Production of 3-hydroxypropionate from biomass

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Production of 3-hydroxypropionate from biomass

By

Netra Rajguru Agarkar

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Program of Study Committee:
Thomas Bobik, Co-major Professor
Alan DiSpirito, Co-major Professor
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Larry Halverson

Iowa State University
Ames, Iowa
2007

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Biorenewable technology is a developing field of science that researches alternative sources for petroleum products like fuels, plastics, paints, etc. The DoE biomass program has identified the 12 top chemicals that can be produced from biomass and further processed to replace many petroleum products; 3-hydroxypropionate (3HP) is a top 12 chemical that can be used to produce plastics, paints, tires, and other consumer products. This thesis describes the attempts to produce 3HP from glucose in *E. coli* and from syngas in *R. rubrum*. Malonyl CoA reductase from *C. aurantiacus* and *R. castenholzii* was cloned and expressed in *E. coli* and *R. rubrum* respectively. This enzyme converts malonyl-CoA (a central metabolic intermediate) to 3-HP. Gas chromatography-mass spectrometry showed recombinant *E. coli* and *R. rubrum* produced 3HP in low levels. The quantity of 3-HP produced is not economical for commercial production so further studies were conducted to increase 3HP production.
CHAPTER 1: INTRODUCTION

In 20th century scientists started genetically engineering microbes to produce chemical compounds like insulin, hormones, drugs, etc. This approach reduced the cost and dependence on specific raw materials. This is very commodious approach as some of the raw material used in chemical industries is very costly and/or nonrenewable. Petroleum is such a raw material. Various starting chemicals used to make plastics, adhesives, fibers, cosmetics, tires, rubbers, etc. are the products of petroleum industry (Brown 2003). But petroleum stocks are diminishing and petroleum use has adverse effects on the environment. Thus, finding alternative starting chemicals that will reduce the dependence on petroleum is a broiling research field. Chemicals that are produced in an environmentally friendly way are called as “green chemicals”. Biotechnological approaches to the production of green chemicals will have far reaching implications. In order to replace petrochemical routes by biosynthetic routes, developing an organism with a pathway that will give the highest yield and developing the catalyst system to produce derivatives of those petroleum products are the main challenges today (T. Werpy 2004). Attempts are being made to generate biofuels and a variety of green chemicals using bacteria. 3-hydroxypropionic acid (3HP) is one such important chemical. It is one of the top 12 platform chemicals recognized by DOE. There is no cost effective method for the production of 3HP from fossil fuels but its derivatives such as acrylic acid are intermediates of the petroleum industry. It has been shown that 3HP can also be converted into 1-3 propanediol, malonic acid, acrylamide and salts and esters of acrylic acid, which are used in large quantities for adhesives, polymers, plastic packaging,
fibers and resins. (Gokarn 2002; Mark Paster July 2003). This thesis will address attempts made to produce 3HP from biomass by bacterial fermentation.

Biomass is material of recent biological origin. It includes all kinds of plants and waste like corn stover, distillers dried grains, etc. The energy in biomass is in the form of high energy chemical bonds. The energy from sunlight is trapped by photosynthesis. This stored energy is a raw material that can be used as a resource for heat and stationary power, transportation fuels, commodity chemicals and fibers. Till now, human beings have exploited this energy by burning biomass as a fuel and by eating plants for the nutritional content. Also, the fossilized biomass has been exploited as coal and oil. But, since it takes eons to convert biomass to fossil fuels these are not renewable within a timescale mankind can use. Burning these fuels converts the old biomass into new CO₂ and adds to the greenhouse effect. But, burning the new biomass contributes no new CO₂ to the atmosphere and replanting the harvested biomass recycles the CO₂ (McKendry 2002a; Brown 2003).

Traditionally, biofuels and green chemicals are produced from biomass by fermentation of carbohydrates in the plant materials. Much of the sugars in the plant material are in the form of polysaccharides such as the storage polysaccharide starch and the structural polysaccharide cellulose which gives shape and strength to the plant. This lignocellulosic material is comprised of cellulose fibers embedded in cross linked lignin hemicelluloses matrix. These sugars have to be released by depolymerization before fermentation, but this lignocellulosic material is highly resistant to chemical or biological attack. The release of sugars is carried out by chemical hydrolysis that employs mineral acids or enzymatic hydrolysis that employs microorganisms. This depolymerization before fermentation is (pretreatment) and is very costly process accounting for about 33% of total
processing costs (Brown 2003). An economically feasible alternative to this process is the
gasification of biomass to produce synthesis gas. And this synthesis gas is used for the
growth of microorganisms and for the production of biofuels and green chemicals by
fermentation.

