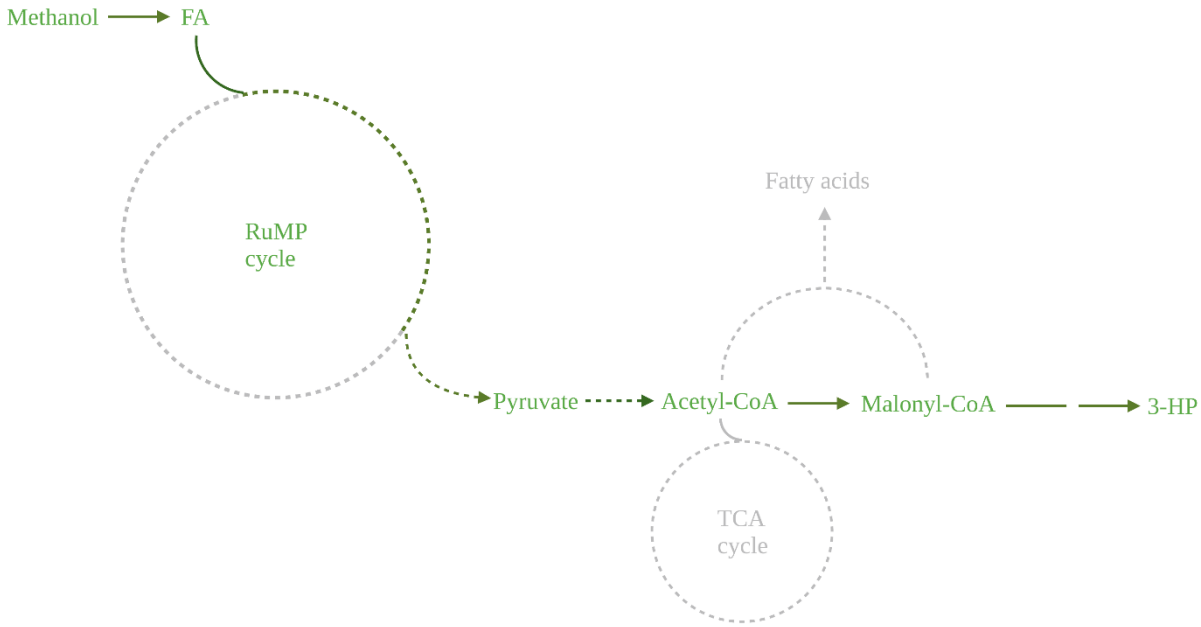

Figure 1.1: Proposed biochemical route for *B. methanolicus* MGA3 of 3-HP production. The methanol is oxidized to FA and then enters the RuMP cycle. From acetyl-CoA the carbon flow is divided into the entrance of the TCA cycle, fatty acid production, and reduction to malonyl-CoA. Two consecutive reductions from malonyl-CoA produce 3-HP. The illustration is created with Biorender.

1.4.1 Malonyl coenzyme A reductase in *Chloroflexus aurantiacus* DSM 636

The catalytic property of the bifunctional MCR was identified in *C. aurantiacus* DSM 636 in 2002 [78]. The bifunctional MCR catalyzes the reduction of malonyl-CoA to 3-HP. In the initial step, malonyl-CoA is reduced to malonic semialdehyde with nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) serving as a cofactor, meaning that reduction of malonyl-CoA to 3-HP requires two moles of NADPH [78]. It is assumed that MCR is only involved in malonyl-CoA reduction as there is no confirmation of MCR activity elsewhere [74].

MCR has been expressed in heterologous hosts such as in *E. coli* [72], *S. cerevisiae* [79], and *Synechococcus elongatus* to establish 3-HP production [80]. Despite the successful strain engineering, the enzyme activity limited the production of 3-HP. The knowledge of the bifunctional nature of MCR, in addition to the recent innovations within synthetic biology has made it possible to split mcr into two distinct functioning fragments; *mcr* (*mcr-C*, amino acids 550-1219) and N-terminal *mcr* (*mcr-N*, amino acids 1-549) [81-83], co-expressed under control

of separate promoters. MCR-C reduces malonyl-CoA to the intermediate malonic semialdehyde, and MCR-N catalyzes the formation of 3-HP from malonic semialdehyde (Figure 1.2). The split MCR has higher catalytic activity than its native counterpart and contributes to increased 3-HP titers [83, 84]. An overview of the reaction mechanism of MCR from *C. aurantiacus* DSM 636 is shown in Figure 1.2.

1.4.2 Malonyl coenzyme A reductase and malonic semialdehyde reductase in autotrophic *Sulfolobales*

Autotrophic members of the *Sulfolobales* such as *Metallosphaera sedula* (*M. sedula*) DSM 5348 and *Saccharolobus solfataricus* (*S. solfataricus*) DSM 1617, previously known as *Sulfolobus solfataricus* P2, also possess an *mcr* gene. There is no significant similarity between *mcr* genes of *M. sedula* DSM 5348 and *S. solfataricus* DSM 1617 and *mcr* of *C. aurantiacus* DSM 636. MCR in autotrophic *Sulfolobales* converts malonyl-CoA to malonic semialdehyde and is not involved further in the process of 3-HP production, which is similar to the function of MCR-C derived from *C. aurantiacus* DSM 636 [75, 76, 83].

The malonic semialdehyde reduction in *M. sedula* DSM 5348 is catalyzed by NADPH-dependent enzyme MSR [85]. *M. sedula* DSM 5348 needs MCR and MSR to convert malonyl-CoA to 3-HP [85]. *Sulfolobales* pass the 3-hydroxypropionic acid/4-hydroxybutyronic acid cycle (HHC), meaning that the *msr* gene is also found in the genome of *S. solfataricus* [86]. Similar to the bifunctional MCR of *C. auranticaus* DSM 636, *mcr* and *msr* from *Sulfolobales* have been transferred to heterologous cell factories to establish a 3-HP production. Even though, the yield was not beneficial in a long-term perspective [80, 87]. An overview of reactions catalyzed by MCR and MSR from *Sulfolobales* is shown in Figure 1.2.

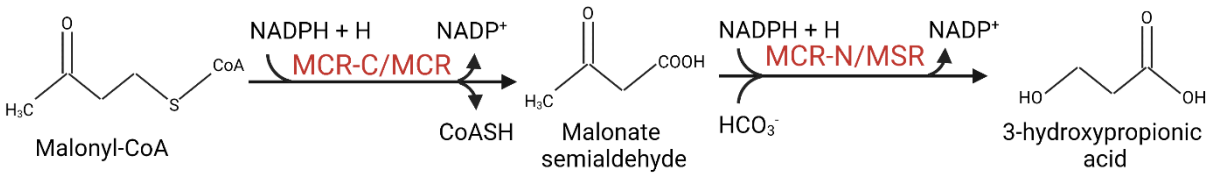

Figure 1.2: Mechanism of bifunctional MCR (MCR-N and MCR-C) and MCR and MSR. The illustration is created with Biorender.

1.4.3 Optimization of 3-hydroxypropionic acid production in recombinant production hosts

There are several ways in how 3-HP production can be improved in recombinant strains. An enzyme with a relatively low catalytic activity can act as a bottleneck and consequently reduce the productivity of the whole pathway as the weakest link of the chain. A solution is to introduce heterologous genes of the rate controlling protein to stabilize flux and increase productivity. Additionally, Metabolic intermediates lost to competing pathways has a negative effect on the efficiency of the desirable pathway [88]. A solution preventing the loss of intermediates is to increase the expression of the genes encoding the pathway for production of the targeted compound [89]. In addition, optimal cofactor regeneration is another issue in genetically modified organisms that limits the metabolic flux of precursors. The cause being the demand of cofactors in the metabolically engineered pathway not matching with the regeneration state of the native cofactor balance [88]. There are methods suitable for cofactor optimization that were shown to contribute to increased yield of high-value byproducts. One such strategy is to overexpress the genes that generate cofactor-producing enzymes or manipulation of transhydrogenase enzymes to balance the oxidation state of NADH/NADPH. Another method replaces the native enzyme with an enzyme of the opposite cofactor specificity. These cofactor adjustments have shown to be a success in recombinant strains such as for *E. coli* [89] and *S. cerevisiae* [73].

1.4.3.1 Cytoplasmic soluble nucleotide transhydrogenase A

E. coli possesses a cytoplasmic soluble nucleotide transhydrogenase (SthA) also known as UdhA that catalyzes the reversible transfer of electrons between NAD^+ and $NADP^+$. SthA balance the NADPH concentration by oxidation (Figure 1.3). This enzyme is catalytically active when more NADPH is present than what is necessary for bacterial growth [90]. A previous study showed that overexpression of *sthA* in recombinant cell factories of *E. coli* decreased the accumulation of acetate and increased the yield of building block chemicals such as poly(3-hydroxybutyrate). This happened due to the high demand of acetyl-CoA when *sthA* balanced NADPH/$NADP^+$ [91].

Figure 1.3: Balancing of NADP/NAD by SthA. The illustration is created with Biorender.

1.4.3.2 Acetyl coenzyme A carboxylase

Acetyl coenzyme A carboxylase (ACC*)* converts acetyl-CoA to malonyl-CoA as shown in Figure 1.4. The reaction catalyzed by ACC is the initial step of the fatty acid biosynthesis in procaryotes; in addition, it is the key-limiting step of the malonyl-CoA pathway. ACC is composed of four subunits, biotin carboxylase (AccA), biotin carboxyl carrier protein (AccB), malonyl-CoA carboxyltransferase 1 (AccC), and malonyl-CoA carboxyltransferase 2 (AccD). ACC works in such a way that AccC transfers CO_2 from bicarbonate to AccB in an ATP-dependent reaction. The active form of AccB requires covalent attachment of biotin assisted by biotinilase (BirA). This action results in the formation of an AccB-CO_2 active structure which convert acetyl-CoA to malonyl-CoA with the assistance of AccA and AccD [92]. Overexpression of ACC was shown to increase the yield of 3-HP in an *E. coli* strain engineered for 3-HP production by outcompeting acetyl-CoA lost to alternative pathways [72, 89].

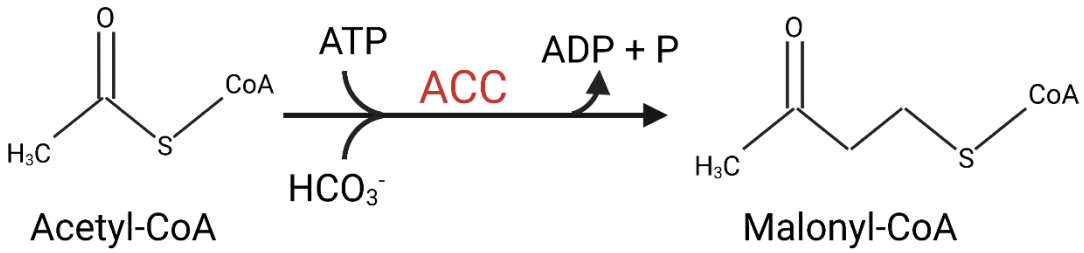

Figure 1.4: Carboxylation of acetyl-CoA to malonyl-CoA by ACC. The illustration is created with Biorender.

1.5 Aim of this project

The aim of this study is to engineer *B. methanolicus* MGA3 for production of 3-HP using the non-food stock C_1 compound methanol. This way, a method for 3-HP production will be established that does not use sugar compounds as feedstock. The 3-HP production strains will be achieved through molecular cloning of *mcr* from *C. aurantiacus* DSM 636 and *mcr* and *msr* from *M. sedula* DSM 5348 and *S. solfataricus* DSM 1617. The cloned genes will be introduced into *B. methanolicus* MGA3 through either electroporation or conjugation using *E. coli* S17-1 cells as conjugant. The so created recombinant *B. methanolicus* MGA3 strains will be analyzed through enzymatic activity of MCR (and MSR) and 3-HP accumulation.

Once production strains are established, the steps will be taken to improve 3-HP yields. To optimize the production of 3-HP in *B. methanolicus* MGA3, additional genes will be expressed in the recombinant strain with the highest 3-HP titers. *sthA* from *E. coli* MG1655 and *Pseudomonas putida* KT2440 will be introduced into production strains in order to restore NADH/NADPH balance. In addition, an attempt at using ACC to improve precursor supply will be performed.

2 Materials & methods

2.1 General protocol for growing *Bacillus methanolicus* MGA3 in liquid culture

All media and restriction enzymes used in this study are shown in section in Appendix A. All primers used in this study are shown in table B.1 in Appendix. *In silico* analysis was conducted with BLAST®. A general protocol was used for all reinoculations in this study. Equation I was used for reinoculation.

$$\frac{Desired\ start\ OD}{Current\ OD} \times Desired\ volume \qquad \text{Equation I}$$

Optical density $(OD)_{600}$ was measured for the preculture while flasks containing new media were prewarmed at 50 °C for *B. methanolicus*. The desired volume for the calculation was six times the volume necessary. The solutions were centrifuged for five minutes at 7830 rounds per minute (rpm), at 40 °C and the supernatant was discarded. The cells were resuspended in 6 ml media and 1 ml was aliquoted to each flask.