Gasification is the high temperature (750-850°C) conversion of biomass to a gaseous
fuel collectively known as producer gas or synthesis gas or syngas. Synthesis gas is
composed mainly of CO (carbon monoxide), CO$_2$ (carbon dioxide), H$_2$ (hydrogen) and CH$_4$
(methane), N$_2$ (nitrogen) and smaller quantities of higher molecular weight hydrocarbons.
Synthesis gas can be used for generation of heat and power and also serves as feedstock for
production of liquid fuels and chemicals (Brown 2003). In addition to these uses, CO and H$_2$
in syngas are potential growth substrates for microorganisms. Certain microorganisms known
as unicarbonitrophs are able to grow on one carbon compounds as their sole energy source
and they can use CO as a growth substrate. These organisms include aerobic
carboxydotrophs, anaerobic acetogens, sulfate reducers, methanogens and phototrophs
(Young S. Do 2007). These organisms have an enzyme to convert CO to CO$_2$ in a water-gas
shift reaction: CO + H$_2$O $\rightarrow$ CO$_2$ + H$_2$. This enzyme is carbon monoxide dehydrogenase a
key enzyme for growth on syngas. Although there are limited number of bacteria available,
BRI Energy Inc has used gasification/fermentation route successfully to produce ethanol and
electricity on commercial level, here the syngas is produced from the organic waste.
Clostridium ljungdahli is a gram positive anaerobic organism isolated from chicken waste
and can grow on syngas. BRI Energy Inc modified this organism to metabolize CO and H$_2$ to
produce acetic acid and ethanol (Gaddy 1998; Gaddy 2002). Thus, microorganisms can be
metabolically engineered to produce value added products while growing on syngas.
Gasification/fermentation route to Biobased products from biomass has several advantages; gasification allows very high conversion of feedstock to usable compounds, approaching 100%. gasification yields uniform products (mixture of CO, CO$_2$, and H$_2$) regardless of the biomass feedstock used and finally gasification yields an inherently asceptic carbon supply since the syngas is produced at very high temperatures. (Brown 2003)

*Rhodospirillum rubrum* is another organism that is being used for syngas fermentation. *R. rubrum* a purple non-sulfur bacterium can utilize CO under anaerobic conditions as a sole carbon source in the presence or absence of light (Kerby, Hong et al. 1992). Two enzymes carbon monoxide dehydrogenase and CO-insensitive hydrogenase in *R. rubrum* are induced on exposure to CO and the combined effect of both enzymes result in the water-gas shift reaction (Ensign and Ludden 1991). Part of CO$_2$ produced is assimilated into cell material and the remaining CO$_2$ along with H$_2$ is released into the environment. *R. rubrum* is studied as a syngas/fermentation organism since metabolically engineered *R. rubrum* can be used to produce value added products such as ethanol, 3HP and at the same time H$_2$ gas can be enriched (Najafpour G 2003; Young S. Do 2007)