2.2 Assessing the toxicity of 3-hydroxypropionic acid towards *Bacillus methanolicus* MGA3

3-HP has not been reported to be a toxic chemical, nonetheless, a test to assess its toxicity on *B. methanolicus* MGA3 was conducted to assure that a recombinant 3-HP-producing strain can survive in presence of the product.

Pre-cultures of WT *B. methanolicus* MGA3 were inoculated at 10:00 am and 04:00 pm in prewarmed MVcMY medium with additives. The cultures were incubated overnight (ON) at 50 °C with shaking at 200 rpm. Pre-cultures were used to inoculate main cultures of 40 ml MVcMY medium with additives and 3-HP (Table 2.2) with an initial OD_{600} of 0.2 calculated with Equation I (see "Media & general information"). The experiment was carried out in triplicates. Start OD_{600} was measured before the flasks were incubated at 50 °C with shaking at 200 rpm. OD_{600} was measured every 2 hours until the majority had no change in OD_{600} between the two last measurements or a decreasing OD_{600}. The outcome of this experiment can be seen in section 3.1 and additional information is found in section C in Appendix.

Table 2.1: Concentrations of 3-HP used in the toxicity assessment.

3-HP [mM]	18.0	15.0	13.0	11.0	9.0	3.6	1.8	0.6	0.0

2.3 Construction of the plasmids pBV2xp-*mcr*, pBV2xp-*mcr-msr* and pTH1mp-*sthA*

Genomic DNA (*mcr* and *msr*), from *C. aurantiacus* DSM 636, *M. sedula* and *S. solfataricus* has been isolated, amplified, and cloned into the vector pTH1mp. The clones were transferred to the host strain *E. coli* DH5α. The same procedure was conducted for genomic DNA (*sthA*) from *E. soli* MG1655 and *P. putida* KT2440 except for cloning into pTH1mp vector. All plasmids, genomic DNA (gDNA), and gene fragments used in this study are shown in Table 2.2. *E. coli* DH5α was used as the general cloning host.

Table 2.2: Plasmids, gDNA, and gene fragments.

Plasmids	**Description**
pBV2xp	Contains a xylose inducible promoter (PxylR) and repressor (XylR), kanamycin and ampicillin resistance by gene *knt* and gene *bla*, a ribosomal binding site (RBS), an origin of replication (*pUC ori*), and two open reading frames (ORFs).
pTH1mp	Contains a methanol dehydrogenase promoter (Pmdh/mp), chloramphenicol and erythromycin resistance by gene *cm-R* and gene *em-R*, and two origins of replication (*ori pTA1060* and *ori puc9*).
gDNA	
$gDNA^{Cau}$	Isolated gDNA from *C. aurantiacus* DSM 636
$gDNA^{Mse}$	Isolated gDNA from *M. sedula* DSM 5348
$gDNA^{Sso}$	Isolated gDNA from *S. solfataricus* DSM 1617
$gDNA^{Eco}$	Isolated gDNA from *E. coli* MG1655
$gDNA^{Ppu}$	Isolated gDNA from *P. putida* KT2440
Fragments	
mcr^{Cau}	*mcr* from *C. aurantiacus* DSM 636
$mcr(GTG)^{Cau}$	*mcr* with GTG start from *C. aurantiacus* DSM 636
$mcr\text{-}N^{Cau}$	N-terminal *mcr* part (amino acids 1-549) from *C. aurantiacus* DSM 636
$mcr\text{-}C^{Cau}$	C-terminal *mcr* part (amino acids 550-1219) from *C. aurantiacus* DSM 636
mcr^{Mse}	*mcr* from *M. sedula* DSM 5348
msr^{Mse}	*msr* from *M. sedula* DSM 5348
mcr^{Sso}	*mcr* from *S. solfataricus* DSM 1617
msr^{Sso}	*msr* from *S. solfataricus* DSM 1617
$sthA^{Eco}$	*sthA* from *E. coli* MG1655
$sthA^{Ppu}$	*sthA* from *P. putida* KT2440

2.3.1 PCR amplification of genes encoding malonyl coenzyme A reductase, malonic semialdehyde reductase, soluble transhydrogenase A and acetyl coenzyme A carboxylase

Polymerase chain reaction (PCR) can, among other things, be used to amplify a region of DNA. The amplification is first initiated when the double-stranded DNA is denatured, and a single-stranded DNA can be used as template. A heat-stable DNA polymerase attaches to the origin of the template DNA guided by a forward primer [93, 94]. The replication stops when the polymerase reaches the position of the reverse primer attached to the template DNA. Each amplification cycle doubles the particular gene sequence [93-95].

Takara Clone Amp Hifi PCR premix was used to amplify the isolated fragments. Volumes of primers, template, and reverse osmosis water (RO-water) were added to the premix according to the Takara Clone Amp Hifi PCR premix protocol. The primers used are Gibson primers, which makes complementary overhangs necessary for ligation by Phusion DNA polymerase during Gibson Assembly (see "Gibson assembly of pBV2xp and pTH1mp with additional isolated fragments"). The amplification program is shown in Table 2.3.

The same method as Liu et al. (2013) was applied when designing Gibson primers to construct the split *mcr* gene from *C. aurantiacus* DSM 636. Liu et al. (2013) determined that the length of *mcr-N* was 549 amino acids, and that *mcr-C* was 670 amino acids. These two primers were designed so that 32 bases complement the end of the genomic sequence, and the remaining bases complement a spacer (5'-AGCCGAGTCAATGGAGCT**AGGAGG**CGCAATAC-3'). The ribosome-binding site (RBS, 5'-AGGAGG-3') locates the start of translation.

Table 2.3: PCR program used to isolate gene sequences for later insertion into vectors.

Temperature [°C]	**Time [seconds]**	**Cycles**
98	10	30
55	15	
72	5 per kb	

Overlap extension PCR (OE-PCR) was conducted to ligate multiple isolated fragments and the protocol is inspired by Kadkhodaei et al. (2016). Two and two fragments were ligated together with an additional PCR to generate a seamless sequence of four fragments. Before another PCR, each PCR product had to be purified (see "Purification & concentration measurements"). Equimolar concentrations of two inserts were added to a 12.5 µl Takara cloneAMP mix to a total volume of 25 µl. The first PCR cycle was without primers. The forward primer of fragment one and the reverse primer of fragment two was included to the PCR reaction past the first PCR run. For additional fragment ligations, the procedure was carried out multiple times. The PCR program for OE-PCR is shown in Table 2.5.

Table 2.5: Program for OE-PCR.

Temperature [°C]	**Time [seconds]**	**Cycles**
First run		
98	10	15
60	15	
72	5 per kb	
Second run		
98	10	20
72	15	
72	5 per kb	

2.3.2 Purification & concentration measurements

Plasmids (pBV2xp and pTH1mp) were isolated using the ZN Plasmid Miniprep - Classic kit protocol with the exception of eluting the plasmids with 50 µl millipore quality (milli-Q) water. Components for digestion of the plasmids can be found in Appendix, in Table A.10. Gel electrophoresis was conducted to ensure successful plasmid isolations and digestions**.** The QIAquick PCR purification kit (250) was used to purify the PCR products according to the manufacturer's protocol with the exception of eluting the plasmids with 30 µl RO-water. The concentration of the isolated plasmids was measured with Thermo Scientific NanoDrop One.

2.3.3 Gibson assembly of pBV2xp and pTH1mp with additional isolated fragments

Gibson cloning is a molecular method that can be used to create constructs of multiple DNA fragments with seamless assembly. T5 exonuclease, Phusion DNA polymerase, and Taq DNA ligase make it possible to construct genetically modified DNA sequences in a one-step interval

at 50 °C for 15 minutes, or at a longer time-interval at lower temperature. The exonuclease has a chew-back mechanism which detaches nucleotides from the 5' ends. The outcome of the removal of nucleotides complementary sticky ends enables annealing of the vector and its inserts. The Phusion DNA polymerase conducts the annealing process. Next, the Taq DNA ligase acts and encloses the modified sequence by sealing the nicks between the fragments to a seamless DNA construct [96]. The isolated fragments were cloned into the sites of cut pBV2xp and cut pTH1mp. Control tubes without inserts were also made which were later used to elucidate successful cloning. The Gibson assembly reactions were incubated for one hour at 37 °C.

2.3.4 Heat shock transformation of *Escherichia coli* DH5α

A heat shock transformation was conducted to introduce the constructs created by Gibson assembly into competent *E. coli* DH5α cells. 100 µl aliquots of competent *E. coli* DH5α cells were set on ice for 10 minutes. 10 µl of the Gibson assembly mixture were added to the competent cells. The solution was incubated on ice for 20 minutes. The competent cells were heat-shocked in a water bath for 45 seconds at 42 °C. The cells were incubated on ice for 2 minutes before 900 µl LB media was added in a sterile environment. The cells were then incubated for 60 minutes at 37 °C with shaking at 200 rpm.

100 µl of cell culture was plated directly on agar plates containing antibiotics while the remaining cell culture was pelleted at 5000 rpm for 2 minutes and resuspended in 100 µl LB media before plating. Antibiotics used were kanamycin (50 µg ml^{-1}) or chloramphenicol (25 µg ml^{-1}) for pBV2xp and pTH1mp constructs, respectively. The agar plates were incubated ON at 37 °C.

2.3.5 Selection of positive clones with colony PCR

Colony PCR can be used to screen colonies carrying the desired construct [97]. A master mix with 5x Green GoTaq reaction buffer, PCR nucleotide mix, primers, GoTaq DNA polymerase, and RO-water was pipetted in PCR tubes as described in the manufacturer's GoTaq PCR protocol. The colony PCR primers used for this study are shown in Appendix, Table B.1. Cells were used as a template in the PCR reaction. An overview of the PCR program is shown in Table 2.4. Potential cells were grown ON in LA-medium and stored at -80 °C in 25 % glycerol.

Table 2.3: PCR program for colony PCR.

Temperature [°C]	Time [minutes]	Cycles
95	2	1
95	1	30
52	1	
72	1 per kb	
72	5	1
4	Indefinite	1

2.4 Sanger sequencing

Sanger sequencing, also known as chain termination sequencing, or dideoxy sequencing, is a molecular method used to read the nucleotides of a DNA sample in an exact order and confirm mutants, insertions, or deletions of a gene. Sanger sequencing works in the way that a DNA polymerase copies the target DNA of a template sequence several times in varying lengths. The final nucleotide for each fragment is targeted with a fluorescent tag, where each of the four nucleotides has their own color. As a final step, all fragments are compared and assembled to a complete read of the target sequence. The sequences to be confirmed can range from a few hundred base pairs to a five-digit number of base pairs [98].

The sequencing of the recombinant strains in this study was conducted by Eurofins Genomics and aligned in Benchling.

2.5 Introduction of *mcr, msr* and *sthA* intro strains of *Bacillus methanolicus* MGA3

Electroporation or conjugation was used as methods of plasmid delivery to create recombinant strains of *B. methanolicus* MGA3.

2.5.1 Electrocompetent *Bacillus methanolicus* MGA3

Electrocompetent cells of *B. methanolicus* MGA3 were made for transformation. *B. methanolicus* MGA3 cells from the -80 C° were brought and harvested in 25 ml of prewarmed Super optimal broth (SOB) media and incubated ON at 50 °C at 200 rpm.

If the ON culture had reached stationary phase, they were reinoculated in 50 ml SOB media and incubated for four hours. Past the potential incubation of four hours, the culture was reinoculated in 4x100 ml SOB media with start OD_{600} of 0.05 (see "General protocol for growing *Bacillus methanolicus* MGA3 in liquid culture"). When the OD_{600} had reached 0.25,

the cultures were centrifuged at 7830 rpm, at 25 °C, for 10 minutes. The cells were resuspended in a 4.5 ml room tempered electroporation buffer (EPB) and centrifuged for another 10 minutes. The cells were resuspended in 9 ml EPB for a final centrifugation of 10 minutes. The supernatant was decanted, the cells were resuspended in the remaining liquid and 100 µl was aliquoted in tubes for storage at -80 °C.

2.5.2 Electroporation of *Bacillus methanolicus* MGA3

Electroporation was conducted to create temporary pores in the cell membrane and introduce plasmid DNA into *B. methanolicus* MGA3. Electrocompetent cells of *B. methanolicus* MGA3 were thawed on ice for 10 minutes before adding 1 µg of plasmid DNA. The cells were incubated on ice for 15 more minutes in a pre-cooled electroporation cuvette. The cells were electroporated using Gene Pulse Xcel with the program shown in Table 2.7.

Table 2.7: Sonication program for *B. methanolicus* MGA3

Voltage [V]	1250
Capacitance [µF]	25
Resistance [Ω]	200
Cuvette [mm]	1

1 ml prewarmed SOB media was added immediately before transferring the cells to 12.5 ml SOB media. The cells were then incubated for a minimum of six hours at 50 °C with shaking at 200 rpm. The cells were centrifuged at 7830 rpm, at 25 °C, for 5 minutes. The cells were resuspended in 100 µl SOB media, plated on SOB agar containing kanamycin (25 µg ml^{-1}), and incubated at 50 °C ON. For co-expression of plasmids with both pBV2xp and pTH1mp, the cells were plated on both kanamycin (25 µg ml^{-1}) and chloramphenicol (5 µg ml^{-1}).

Colonies were picked and resuspended in 25 ml pre-warmed MVcMY medium with additives. The culture was incubated ON at 50 °C with shaking at 200 rpm. The ON culture was stored at -80 °C in a final concentration of 21.75 % glycerol.

2.5.3 Conjugation of *Bacillus methanolicus* MGA3

E. coli S17-1 has previously been used to transfer plasmids to bacteria [99] and was used as another alternative to construct recombinant strains of *B. methanolicus* MGA3. *E. coli* S17-1 has a transfer plasmid, RP4, which transfers the DNA plasmid of interest to the recipient WT *B. methanolicus* MGA3 cells during conjugation. The constructs were inserted by heat shock transformation (see 'Heat shock transformation of clones to *Escherichia coli* DH5α', apart from adding 5 µl construct instead of Gibson assembly master mix). The cell culture containing the pBV2xp vector was plated on kanamycin (50 ug ml^{-1}) plates ON at 37 °C. Cell culture containing the pTH1mp vector was plated on chloramphenicol (25 µg ml^{-1}).

WT *B. methanolicus* MGA3 were grown in 25 ml SOB media ON at 50 °C, in addition, the recombinant *E. coli* S17-1 strain with the gene of interest in LA media with their respective antibiotics, kanamycin (50 µg ml^{-1}) or chloramphenicol (25 µl ml^{-1}) at 37 °C.

The ON cultures were diluted with ratio 1:100 in 25 ml in their respective media. WT *B. methanolicus* MGA3 was incubated at 50 °C for 4 hours, and recombinant *E. coli* S17-1 at 37 °C for two hours. 9 ml WT *B. methanolicus* MGA3 was mixed with 3 ml recombinant *E. coli* S17-1, in addition, 900 µl WT *B. methanolicus* MGA3 with 300 µl recombinant *E. coli* S17-1. WT *B. methanolicus* MGA3 was cooled at room temperature for 15 minutes in advance of mixing with the culture of recombinant *E. coli* S17-1. The cells were spun down at 7830 rpm, for 3 minutes for the 300! µl cell suspension, and for five minutes for the 3! ml cell suspension. The supernatants were decanted, and the cells were resuspended in the remaining media in the flasks. The cell suspension was decanted on non-selective SOB plates without plating out and incubated at 40 °C ON.

The droplet with cell mass was collected and resuspended in 200 µl prewarmed SOB media. 20 µl of the 300! µl sample was mixed with 200 µl prewarmed SOB media. The resuspended cells were plated on selective kanamycin (25 µg ml^{-1}) or co-expressing plates with both chloramphenicol (5 µg ml^{-1}) and kanamycin (25 µg ml^{-1}) plates. Colonies were collected and transferred to MVcMY media with additives, incubated at 50 °C at 200 rpm ON, and stored at -80 °C in 21.75 % glycerol when OD_{600} ~ 2.0. The recombinant strains of *B. methanolicus* MGA3 are shown in section 3.2.

2.6 Catalytic activity measurements of malonyl coenzyme A reductase, malonic semialdehyde reductase and soluble transhydrogenase A in *Bacillus methanolicus* MGA3

Enzyme assay is a quantitative method to measure the catalytic activity of an enzyme [100]. Bradford assay measure concentration of proteins by measuring the change of absorbance of dye (Coomassie Brilliant Blue G-250). Higher protein content gives a darker color [101].

2.6.1 Preparation for enzyme- and Bradford assay

B. methanolicus MGA3 strains were inoculated at 10:00 am and 04:00 pm in prewarmed MVcMY medium with additives. The cultures were incubated ON at 50 °C, at 250 rpm.

The cultures that had OD_{600} between 2-3 were further used. Triplicates for each strain were made in 40 ml prewarmed MVcMY medium with additives with a start OD_{600} of 0.2 (see "Media and general information"). The cultures were incubated at 50 °C, at 200 rpm until the criteria of minimum of two hours incubation and a doubling in OD_{600} were fulfilled. A final concentration of 0.5 % xylose was added and cultures was incubated for another two hours. 20 ml of each culture was centrifuged at 7830 rpm, at 4 °C, for 10 minutes, and washed twice with 50 mM tris hydrochloride (tris-HCl) buffer (pH 7.5). The cells were stored at -80 °C for later use.

2.6.2 Enzyme assay for malonyl-coenzyme A reductase/malonic semialdehyde reductase- and cytoplasmic soluble nucleotide transhydrogenase A activity

The cells were thawed on ice and resuspended in a 50 mM tris-HCl buffer (pH 7.5). The resuspended cells were further lysed with the Fisherbrand Sonic Dismembrator (FB-505). The sonicator had a five-minute program with an amplitude of 25 %, an interval of 2 seconds of pulse, and 1 second pause. The cells were on ice for the whole procedure. The samples were centrifuged for 1 hour at 14 000 g, at 4 °C to remove cell debris. The supernatant was transferred to a new container to be used as crude extract.

SpectraMax Plus 384 Microplate spectrophotometer was used for the assay with the program measuring kinetics, with a time interval of three minutes, measuring every five seconds. The master-mixes were prewarmed at 50 °C. 100 µl crude extract was added and measured first (background), the co-factor was added for a second measurement (reaction).

The spectrophotometer measured at 365 nm when degradation of NADPH was measured. A reaction mix for each sample was prepared containing 750 µl of 50 mM tris HCl-buffer (pH 7.5), 50 µl of 8 mM NADPH, and 50 µl of 100 mM magnesium chloride ($MgCl_2$). The assay is illustrated in Figure 2.1.

Figure 2.1: The reaction is initiated when the cofactor NADPH is included in the solution with malonyl-CoA and the enzyme (crude extract). MCR/MSR activity was validated by measuring the degradation of NADPH. The illustration is created with Biorender.

The spectrophotometer measured at 400 nm when reduction of thio-NAD^+ was measured. A reaction mix for each sample was prepared containing 800 µl of 50 mM tris HCl-buffer (pH 7.5), 50 µl of 2 mM thio-NAD+. The assay is illustrated in Figure 2.2.

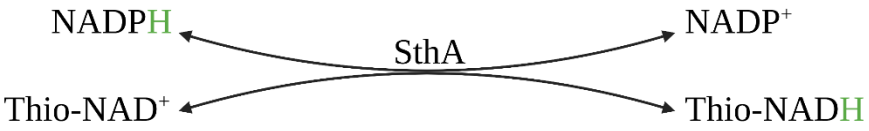

Figure 2.2: The reaction is initiated when NADPH is included in the solution with thio-NAD^+ and the enzyme (crude extract). SthA activity was validated by measuring the reduction of thio-NAD^+. The illustration is created with Biorender.

2.6.3 Bradford assay for protein concentration measurements

750 µl 5X protein assay dye reagent concentrate (Bio-Rad) was diluted five times with milli-Q water and mixed with 50 µl 10x diluted crude extract. 50 µl Bovine serum albumin (BSA) was mixed with 750 µl of the Bio-Rad reagent dye to assemble a standard curve with concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg ml^{-1} BSA. This was conducted according to the Bio Rad™ Quick start™ Bradford Protein Assay protocol.

These measurements were conducted with the SpectraMax Plus 384 Microplate spectrophotometer. The spectrophotometer measured at 595 nm with the program measuring endpoint. The standard solutions assembled a linearly fitted trend line with a formula used to calculate protein content in each sample. The measured catalytic activities can be found in section 3.3 and additional information is found in section D and section E in Appendix.

3 Result

In this study, the assembly of recombinant strains of *B. methanolicus* MGA3 were intended to introduce its capacity to produce 3-HP. Also, future improvements of *B. methanolicus* MGA3 to optimize 3-HP production.

First, it has been tested suitability of *B. methanolicus* MGA3 as a cell factory for production of 3-HP. Next, engineered *B. methanolicus* MGA3 strains has been assembled for 3-HP production. To analyze the newly created strains, the enzyme activity of the heterologous enzymes was measured.

3.1 *Bacillus methanolicus* MGA3 traits beneficial for 3-hydroxypropionic acid production

An *in silico* analysis was conducted to investigate whether a 3-HP degradable pathway is present in the genome of *B. methanolicus* MGA3. A protein BLAST search of (methyl)malonic-semialdehyde dehydrogenase from *Psaudomonas aueruguonosa* PAO1 (gene ID: 878814), 3-hydroxybutyrate dehydrogenase from *Pseudomonas putida (P. putida)* NBRC 14164 (gene ID: 45523943), 3-hydroxy acid dehydrogenase from *E. coli* MG155 (gene ID: 946085) and the transcriptional regulator protein LysR from *E. coli* MG1655 (gene ID: 947311) was conducted against the genome of *B. methanolicus* MGA3. No homologues of *hpdH*, *hbdH-4*, *mmsA* or *lysR* were present in the genome of *B. methanolicus* MGA3 which means that *B. methanolicus* does not possess the 3-HP degradation pathway and can be used as a host for 3-HP production without previous genetic modifications to prevent 3-HP degradation.

A growth experiment was conducted to find out whether *B. methanolicus* MGA3 tolerates high 3-HP concentrations. The growth experiment was conducted in triplicates. *B. methabolicus* MGA3 was cultivated in MVcMY minimal medium supplemented with 3-HP at concentrations of 0.6 mM, 1.8 mM, 3.6 mM, 9 mM, 11 mM, 13 mM, 15 mM, 18 mM, and control without 3-HP. The OD_{600} was measured every two hours until the stationary phase, with two last measurements at 24 and 26 hours after inoculation. The OD_{600} measurements are listed in Table C.1 in Appendix. *B. methanolicus* MGA3 grows exponentially for 12 hours in the medium supplemented with 3-HP at concentrations $\leq$ 9 mM at growth rates in the range of 0.39-0.34 h^{-1}, and with 3-HP at 9-15 mM, for 10 hours at growth rates in the range of 0.34-0.23 h^{-1}. When the growth medium was supplemented with 18 mM 3-HP the growth of *B. methanolicus* MGA3 was inhibited after first 3 doublings and is therefore considered it as a lack of growth and growth

rate of 0 h^{-1}. The average growth rates of *B. methanolicus* MGA3 are presented in Figure 3.1. A half-maximal inhibitory concentration (IC_{50}) of 8.82 mM was calculated based on the measured *B. methanolicus* growth rates at different 3-HP concentrations (Table C.2 in Appendix) This data suggest that *B. methanolicus* MGA3 can sustain moderate concentrations of 3-HP in the growth medium, and if high titers of this compound are achieved in the future, it may be necessary to develop strains more resistant to 3-HP, for example through adaptive laboratory evolution, as was done before for 5AVA producing *B. methanolicus* strains [54].

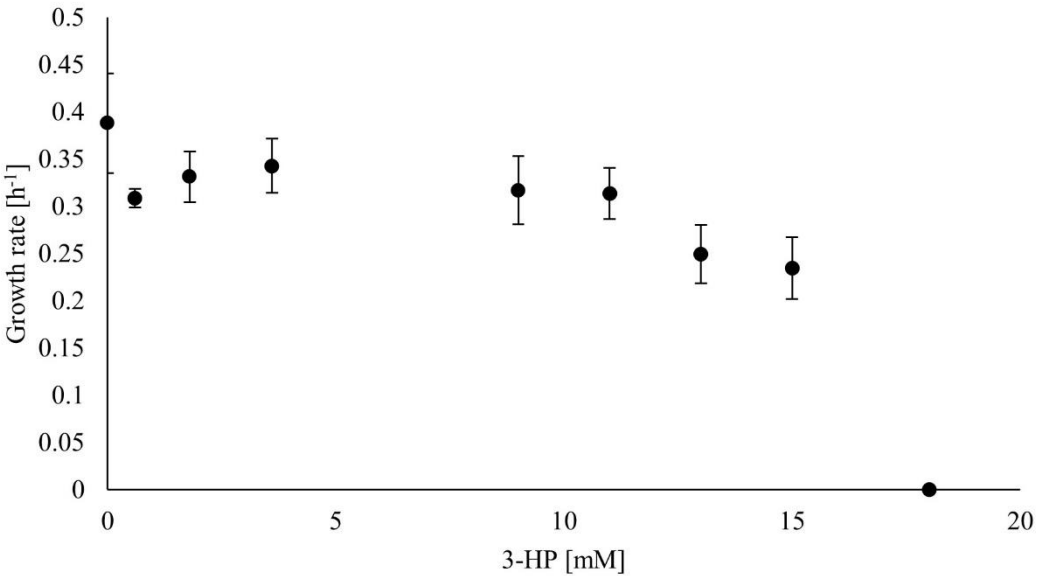

Figure 3.1: Average growth rates of WT *B. methanolicus* MGA3 in medium with varying concentrations of 3-HP.