The syngas fermentation requires autotrophic CO$_2$ fixation. Four pathways of autotrophic CO$_2$ fixation have been described, 1) the reductive pentose phosphate cycle (Calvin cycle), 2) The reductive TCA cycle and 3) the reductive non cyclic acetyl-CoA pathway and 4) 3-hydroxypropionic acid cycle. The Calvin cycle is the only autotrophic pathway in plants and occurs in aerobic bacteria as well as purple nonsulfur bacteria (*Rhodobacter, Rhodospirillum, Rhodopseudomonas*), purple sulfur bacteria (*Chromatium*), cyanobacteria (*Anabena, Synechococcus*), hydrogen bacteria (*Ralstonia, Hydrogenvibrio*), and other chemoautotrophs (*Thiobacillus*). The Calvin cycle consists of 13 enzymatic
reactions and the reaction catalyzed by ribulose-1, 5- bisphosphate carboxylase/oxygenase is the only reaction that catalyzes the fixation of CO$_2$. The unique enzymes of the Calvin cycle are ribulose-1, 5- bisphosphate carboxylase/oxygenase which is the most abundant protein on the earth and phosphoribulosekinase and sedoheptulose bisphosphatase. (Atomi 2002). The 2$^{nd}$ and 3$^{rd}$ pathways found in anaerobic or microaerophilic bacteria. The green sulfur bacteria use reductive TCA cycle to fix CO$_2$. This pathway was first proposed in 1966 and the discovery of the ferredoxin pyruvate synthase and 2-oxoglutarate synthase as well as ATP dependant citrate lyase confirmed the presence of reverse flux of the TCA cycle. One complete turn of RTCA cycle results in the incorporation of four CO$_2$ molecules and the formation of oxaloacetate. (Evans, Buchanan et al. 1966; Atomi 2002). And finally the non cyclic acetyl-CoA pathway is found in methanogens, acetogenic bacteria and most autotrophic sulphate reducing bacteria. The key enzyme for this pathway is carbon monoxide dehydrogenase-acetyl-CoA synthase (White 2007). Holo et al proposed the presence of another CO$_2$ fixation pathway in thermophilic photosynthetic bacterium Chloroflexus aurantiacus and demonstrated that 3HP is excreted into the culture at the late growth phase of autotrophically growing bacteria. This production of 3HP is unique to this cycle. The Calvin cycle does not operate in this organism. 3HP cycle has also been reported in the branch of archaeabacteria comprising Acidianus, Sulfolobus and Metallosphaera species(Hugler, Krieger et al. 2003; Ishii, Chuakrut et al. 2004). The cycle starts with combining CO$_2$ with acetyl-CoA forming malonyl-CoA using ATP-dependant biotin containing enzyme acetyl-CoA carboxylase. This malonyl-CoA is then converted to 3HP by malonyl-CoA reductase enzyme, and cycle continues to produce acetyl-CoA and glyoxylate. Malonyl-CoA reductase is a key enzyme in this cycle and not required in any other known
bacterial pathway. The enzyme has been characterized by Hügler et al (Hugler, Menendez et al. 2002). It is a large bifunctional enzyme harboring an aldehyde dehydrogenase and alcohol dehydrogenase domains and has molecular weight of about 265 KDa. Conversion of malonyl-CoA to 3HP proceeds via malonate semialdehyde intermediate, which remains bound to the enzyme. Two molecules of NADPH are oxidized to NADP per molecule of 3HP formed. This enzyme is highly specific for its substrates and NADH is not oxidized. Also acetyl-CoA, propionyl-CoA and succinyl-CoA can not be substituted for malonyl-CoA. MCR follows Michaelis-Mentent kinetics and thus it is not an allosteric enzyme. It does not require any cofactor for activity. So far the complete 3HP cycle has been studied only in C. aurantiacus and the research is going on to study this cycle in Sulfolobus spp. The malonyl-CoA reductase enzyme from Sulfolobus spp. is also being studied (Hugler, Krieger et al. 2003; Alber, Olinger et al. 2006). This archaeal version of the enzyme is not bifunctional and has no resemblance with the bacterial enzyme. When a NCBI database was searched with C. aurantiacus malonyl-CoA reductase only 5 other similar proteins were found from Chloroflexus aggregans (Genbank accession number EAV11821.1), Roseiflexus castenholzii (Genbank accession number EAV26845.1), Roseiflexus sp strain RS-1 (Genbank accession number EAT28741), uncharacterized protein from α-proteobacterium Erythrobacter sp. strain NAP-1 (Genbank accession number EAQ29650) and marine gamma proteobacterium HTCC2080 (Genbank accession number EAW40916.1), but no similar archaeal proteins were found and showed little similarity to known oxidoreductases. The archaeal enzyme is monofunctional, smaller in size and has only aldehyde dehydrogenase activity. It converts malonyl-CoA to malonate semialdehyde and has resemblance to aspartate-semialdehyde dehydrogenase.
In this work malonyl-CoA reductase from *C. aurantiacus* and *R. castenholzii* are used to produce 3HP in *E. coli* and *R. rubrum*. 
CHAPTER 2: MALONYL COA REDUCTASE CLONING IN *E. coli*

2.1 INTRODUCTION

Gokarn et al. have proposed five possible routes to produce 3HP biosynthetically from sugars or intermediates of central carbon metabolism (Gokarn 2002) (Fig 1). All the pathways start with pyruvate but proceed through different intermediates to produce 3HP. One pathway starts with pyruvate and forms β-alanine intermediate, it is converted to malonate semialdehyde which is converted to 3HP. The β-alanine formed in the above pathway can also be diverted to form β-alanyl-CoA, which is converted to acryloyl-CoA and then to 3HP. In another pathway, pyruvate is converted to lactate then to acryloyl-CoA via lactoyl CoA and then to 3HP. In last pathway, pyruvate is converted to 3HP via succinate.