3.2 Engineered recombinant strains of *Bacillus methanolicus* MGA3

In order to introduce 3-HP production into *B. methanolicus* MGA3, recombinant strains were created that harbor the *mcr* from *C. aurantiacus* DSM 636, *mcr* and *msr* from M. sedula 5348, and *mcr* and *msr* from *S. solfataricus* DSM 1617 (Table 3.1). In addition, strains with *sthA* from *E. coli* MG1655 and *P. putida* KT440 (Table 3.1) used for regeneration of the cofactor necessary for 3-HP production, were created to test the activity of SthA in *B. methanolicus* MGA3. The recombinant strains of *B. methanolicus* MGA3 with both *sthA* and *mcr* (and *msr*) were not engineered based on the information acquired in preliminary screening of enzymatic activity in recombinant strains.

The initial step in assembling the recombinant strains was to isolate the genes of interest from gDNA with specific primers presented in Table B.1 in Appendix. Next, an appropriate vector, pBV2xp or pTH1mp, was isolated from respective *E. coli* strain and digested. This was followed by insertion of the previously PCR-amplified gene(s) of interest into the empty vector using the Gibson assembly method. *E. coli* DH5α was used as a host strain for the cloned plasmids. Colony PCR was conducted to select positive clones and gel electrophoresis was used to identify clones with expected product size that were chosen for Sanger sequencing (Figure 3.2).

Figure 3.2 A represents sequencing results from *E. coli* DH5α with *mcr* from *C. aurantiacus* DSM 636. B represents sequencing results from *E. coli* DH5α with *mcr2* from *C. aurantiacus.* C represents sequencing results from *E. coli* DH5α with *mcr* and *msr* from *M. sedula* DSM 5348. D represents sequencing results from *E. coli* DH5α with *mcr* and *msr* from *S. solfataricus* DSM 1617. E represents sequencing results from *E. coli* DH5α with *sthA* from *E. coli* MG1566. F represents sequencing results from *E. coli* DH5α with *sthA* from *P. putida* KT2440.

The plasmids were isolated from *E. coli* DH5α and introduced into *B. methanolicus* with electroporation of electrocompetent cells of *B. methanolicus* MGA3 or by conjugation with *E. coli* S17-1 as host strain (Table 3.1).

Table 3.1: Recombinant strains of *B. methanolicus* MGA3.

Strains	**Description**
MGA3 pBV2xp	Carrying pBV2xp vector
MGA3 pBV2xp-*mcr*^{Cau}	Carrying pBV2xp vector and *mcr* from *C. aurantiacus* DSM 636
MGA3 pBV2xp-*mcr2*^{Cau}	Carrying pBV2xp vector and split *mcr* from *C. aurantiacus* DSM 636
MGA3 pBV2xp-*mcr-msr*^{Mse}	Carrying pBV2xp vector and *mcr* and *msr* from *M. sedula* DSM 5348
MGA3 pBV2xp-*mcr-msr*^{Sso}	Carrying pBV2xp vector and *mcr* and *msr* from *S. solfataricus* DSM 1617
MGA3 pTH1mp-*sthA*^{Eco}	Carrying pTH1mp vector and *sthA* from *E. coli* MG1655 for optimization of 3-HP production. *Mcr (*and *msr*) are not engineered to this strain.
MGA3 pTH1mp-*sthA*^{Ppu}	Carrying pTH1mp vector and *sthA* from *P. putida* KT2440 for optimization for 3-HP production. *Mcr* (and *msr*) are not engineered to this strain.

3.3 Catalytic activity in recombinant strains of *Bacillus methanolicus* MGA3

After selection of positive clones of *E. coli* DH5α, the newly created plasmids were introduced into *B. methanolicus* MGA3 in order to create production strains listed in Table 3.1. An enzyme assay was conducted to check the catalytic activity of heterologously produced enzymes in the recombinant strains of *B. methanolicus* MGA3. The cells were lysed, and the supernatant was collected after a centrifugation and used as crude extract for the assay. The change in absorbance at 340 nm was measured with a spectrophotometer over a period of three minutes in two rounds, first with no substrate (malonyl-CoA) as a measurement of background activity, and secondly with malonyl-CoA measuring the degradation of NADPH. The protein concentration in the samples were measured with Bradford assay and these values were used to calculate the catalytic activity of the enzyme per mg of protein in the samples.

3.3.1 Catalytic activity of malonyl coenzyme A reductase and malonic semialdehyde reductase

The catalytic activity of MCR or MCR/MSR in recombinant *B. methanolicus* MGA3 strains is shown in Figure 3.3. Low background MCR activity was measured for the empty vector control strain MGA3 pBV2xp, and similar values were observed for MGA3 pBV2xp-*mcr*^{Cau} and MGA3 pBV3xp-*mcr-msr*^{Sso}, including that these strains do not have or have very low MCR or MCR/MSR activity and potentially do not produce 3-HP. Meanwhile MCR or MCR/MSR activity of MGA3 pBV2xp-*mcr-msr*^{Mse} and MGA3 pBV2xp-*mcr2*^{Cau} strains is 0.6 U mg^{-1} and 0.4 U mg^{-1}, respectively, indicating their potential as 3-HP producers. Raw data is available in section D in Appendix.

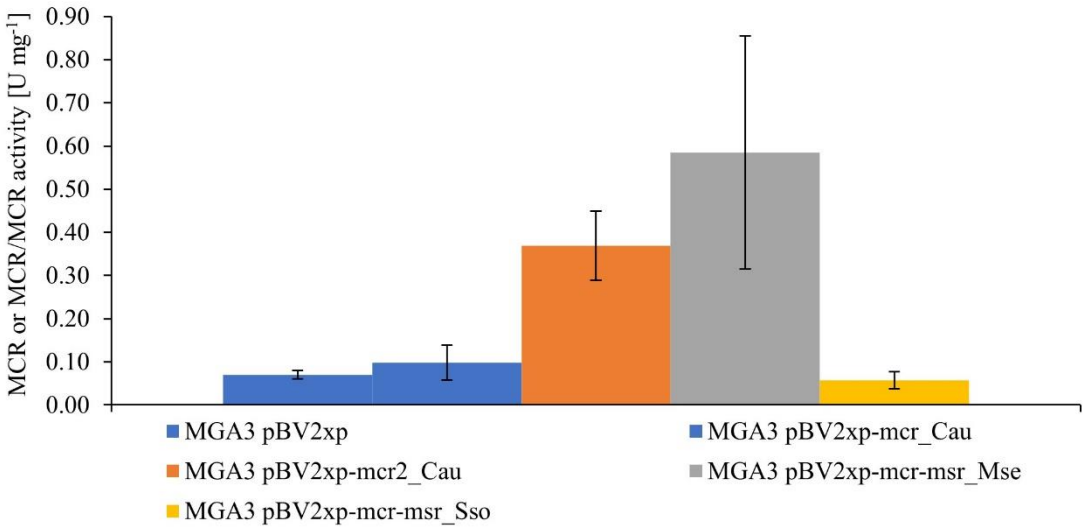

Figure 3.3: Catalytic activity of recombinant strains of *B. methanolicus* MGA3 with recombinant genes coding malonyl-CoA reductase and malonic semialdehyde reductase and with empty vector.

3.3.2 Catalytic activity membrane soluble transhydrogenase A

Enzyme assay to quantify activity of SthA in recombinant strains MGA3 pTH1mp-*sthA*^{Eco} and MGA3 pTH1mp-*sthA*^{Ppu} were conducted at 45 °C and 37 °C. No significant reduction of thio-NADPH in crude extracts was observed from these strains (Table 3.3). After revision of the enzyme assay protocol, I have found out the error that explains why no SthA activity was detected, namely because NAD^+ was used instead of thio-NAD^+.

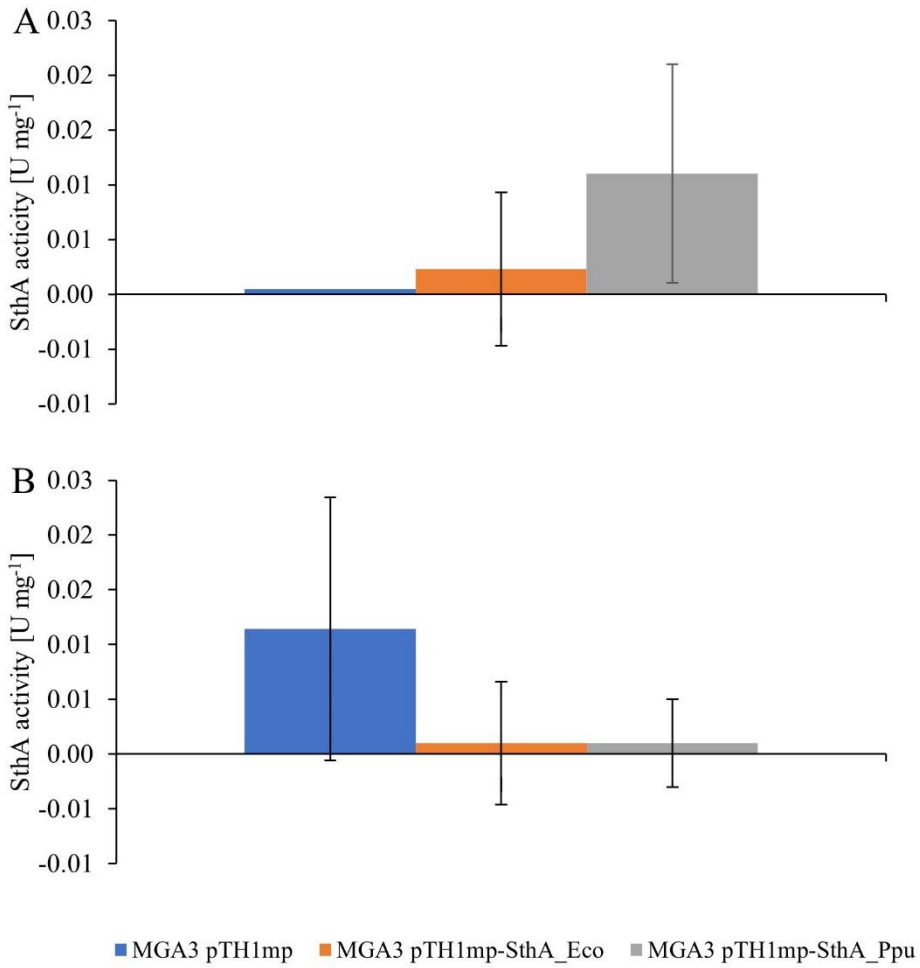

Figure 3.3: Catalytic activity of recombinant strains of *B. methanolicus* MGA3 with recombinant genes coding soluble nucleotide transhydrogenase and with empty vector. Graph A represents when the enzyme assay was run at 45 °C and graph B represents when the enzyme assay was run at 37 °C.

4 Discussion

In previous studies, wild type (WT) and recombinant *Bacillus methanolicus* (*B. methanolicus*) MGA3 has been proven to be a promising host for production of valuable chemicals such as L-glutamate, L-lysine [31], cadaverine [32], γ-aminobutyric acid [33], (R)-acetoin and 5-aminovalerate [34, 54]. In this study, the aim was to establish production of 3-hydroxypropionic acid (3-HP) from methanol by creating recombinant *B. methanolicus* MGA3 strains.

4.1 Evaluating *Bacillus methanolicus* MGA3 for production of 3-hydroxypropionic acid

To evaluate the potential of *B. methanolicus* MGA3 to become a production host of 3-HP, the genetic landscape of *B. methanolicus* MGA3 was investigated in order to detect potential 3-HP degradation pathways. 3-HP degradation was previously described for *Pseudomonas denitrificans*, where it was shown that 3-hydroxypropionic acid dehydrogenase (encoded by *hpfh),* (methyl)malonic semialdehyde dehydrogenase (encoded by *mmsA*) and 3-hydroxyisobutyrate dehydrogenase (encoded by *hbdh*-4) are involved in 3-HP degradation. The expression of *hpdH* is controlled by the transcriptional regulator protein LysR. The 3-HP degradation pathway is expressed in presence of 3-HP under aerobic conditions. The *in silico* analysis of the genome of *B. methanolicus* MGA3 conducted in this study did not lead to detection of homologues of *hpdH, mmsA, hbdH* or the transcriptional regulator gene *lysR* in the genome of *B. methanolicus* MGA3. This indicates that *B. methanolicus* MGA3 does not have the genetic background necessary for 3-HP degradation and is potentially a feasible host for its production.

Next, a growth experiment was conducted to test its resistance to different 3-HP concentrations. The growth rate of WT *B. methanolicus* MGA3 without 3-HP supplementations is 0.39 ± 0.05 h^{-1} which is in accordance with previous studies [32-34]. Supplementation of 3-HP in the upper limit of ≤ 11 mM, led to decrease of growth rates to 0.31-0.34 h^{-1} (Figure 3.1), and with further increase of 3-HP concentration, the growth rates decreased to 0.13 h^{-1} at 18 mM (Figure 3.1).

Based on the comparison of the growth rates of *B. methanolicus* at different 3-HP concentrations (Figure 3.1), the half-maximum inhibitory concentration (IC_{50}) of 3-HP in *B. methanolicus* MGA3 was calculated to be equal to 8.82 mM, which corresponds to 1.2 g L^{-1}, this is considerably lower than IC_{50} for acetoin in *B. methanolicus* MGA3 which equals to 26 g L^{-1} [34]. However, considering that production titers of less than 1 g L^{-1} are typically achieved

for different compounds in *B. methanolicus* during flask cultivation [34, 54], it can be expected that *B. methanolicus* MGA3 growth will not be affected by 3-HP accumulation in flasks. It was shown before for 5-aminovalerate [54], that tolerance of *B. methanolicus* MGA3 to this compound can be increased through adaptive laboratory evolution, and this approach could be a feasible solution also for 3-HP in case high titers of this compound are produced by *B. methanolicus* MGA3 [33, 54]. Compared to other organisms, tolerance of *B. methanolicus* MGA3 to 3-HP seems to be relatively low, for example for *E. coli* it equals to 100 mM 3-HP [102].

To summarize, *B. methanolicus* MGA3 has several features that indicate it is a suitable host for 3-HP production such as synthesis of 3-HP precursor, malonyl-CoA, the lack of a degradation pathway of 3-HP in the genome and a tolerance of 3-HP (Figure 3.1) [45, 65]. In addition, since *B. methanolicus* grows at relatively high temperatures (50 °C) and uses methanol as carbon source, it may lead to reduced costs to cooling and decreased chances of contamination during fermentations [36-38].

4.2 Construction of *Bacillus methanolicus* MGA3 strains for 3-hydroxypropionic acid production

The aim of this thesis was to assemble 3-HP-producing strains by heterologously expressing the enzymes malonyl coenzyme A reductase (MCR) and malonic semialdehyde reductase (MSR) in *B. methanolicus* MGA3. The *mcr* and *msr* genes encoding MCR and MSR were cloned into vector pBV2xp, transformed in *Escherichia coli* (*E. coli*) DH5α host cells and introduced into *B. methanolicus* MGA3 [85].

The constructs with *mcr* (and *msr*) transformed in host cells of *E. coli* DH5α were aligned with the WT template of the genes (Figure 3.3A-D) which showed no mutations. Therefore, it is assumed that mutations on the mcr and msr genes have not occurred after introduction into *B. methanolicus* MGA3.

The average growth rate for all recombinant strains of *B. methanolicus* MGA3 for 3-HP production (Table F.2) has corresponding growth rate as for WT *Bacillus methanolicus* MGA3 (Table C.2). It was expected that the growth rate for the recombinant strains should be decreased. Since 3-HP is relatively toxic, production would probably affect the cell and decrease growth as it did for recombinant *Synechococcus elongatus* under 3-HP production

[80]. Based on this observation, there is still potential for production of 3-HP in the recombinant *B. methanolicus* MGA3 production strains, but it may not be frequent.

4.3 Catalytic activity of malonyl coenzyme A reductase and malonic semialdehyde reductase in recombinant *Bacillus methanolicus* MGA3

The reductase enzyme activities of MCR and MSR in recombinant strains of *B. methanolicus* MGA3 were evaluated with an enzyme assay described in section 2.6. The method by Liu et al. (2013) was used to measure catalytic activity of MCR and MSR. The assay was conducted at 45 C° so that the enzyme activity is analyzed under physiologically relevant conditions [83, 103]. All production strains have higher MCR or MCR/MSR activity than the empty vector.

MGA3 pBV2xp-*mcr2*^{Cau} and MGA3 pBV2xp-*mcr-msr*^{Mse} have a significant catalytic activity of MCR and MSR, at 0.37 ± 0.08 and 0.58 ± 0.27 U mg^{-1}, respectively (Figure 3.3). MGA3 pBV2xp-*mcr*^{Cau} and MGA3 pBV2xp-*mcr-msr*^{Sso} have low catalytic activity of MCR/MSR (Figure 3.3).

The difference between the catalytic activity of MGA3 pBV2xp-*mcr*^{Cau} and MGA3 pBV2xp-*mcr2*^{Cau} is corresponding with a study done by Liu et al. (2013). In their work, Liu et al. (2013) split the C-terminal and the N-terminal domains of MCR from *C. aurantiacus* DSM 636 into two distinct catalytically functioning parts (MCR-C and MCR-N), which correspondingly led to increased catalytic activity of MCR. In this study, the catalytic activity increased four-fold between MGA3 pBV2xp-*mcr*^{Cau} and MGA3 pBV2xp-*mcr2*^{Cau}. Liu et al. (2013) assumed that the N-terminal part in native MCR inhibits the C-terminal part converting malonyl-CoA to malonic semialdehyde. Presumably, the contrast between catalytic activity of MCR in MGA3 pBV2xp-*mcr2*^{Cau} and MGA3 pBV2xp-*mcr*^{Cau} has similar background.

Even though the catalytic activity of MCR from *C. aurantiacus* was improved by separating the domains, Liu et al. (2013) observed that the protein levels were significantly higher for the MCR-N fragment and concluded with a natural imbalance between activities of the two domains. In another study, Liu et al. (2016) adjusted the expressions between the N- and C-terminal parts of split *mcr*. This led to a 270-fold increase in 3-HP production, from 0.15 g L^{-1} by original split *mcr* to 40.6 g L^{-1}. The natural activity imbalance between MCR-N and MCR-C was adjusted by two strategies; improving the MCR-C activity by directed evolution and decreasing the MCR-N expression level by chromosomal integration of *mcr-N*. Even though the catalytic activity of 0.37 U mg^{-1} ± 0.08 for MGA3 pBV2xp-*mcr2*^{Cau} is promising, based on

the results of Liu et al. (2016), it seems that there is still room for further improvements through adjusting the activity of two MCR domains. The possibility of a natural imbalance in expression levels between *mcr* and *msr* from *M. sedula* DSM 5348 has been mentioned in another study [87]; it was also suggested that correcting of the imbalance could lead to higher 3-HP titers. This is of relevance for further improvement of the MGA3 pBV2xp-*msr*-*mcr*^{Mse} strain that had the highest catalytic activity in this study, at 0.58 ± 0.27 U mg^{-1} protein (Figure 3.3) [84, 87].

The alternatives and use of codons for amino acids vary significantly between organisms, protein expression levels within the same organism, and even within the same operon. Variations in codon usage profoundly impact heterologous protein expression as there is an interplay between the abundance of transcriptional RNA and the codons. Such differences in codon usage have a profound impact on heterologous protein expression. There is a relationship between the abundance of transcriptional RNA and the codons. Imbalance within this relationship, which can occur for heterologous genes, can reason for decreased expression levels [104]. *mcr* from *C. aurantiacus* DSM 636 have a GC content of 57.70 %, *mcr* and *msr* from *M. sedula* DSM 5348 have a GC content of 49.27 %, and *mcr* and *msr* from *S. solfataricus* DSM 1617 have a GC content of 37.47 %. MGA3 pBV2xp-*mcr*-*msr*_Sso has also the lowest catalytic activity, at 0.06 ± 0.02 U mg^{-1} protein. Based on these observations, the low catalytic activity of MCR and MSR from *S. solfataricus* DSM 1617 may be due to codon usage bias in *B. methanolicus* MGA3. On the other hand, The GC content of the complete genome of *B. methanolicus* MGA3 is 38.5 % [39], 56.5 % for *C. aurantiacus* DSM 636 [105], 46 % for *M. sedula* DSM 5348 [106], and 35.5 % for *S. solfataricus* DSM 1617 [107]. The GC content of the whole genome of *B. methanolicus* MGA3 reflects the GC content in *S. solfataricus* DSM 1617. To increase the expression levels, *mcr* and *msr* genes can be codon-optimized and synthesized by Twist Bioscience or GeneArt (Thermo Fisher Scientific) so that the codons are synonymous with the frequently used transcriptional RNA in *B. methanolicus* MGA3. The use of synthetic genes with optimized sequences is likely to increase the activity levels of MCR and MSR and improve potential production of 3-HP.

4.4 Optimization of 3-hydroxypropionic acid production in *Bacillus methanolicus* MGA3 through co-factor regeneration and improvement of precursor supply

The soluble transhydrogenase A (SthA) activity in recombinant strains of *B. methanolicus* MGA3, not 3-HP producers, was evaluated with an enzyme assay, the methods of which are described in section 2.6. This is measured by the degradation of thio-nicotinamide adenine

dinucleotide (thio-NAD^+). Strains with combined properties were planned to be created based on the initial screening of catalytic measurements. An attempt in cloning of *accBCDA-birA*, which code for acetyl coenzyme a carboxylase (ACC-BirA) was conducted, but sequencing results showed that this was not successful.

Pathway efficiency can be constrained by enzymes with low inherent catalytic activity which act as a bottleneck, metabolic intermediates lost to competing pathways or low cofactor regeneration [88, 89]. Such obstacles can be resolved by introducing new genes or overexpressing genes that encode advantageous compounds for the desirable pathway. Some of the ways how 3-HP accumulation can potentially be increased in *B. methanolicus* MGA3 is through increase of metabolic flux towards precursor biosynthesis or rapid NADPH regeneration [72, 90, 92].

In this study *sthA* from *E. coli* MG1655 and *P. putida* KT2440 were successfully cloned into the pTH1mp plasmid, transformed in *E. coli* DH5α host cell (Figure 3.5E and Figure 3.5F) and introduced into *B. methanolicus* MGA3, rendering two strains MGA3 pTH1mp-$sthA^{Eco}$ and MGA3 pTH1mp-$sthA^{Ppu}$, respectively. The pathway of malonyl-CoA to 3-HP uses NADPH as a cofactor, and 3-HP biosynthesis can be limited by NADPH supply. A previous study in *E. coli* showed that expression of *sthA*, which code for SthA, leads to increased production of poly(3-hydroxybutyrate) by 82 % compared with the control strain [91]. No catalytic activity of SthA in recombinant *B. methanolicus* MGA3 was detected either at 50 C° or 37 °C (Figure 3.3). After following evaluation, an error was detected in the assay, which affected the results but was not corrected due to time constrains.

Even though there has been catalytic activity, this has not been detected as it was used NAD^+ instead of thio-NAD^+ for the assay. Nicotinamide adenine dinucleotide(hydrogen) NAD(H) and NADP(H) are measured at the same wavelength (365 nm), and even though there has been catalytic activity, this has not been detected. Thio-NAD^+ has to be used for the assay at a later timepoint to evaluate the catalytic activity of SthA. thio-NAD(H) is used because it is measured at another wavelength (400 nm) than NADP(H), therefore reduction of thio-NAD^+ will be measured (Figure 2.2) and give the correct measurements that represents SthA activity.

Originally, *sthA* isolated from WT *Azotobacter vinelandii* (*A. vinelandii*) was also intended to be part of the *B. methanolicus* MGA3 strain collection to optimize 3-HP production but failed to be cloned into the expression vector pTH1mp. This cloning will not be continued because SthA derived from *A. vinelandii* is inactive after 20 minutes of incubation at 55 °C [108].

Additionally, previous heterologous expression of *sthA* from WT *A. vinelandii* has only been found to be carried out in a mesophilic (growth at 20-45 °C) procaryote [109]. Membrane bound transhydrogenase (PntAB), can also be considered a target for improving 3-HP production. This was reported by Rathnasing et al. (2012) where they found that overexpression of *pntAB* and *accBCDA-birA* (discussed later) and subsequent conversion of NADH to NADPH increased the intracellular substrate for MCR for 3-HP production [72, 90].

There are several competing pathways to 3-HP biosynthesis, some of which are shown in Figure 1.1. Notably, acetyl-CoA is the precursor for several pathways, such as the TCA cycle in *B. methanolicus* MGA3. To increase flux in the 3-HP producing pathway, it was desirable to redirect metabolic flux from competitive pathways towards synthesis of 3-HP precursor, malonyl-CoA. ACC-BirA catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. The effect of overexpressing *accBCDA-birA* favors the malonyl-CoA pathway and decreases production of byproducts [72, 89]. ACC-BirA is encoded by *accB* and *accC* which is located in one operon, and by *accA, accD,* and *birA,* which are originally located in different regions of the chromosome. In this study, *accB, accD, accA, accD,* and *birA* were PCR-amplified from the genomic DNA of *Bacillus methanolicus* MGA3. The isolation of the three DNA fragments was successful; however, obstacles occurred when the fragments were to be inserted into the vector (pTH1mp). The first cloning attempt was to simultaneously insert all three fragments into the pTH1mp vector using Gibson cloning. Gibson cloning did not succeed in cloning all three fragments (*accBC, accDA* and *birA*) into pBV2xp. When multiple attempts of Gibson cloning were unsuccessful, overlap extension PCR (OE-PCR) followed by Gibson cloning was suggested. The plan was to construct all three fragments (*accBC, accDA* and *birA*) into one fragment before cloning. A gel electrophoresis was performed after each PCR cycle which ensured successful construction of *accBCDA.* After the second round of OE-PCR the fragment seemed promising. A Gibson cloning was performed, and the plasmid was transferred to the host strain *E. coli* DH5α. A colony PCR was performed, and the plasmid was sent to sequencing. As a result of these attempts, the sequencing results (not presented) revealed that neither *accBCDA* nor *birA* was inserted, and due to time constraints further attempts at cloning were not undertaken. Gibson can theoretically assemble up to six DNA fragments and OE-PCR is said to manage to combine up to eight DNA fragments [96, 110]. Therefore, it is assumed that continuous attempts of Gibson cloning and OE-PCR will successfully result in the assembly of *accBCDA-birA* in *E. coli* DH5α. In addition, other studies have previously conducted assemblies of *accBCDA-birA* which used two alternative methods [72, 89]. The first study had

multiple steps of cloning where one fragment was cloned for each cycle. The PCR products generated for cloning contained an internal restriction site and a ribosomal binding site (RBS) at the 3' end where the RBS was either overlapping with the terminal base pair of the stop codon of the previous gene or placed 2 base pairs downstream of the stop codon. The plasmid was digested with blunt ends and ligated with the first PCR product and digested. The rest of the fragments was cloned in the same manner into the plasmid [72, 111]. The second study used a cloning set, Clone Express® MultiS One Step Cloning kit, to clone *accBCDA-birA* [89]. Once overexpression of *accBCDA-birA* is achieved in *B. methanolicus* MGA3, further work on NADPH supply would be beneficial. Replacing the native NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 3-HP producing *B. methanolicus* MGA3 with non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GapN) could lead to increased supply of NADPH at the expense of NADH and increase 3-HP accumulation as shown in *S. cerevisiae* [73].

5 Future perspectives

In this study, *Bacillus methanolicus* MGA3 is considered a potential production host for 3-hydroxypropionic acid, as of inherent synthesis of malonyl-CoA, tolerance to 3-hydroxypropionic acid and the lack of a degradation pathway for 3-hydroxypropionic acid. Recombinant *Bacillus methanolicus* MGA3 strains expressing heterologous malonyl coenzyme A reductase and malonic semialdehyde reductase were constructed and measured catalytic activities up to 0.57 U mg^{-1}. As far as is known, this study is the first to report catalytic activity malonyl coenzyme A reductase and malonic semialdehyde reductase in *B. methanolicus* MGA3. It is yet to be confirmed production of 3-hydroxypropionic acid before introducing co-regulating genes into *Bacillus methanolicus* MGA3 for optimization.

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Appendix

A. Media & growth medium

All media and solutions used in this study is presented below. Media and solutions were sterilized by autoclaving at 121 °C for 20 minutes, alternatively by filter sterilization with 0.2 µl filters.

Table A.1: LB/LA medium.

Component	**Mass [g L^{-1}]**	**Volume [ml L^{-1}]**
Tryptone	10	
Yeast extract	5	
Natrium chloride (NaCl)	5	
RO-water		1000
If making LB-agar: Agar	15	
➔ Autoclave		

Table A.2: SOB medium.

Component	**Mass [g L^{-1}]**	**Volume [ml L^{-1}]**
SOB	28	
RO-water		1000
If making SOB-agar: Agar	15	
➔ Autoclave		

Table A.3: MVcM high salt buffer 10x used for MVcM medium and MVcMY medium.

Component	**Concentration [M]**	**Mass [g L^{-1}]**	**Volume [ml L^{-1}]**
Dipotassium phosphate (K_2HPO_4)	0.235	40.93	
Sodium phosphate monobasic dihydrate ($NaH_2PO_4*H_2O$)	0.108	14.9	
Ammonium sulfate ($(NH_4)_2SO_4$)	0.16	21.14	
RO-water			1000
➔ Autoclave			

Table A.4: MVcMY/MVcM medium

Component	**Mass [g L^{-1}]**	**Volume [ml L^{-1}]**
MVcM high salt buffer 10x		100
For MVcMY add yeast extract	0.25	
RO-water		1000
➔ Adjust pH to 7.2 with HCl or NaOH and autoclave.		
Nutrient additions		
Magnesium sulfate ($MgSO_4$) of stock solution		0.1
Trace metals stock solution		0.1
Complete vitamins 1000x stock solution		0.1
Methanol (CH_3OH)		0.811

Table A.5: Trace metals stock solution

Component	**Molecular weight [g $mole^{-1}$]**	**Concentration [M]**	**Volume [ml L^{-1}]**	**Mass [g L^{-1}]**
$FeSO_4{}^*7H_2O$	270.02	0.020		5.56
$CuCl_2{}^*2H_2O$	170.49	0.00016		0.027
$CaCl_2{}^*2H_2O$	147.02	0.050		7.35
$CoCl_2{}^*4H_2O$	237.93	0.00017		0.040
$MnCl_2{}^*4H_2O$	197.91	0.050		9.90
$ZnSO_4{}^*7H_2O$	287.54	0.0010		0.288
$Na_2MoO_4{}^*2H_2O$	241.98	0.0002		0.048
H_3BO_3	61.83	0.0005		0.031
Milli-Q water			500	
Concentrated HCl	36.46	1.99	80	
Milli-Q water			Up to 1000	

Table A.6: Complete vitamin stock solution.

Component	**Molecular weight [g mole^{-1}]**	**Concentration [M]**	**Volume [ml L^{-1}]**	**Mass [g L^{-1}]**
d-Biotin	244.31	0.00041		0.100
RO-water			800	
➔ Mix and dissolve biotin using heat (not boiling). Cool flask a little before adding other components.				
Thiamine*HCl (vitamin B_1)	300.81	0.00033		0.100
Riboflavin (vitamin B_2)	376.37	0.00027		0.100
Pyridoxine*HCl	169.18	0.00059		0.100
Pantothenate	219.24	0.00046		0.100
Nicotinamide (Vitamin B_3)	122.13	0.00082		0.100
Aminobenzoic acid (vitamin L_1)	137.14	0.00015		0.020
Folic acid (vitamin B_{11})	441.4	0.00002		0.010
Alphamine (Vitamin B_{12})	1355.38	0.00001		0.010
Lipoic acid	206.32	0.0005		0.010
➔ Mix and add 6 M NaOH until riboflavin and lipoic acid are completely solved				
RO-water			Up to 1000	

Table A.7: MVcMY medium with additives.

Components	**Volume [ml]**
MVcMY media	1500
Methanol	12.17
Trace metals	1.5
Vitamins	1.5
$MgSO_4$	1.5

Table A.8: Electroporation buffer (EPB) for preparation of electrocompetent cells.

Component	**Concentration [M]**	**Mass [g]**	**Volume [ml]**
HEPES	0.001	0.06	
PEG_{8000}		62.5	
RO-water			250
➔ Sterile filtrate			

Table A.9: 1 % Agarose gel for gel electrophoresis.

component	Mass (g 300ml^{-1})	Volume (ml)
Agarose	3	
Tae 1x buffer		300
➔ Microwave 5 minutes at 700 W. continue when cooled down in heat cabinet (60 °C)		
GelRed or GelGreen		0.015

The restriction enzymes BamHI HF and SacI HF with Cutsmart buffer were used to digest pBV2xp. The restriction enzymes PciI and BamHI with r3.1 buffer were used for pTH1mp digestion. The plasmid and its restriction enzymes and buffer were incubated in a water bath for 4 hours at 37 °C. An overview of the added volumes can be seen in Table A.9.

Table A.10: volumes of plasmid, buffer, and restriction enzymes for digestion of pBV2xp and pTH1mp.

Contents	Volume [µl]
Uncut plasmid	45
Buffer	10
Reverse restriction enzyme	2
Forward Restriction enzyme	2
RO-water	42
Total	100

B. Primers

An overview of all primers that has been necessary in this study is listed in this section. The list of primers is shown in Table B.1.

Table B.1: Primers used for amplification of specific gene fragments, for sequencing samples of the isolated gene fragments, Gibson assembly and colony PCR.

Name	**Sequence**	**Description**
HP01	TTCACTTAAGGGGGAAATGGCAA ATGAGCGGAACAGGACGACTGGC AGGAAAG	Forward *mcr C. aurantiacus* DSM 636
HP23	ACGACGGCCAGTGAATTCGAGCT TTACACGGTAATCGCCCGTCCGCG ATG	Reverse *mcr C. aurantiacus* DSM 636
HP27	TTCACTTAAGGGGGAAATGGCAA GTGAGCGGAACAGGACGACTGGC AGGAAAG	Forward GTG start *mcr C. aurantiacus* DSM 636
HP44	AGCCGAGTCAATGGAGCTAGGAG GCGCAATACATTAGCGCCACCAC CGGCGCACGCAGTGCATC	Forward for *mcr-C C. aurantiacus* DSM 636
HP43	GTATTGCGCCTCCTAGCTCCATTG ACTCGGCTGTTGGCAGGGATGTTG AGGGTAATCTCATTGA	Reverse for *mcr-N C. aurantiacus* DSM 636
HP36	AAGTGATCGCATCCGTACAGTG	Sequencing primer *mcr C. aurantiacus* DSM 636
HP37	AATACCGGCGGGATTCATGCA	Sequencing primer *mcr C. aurantiacus* DSM 636
HP38	ATCGGCATAAGCTCGAACAGATG C	Sequencing primer *mcr C. aurantiacus* DSM 636
HP39	AATGATGTGGCCGCACTAGAGCA	Sequencing primer *mcr C. aurantiacus* DSM 636
HP40	ATCAGATCGAAGCCGCTATC	Sequencing primer *mcr C. aurantiacus* DSM 636
HP28	TTCACTTAAGGGGGAAATGGCAA ATGAGGAGAACGCTAAAGGCCGC AAT	Forward *mcr M. sedula* DSM 5348
HP29	TCATCTCTTGTCTATGTAGCCCTTC TCCAC	Reverse *mcr M. sedula* DSM 5348

HP30	AGGGCTACATAGACAAGAGATGAtacATAAATAGGAGGTAGTAagaATGACTGAAAAGGTATCTGTA	Forward *msr M. sedula* DSM 5348
HP31	ACGACGGCCAGTGAATTCGAGCTTTATTTTTCCCAAACTAGTTTGT	Reverse *msr M. sedula* DSM 5348
HP41	TGCAATACCCCTAACGCCCATT	Sequencing primer *mcr-msr M. sedula* DSM 5348
HP32	TTCACTTAAGGGGGAAATGGCAAATGAGGAGAACACTTAAAGCAGCTAT	Forward *mcr S. solfataricus* DSM 1617
HP33	TTACCTTTTATCAATATATCCTTTCTCAATTAAC	Reverse *mcr S. solfataricus* DSM 1617
HP34	AGGATATATTGATAAAAGGTAATACATAAATAGGAGGTAGTAAGAATGTTTATACATATGAAATCAATTAATAAG	Forward *msr S. solfataricus* DSM 1617
HP35	ACGACGGCCAGTGAATTCGAGCTTTATGAATTACATTTTTCCTTATATACT	Reverse *msr S. solfataricus* DSM 1617
HP42	TCAATCGTTATCTGGTGCCGGT	Sequencing primer *mcr-msr S. solfataricus* DSM 1617
HP05	TAAACAATTACATAAATAGGAGGTAGTACATATGTTAAAAATACAAGAAATTCGTGAATTGA	Forward a*ccBC B. methanolicus* MGA3
HP06	TTAATCGTCTGATTTCATGACATCATACATT	Reverse *accBC B. methanolicus* MGA3
HP07	CATGAAATCAGACGATTAATACATAAATAGGAGGTAGTAAGAATGCTTAAAGACATTTTTACAAA	Forward *accDA B. methanolicus* MGA3
HP08	CTAATTCACTTCTACATATTCAGTA	Reverse *accDA B. methanolicus* MGA3
HP09	TGAATATGTAGAAGTGAATTAGTACATAAATAGGAGGTAGTAAGAATGCAATCAGAATTGAGAAAGAAAC	Forward *birA B. methanolicus* MGA3
HP24	TAGACCTATGGCGGGTACCATATGTTATATTTTTCCGGATAAATCAATATCAGCAGAG	Reverse *birA B. methanolicus* MGA3
$sthA^{Ppu}$ fwd	TAAATAGGAGGTAGTACATGATGGCTGTCTACAACTACGA	Forward *sthA p. putida* KT2440

sthA^{Ppu} rev	TGGCGGGTACCATATGGATCTCAA AAAAGCCGGTTCAGGC	Reverse *sthA p. putida* KT2440
sthA^{Avi} fwd	TAAATAGGAGGTAGTACATATGG CTGTATATAACTACGATGT	Forward *sthA* WT *A. vinelandii*
sthA^{Avi} rev	TGGCGGGTACCATATGGATCTCAA AAAAGCCGATTGAGAC	Reverse *sthA* WT *A. vinelandii*
sthA^{Eco} fwd	TAAATAGGAGGTAGTACATATGC CACATTCCTACGATTA	Forward *sthA E. coli* MG1655
sthA^{Eco} rev	TGGCGGGTACCATATGGATCTTAA AACAGGCGGTTTAAACC	Reverse *sthA E. coli* MG1655
PXPF	TGTTTATCCACCGAACTAAG	Forward pBV2xp Colony PCR
BVXR	CCGCACAGATGCGTAAGGAG	Reverse pBV2xp Colony PCR
VPJF	TCTAATCCTTCTAAAAAATATAAT TTAGAAAACTAAG	Forward pTH1mp Colony PCR
VPJR	GGTGCGGGCCTCTTCGCTATTACG	Reverse pTH1mp Colony PCR

C. Growth experiment

OD_{600} measurements for growth of WT *B. methanolicus* MGA3 in environments with given concentrations of 3-HP. The ODs of the triplicates at given time points are shown in Table C.1 OD_{600} was measured every second hour in a period of 12 hours, with final measurements after 24 and 26 hours respectively.

Table C.1: OD_{600} measurements of growth experiment with WT *B. methanolicus* MGA3 in different concentrations of 3-HP.

Hours	**18_A**	**18_B**	**18_C**	**18_D**	**18_E**	**18_F**	**15_A**	**15_B**	**15_C**
0	0.30	0.19	0.21	0.18	0.22	0.17	0.20	0.21	1.18
2	0.55	0.35	0.35	0.31	0.32	0.26	0.40	0.57	0.29
4	0.65	0.50	0.60	0.42	0.33	0.44	0.60	1.20	0.50
6	0.80	0.60	0.80	0.49	0.29	0.70	0.70	1.90	0.80
8	0.80	0.60	0.80	0.50	0.25	0.80	0.90	2.50	1.10
10	0.78	0.59	0.81	0.58	0.29	1.10	1.30	2.90	1.70
12	0.80	0.55	0.76	0.55	0.28	1.21	1.40	2.80	2.20
24	0.78	0.43	0.96	0.30	0.05	1.27	1.60	2.00	1.60
26	0.72	0.43	1.03	0.37	0.12	1.37	1.80	1.80	1.80
	13_A	**13_B**	**13_C**	**11_A**	**11_B**	**11_C**	**9_A**	**9_B**	**9_C**
0	0.21	0.21	0.17	0.19	0.23	0.18	0.23	0.21	0.20
2	0.49	0.59	0.29	0.48	0.66	0.35	0.50	0.45	0.40
4	1.70	1.25	0.60	1.09	1.50	0.85	1.10	1.00	1.10
6	1.90	2.40	0.90	2.40	2.90	1.80	2.20	2.40	2.20
8	2.60	3.00	1.60	3.80	3.60	3.40	4.00	3.60	4.00
10	3.00	3.60	2.60	4.40	3.80	4.20	5.40	5.60	5.40
12	3.20	3.20	2.80	4.00	3.40	3.80	5.00	5.20	4.60
24	1.80	1.60	2.00	1.80	1.80	2.00	2.00	1.80	2.00
26	2.00	2.00	1.80	2.00	2.00	2.20	2.00	1.60	2.00
	9_D	**9_E**	**9_F**	**3.6_A**	**3.6_B**	**3.6_C**	**1.8_A**	**1.8_B**	**1.8_C**
0	0.21	0.24	0.19	0.23	0.20	0.21	0.21	0.19	0.21
2	0.51	0.72	0.38	0.50	0.40	0.40	0.40	0.40	0.40
4	1.25	1.75	0.90	1.20	0.95	1.05	1.05	0.90	1.05
6	2.80	3.70	2.10	2.00	2.00	2.40	2.20	2.00	2.00
8	4.40	4.40	4.20	4.20	3.40	4.00	3.60	3.80	4.00
10	4.20	4.60	4.80	7.20	6.60	8.00	6.60	6.20	7.40
12	3.80	4.40	4.60	7.20	7.00	7.00	7.60	7.00	6.80
24	2.00	2.20	1.80	2.20	2.60	2.40	2.20	1.80	2.20

26	2.20	2.60	2.60	2.00	2.60	2.40	2.00	1.60	2.00
	0.6_A	**0.6_B**	**0.6_C**	**0_A**	**0_B**	**0_C**	**0_D**	**0_E**	**0_F**
0	0.25	0.21	0.20	0.23	0.18	0.20	0.21	0.20	0.20
2	0.45	0.40	0.35	0.50	0.40	0.40	0.47	0.62	0.25
4	1.05	1.05	0.95	1.10	1.00	1.15	1.15	1.60	0.90
8	2.00	2.40	1.80	2.20	2.20	2.20	2.80	4.30	2.20
10	3.40	4.20	3.40	4.00	4.20	3.80	7.20	7.20	6.00
12	6.20	6.60	6.00	6.80	6.80	7.00	5.80	5.20	6.60
24	7.60	7.40	6.80	7.20	6.80	7.00	5.00	3.60	5.00
26	1.60	1.20	1.40	1.40	1.60	1.20	4.00	3.60	4.20
	1.20	1.00	1.20	1.00	1.40	1.00	4.60	4.20	4.60

The average growth rates of WT *B. methanolicus* MGA3 for the respective environments with 3-HP was found graphically. The interval of increasing OD_{600} was selected as a function of time and the equation of its trendline gave the growth rate. An example for one of the triplicates can be seen in Figure C.2.

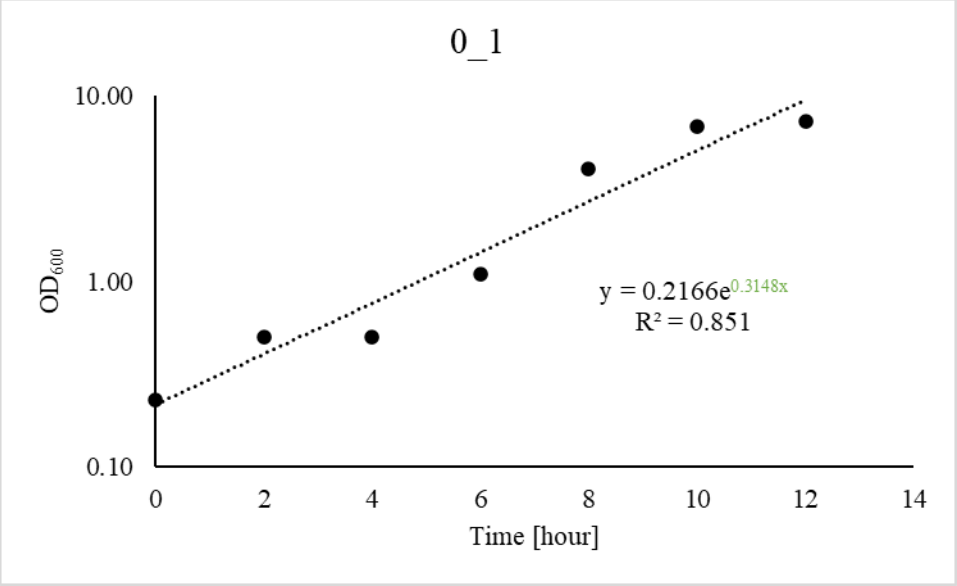


Figure C.2: Graphic view of one of the triplicates for growth without 3-HP. The green number represents the growth rate for WT *B. methanolicus* MGA3 at this condition.

IC_{50} was calculated based on the equation, y = ax + b, of the trendline for the average growth rates shown in Figure C.3 with Equation II.

$$IC_{50} = {}^{(0.5 - b)}/_{a} \qquad \text{Equation II}$$

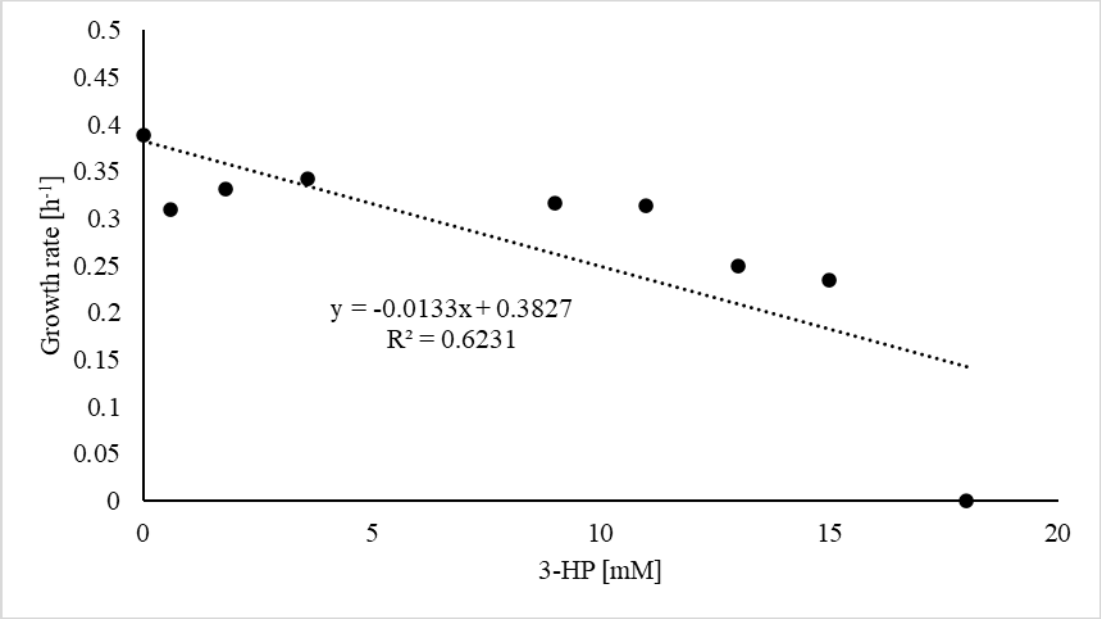


Figure C.3: Average growth rate of WT *B. methanolicus* MGA3 in medium with varying concentrations of 3-HP with a fitted trend line.

Table C.2: Growth rate of WT *B. methanolicus* MGA3 in growth medium with certain concentrations of 3-HP.

Concentration 3-HP [mM]	**Average growth rate [h^{-1}]**	**Standard deviation [h^{-1}]**
0	0.39	± 0.05
0.6	0.31	± 0.01
1.8	0.33	± 0.03
3.6	0.34	± 0.03
9	0.32	± 0.04
11	0.31	± 0.03
13	0.25	± 0.03
15	0.23	± 0.03
1**8**	0.13	± 0.04

D. Enzyme- & Bradford assay for strains with malonyl coenzyme A reductase/malonic semialdehyde reductase

Dilutions of BSA for a calibration curve was proposed according to the Bio Rad™ Quick start™ Bradford Protein Assay protocol which can be seen in Table D.1.

Table D.1: Dilutions of BSA for the calibration curve.

Protein concentration [mg ml^{-1}]	**Absorbance [595 nm]**
1	2.50
0.5	2.05
0.25	1.43
0.125	1.10
0.625	0.90
0.00	0.61

The calibration curve from measured absorbance can be seen in Figure D.1 with OD_{595} as a function of time.

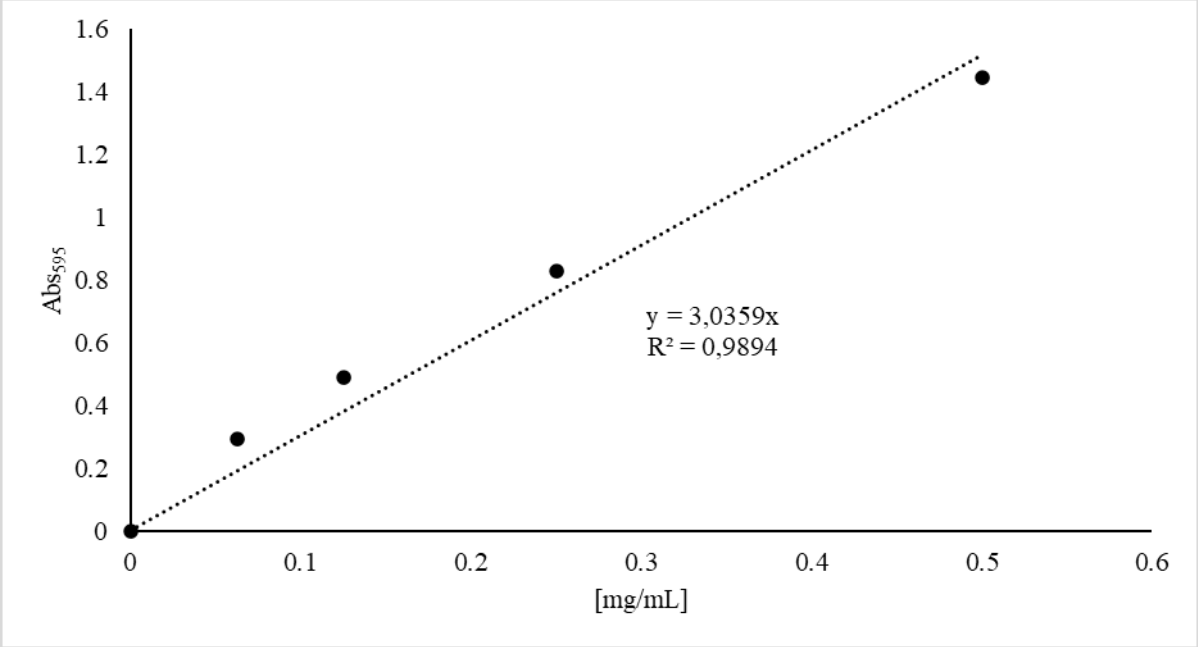


Figure D.2: Calibration curve for Bradford protein assay.

Equation III was assembled with the formula of the linear fitted trend line in Figure D.2 and used to get an estimate of protein content in the crude extracts for the enzyme assays (MGA3 pBV2xp-*mcr*^{Cau}, MGA3 pBV2xp-*mcr2*^{Cau}, MGA3 pBV2xp-*mcr-msr*^{Mse}, MGA3 pBV2xp *mcr-msr*^{Sso}, MGA3 pBV2xp).

$$C = \frac{OD_{595}}{3.0359} \qquad \text{Equation III}$$

Equation IV was used to calculate U ml^{-1} of protein for each sample

$$U/_{ml} = \frac{(-1 \times \text{Abs activity}) - (-1 \times \text{Abs Baseline})) \times \text{volume in cuvette} \times \text{ratio of sample}}{\text{Volume crude extract} \times \text{Molar extinction coefficient}} \qquad \text{Equation IV}$$

Table D.3: catalytic activity of recombinant 3-Hp production strains of *B. methanolicus* MGA3.

Strain	**Protein concentration [U mg^{-1}]**	**Standard deviation [U mg^{-1}]**
MGA3 pBV2xp-*mcr*^{Cau}	0.10	± 0.04
MGA3 pBV2xp-*mcr2*^{Cau}	0.37	± 0.08
MGA3 pBV2xp-*mcr-msr*^{Mse}	0.58	± 0.27
MGA3 pBV2xp-*mcr-msr*^{Sso}	0.06	± 0.02
MGA3 pBV2xp	0.01	± 0.01

E. Enzyme- & Bradford assay for strains with *sthA*

An Enzyme assay was conducted to quantify activity of recombinant SthA in recombinant strains MGA3 pTH1mp-*sth*^{Eco} and MGA3 pTH1mp-*sthA*^{Ppu}. The enzyme assay was conducted at 45 °C and 37 °C. No catalytic activity was measured for these two strains. The catalytic activity of SthA in the recombinant strains of *B. methanolicus* MGA3 can be seen in Figure E.2. All calculations for the catalytic activity of MGA3 pTH1mp-*sthA*^{Eco} and MGA3 pTH1mp-*sthA*^{Ppu} was conducted in the same way as shown in section D for recombinant strains of *B. methanolicus* MGA3 measuring catalytic activity of MCR and MSR. The curve to calculate catalytic activity can be seen in Figure E.1. Table E.1 and table E.2 show catalytic activity of sthA in recombinant strains of *B. methanolicus* MGA3, when the enzyme assay was run at 45 °C and 37 °C degrees respectively.

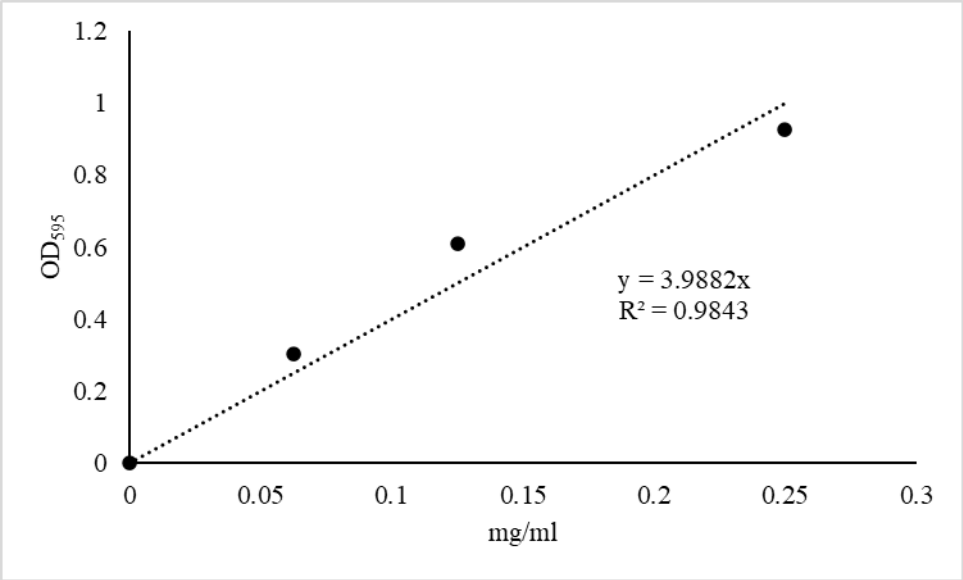


Figure E.1: Calibration curve for Bradford protein assay.

Table E.1: Catalytic activity of SthA from recombinant *B. methanolicus* MGA3 when the enzyme assay was run at 45 °C.

Strain	Protein concentration [U mg^{-1}]	Standard deviation [U mg^{-1}]
MGA3 pTH1mp-*sthA*^{Eco}	0.00	± 0.00
MGA3 pTH1mp-*sthA*^{Ppu}	0.01	± 0.00
MGA3 pTH1mp	0.00	± 0.00

Table E.2: Catalytic activity of SthA from recombinant *B. methanolicus* MGA3 when the enzyme assay was run at 37 °C.

Strain	**Protein concentration [U mg^{-1}]**	**Standard deviation [U mg^{-1}]**
MGA3 pTH1mp-*sthA*^{Eco}	0.00	± 0.00
MGA3 pTH1mp-*sthA*^{Ppu}	0.00	± 0.00
MGA3 pTH1mp	0.01	± 0.00

F. Growth of recombinant *Bacillus methanolicus* MGA3 with *mcr* and *msr*

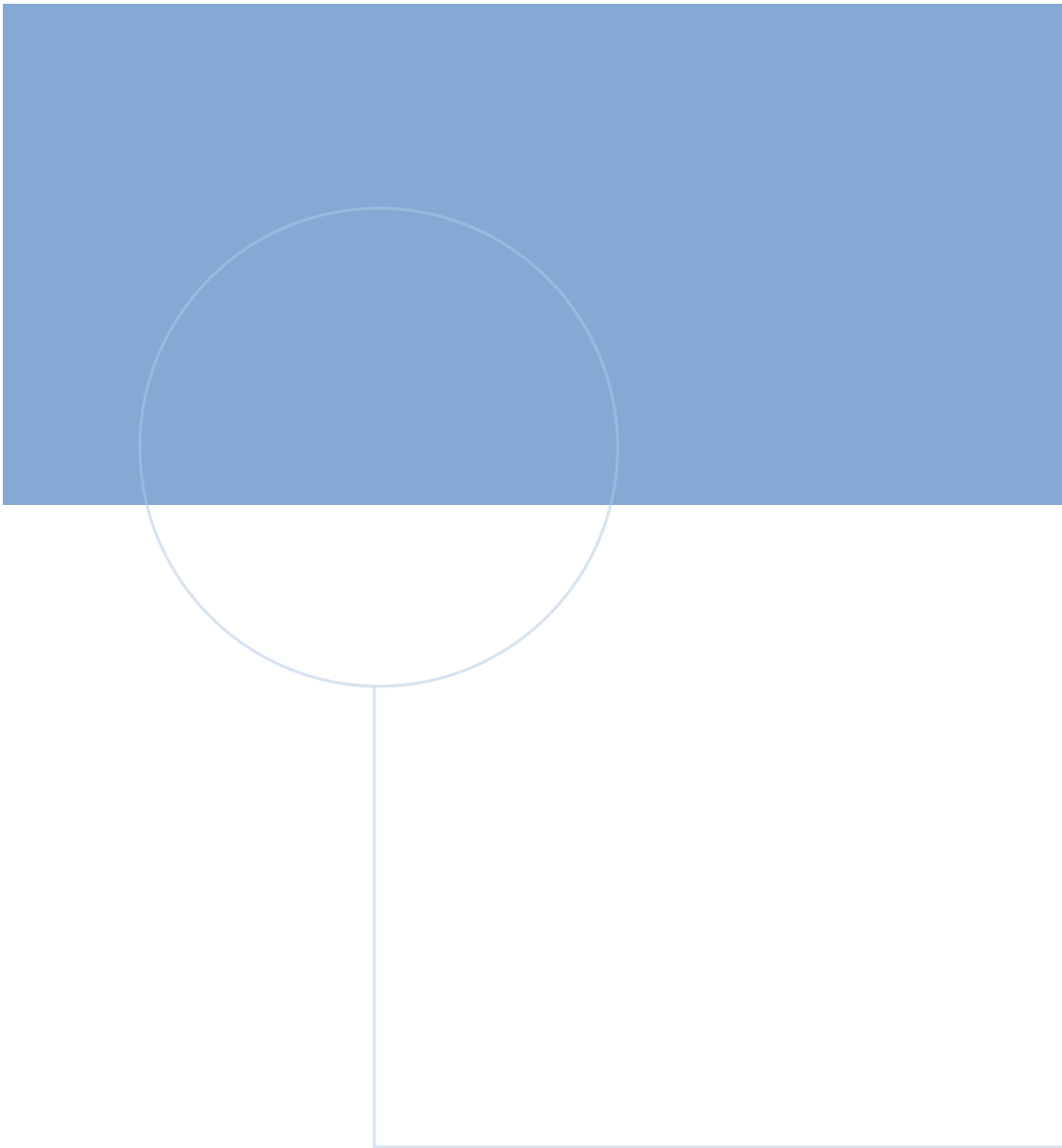
The measured OD_{600} during incubation of all engineered recombinant strains of *B. methanolicus* MGA3 for production of 3-HP can be seen in Table F.1. The growth rates of the strains can be seen in Table F.2 and was calculated in the same way as for the growth experiment (section C).

Table F.1: OD_{600} measurements of the recombinant strains *of B. methanolicus* MGA3.

Hours	**Ev_A**	**Ev_B**	**Ev_C**	***mcr*_A**	***mcr*_B**	***mcr*_C**	***mcr2*_A**	***mcr2*_B**	***mcr2*_C**
0	0.21	0.21	0.22	0.20	0.23	0.22	0.21	0.21	0.20
2	0.46	0.44	0.46	0.40	0.46	0.43	0.43	0.44	0.44
4	1.00	1.05	1.10	1.00	1.10	0.95	1.00	1.15	1.10
8	5.20	5.40	5.90	5.20	6.20	5.30	5.70	6.60	5.80
10	8.40	8.80	8.40	9.20	9.00	8.40	8.80	8.40	8.80
24	4.80	5.20	5.00	5.40	6.20	4.40	5.20	5.20	4.40
	***Sso*_A**	***Sso*_B**	***Sso*_C**	***Mse*_A**	***Mse*_B**	***Mse*_C**			
0	0.29	0.28	0.27	0.21	0.20	0.20			
2	0.59	0.56	0.54	0.48	0.48	0.49			
4	1.30	1.30	1.25	1.20	1.20	1.25			
8	7.90	7.80	7.40	6.20	6.40	6.60			
10	8.20	7.40	8.20	7.40	8.60	8.20			
24	3.80	3.00	3.00	5.20	5.80	4.60			

Table F.2: Average growth rates of recombinant strains of *B. methanolicus* MGA3 for production of 3-HP.

Strains	**Average growth rate [h^{-1}]**	**Standard deviation [h^{-1}]**
MGA3 pBV2xp-*mce*^{Cau}	0.39	± 0.01
MGA3 pBV2xp-*mcr2*^{Cau}	0.39	± 0.00
MGA3 pBV2xp-*mcr-msr*^{Mse}	0.38	± 0.01
MGA3 pBV2xp-*mcr-msr*^{Sso}	0.38	± 0.03
MGA3 pBV2xp	0.38	± 0.00



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