![Diagram of biosynthetic pathways for 3HP](image)

*Figure 1*: Possible biosynthetic pathways for the synthesis of 3HP, as described by Cargill Inc. in patent application PCT WO 02/42418 A2.
In this study *E. coli* and *R. rubrum* were genetically engineered to produce 3HP from glucose or syngas respectively. This was done by introduction of a single enzyme Malonyl-CoA Reductase (MCR). *C. aurantiacus* MCR has been characterized in detail by Hügler et al. It is a large bifunctional enzyme harboring an aldehyde dehydrogenase and alcohol dehydrogenase domains and has molecular weight of about 265 KDa. Conversion of malonyl-CoA to 3HP proceeds via malonate semialdehyde intermediate, which remains bound to the enzyme. Two molecules of NADPH are oxidized to NADP per molecule of 3HP formed. This enzyme is highly specific for its substrates and NADH is not oxidized, also acetyl-CoA, propionyl CoA and succinyl CoA can not be substituted for malonyl-CoA. MCR follows Michaelis-Mentent kinetics and thus it is not an allosteric enzyme. It does not require any cofactor for activity (Hugler, Menendez et al. 2002; Hugler, Krieger et al. 2003). The MCR from *R. castenholzii* has not been characterized. The genbank blast search shows 58% protein identity with *C. aurantiacus* MCR. Based on 16s rDNA sequencing *R. castenholzii* is placed in the family Chloroflexaceae represented by anoxygenic filamentous phototrophic bacteria including *C. aurantiacus*. (Hanada, Takaichi et al. 2002).

In this section *E. coli* is engineered to produce 3HP. MCR from *C. aurantiacus* and *R. castenholzii* is expressed in *E. coli*. The specific activity of *C. aurantiacus* and *R. castenholzii* MCR was 75 and 11 nmol min\(^{-1}\) mg\(^{-1}\) respectively. The production of 3HP during growth of recombinant *E. coli* expressing MCR was measured under various conditions.

2.2 MATERIALS AND METHODS

**Chemicals and reagents:** Malonyl-CoA and NADPH were from Sigma. IPTG was from
Anatrace and DTT was from ICN. Restriction enzymes, Taq polymerase, Pfu polymerase, T4 ligase were from New England Biolabs. 3HP was obtained from Cargill. Other chemicals were from Fisher Scientific.

**Bacterial Strains and Media:** The bacterial strain used in this study was *E. coli* DH5α (F^-λ^- endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1 Δ (lacZYA-argF) U169 (Φ80dlacZΔM15)). LB (Luria-Bertani) medium was the rich medium used (Miller, 1972). Minimal medium was NCE (Berkowitz, Hushon et al. 1968) supplemented with 1mM MgSO_4_ and a carbon source. The carbon sources used were 0.2% glucose, 0.2% glycerol and 1.0% succinate. Ampicillin was used at 100 µg/ml Kanamycin at 25 µg/ml and IPTG was used at 0.5mM conc.

**General molecular methods:** Agarose gel electrophoresis was performed as described by Maniatis et al. (Maniatis, Fritsch et al. 1982). Plasmid DNA was purified by the alkaline lysis procedure (Maniatis, Fritsch et al. 1982) or by using Qiagen kits according to the manufacturer’s instructions. Following restriction digestion or PCR amplification, DNA was purified using Promega or Qiagen PCR purification kits. Restriction digestions were carried out using standard protocols (Maniatis, Fritsch et al. 1982). For ligation of DNA fragments, T4 DNA ligase was used according to manufacturer’s instructions. Electroporation was used for bacterial transformation. A Gene Pulser (Bio-Rad, Richmond, Calif.) was used according to manufacturer’s instructions and at the following settings; capacitance, 25μF; pulse controller, 200Ω; voltage, 2.5kV. LB medium with containing the appropriate antibiotic was used to select for transformed cells and, prior to the analysis of transformants, pure cultures were prepared.
**General Protein methods:** SDS PAGE was performed for using Bio-Rad ready gels and Mini-protean II electrophoresis cells. PAGE was run at 200V for 45 min using a Bio-Rad Powerpac 300. Following gel electrophoresis, Coomassie brilliant blue R-250 was used to stain proteins. Protein concentration was determined using Bio-Rad Protein assay reagent according to the manufacturer’s instructions with Bovine serum albumin (BSA) as the standard.

**DNA sequencing and analysis:** DNA sequencing was carried out at the DNA sequencing and Synthesis Facility of the Iowa State University, using Applied Biosystem Inc. ABI 3730 DNA analyzer. The template for DNA sequencing was plasmid DNA purified using Qiagen Hispeed plasmid midi prep kit.

**Cloning malonyl-CoA reductase gene:** PCR was used to amplify the malonyl-CoA reductase gene from the genomic DNA of *Chloroflexus aurantiacus* strain OK-70-fl and *Roseoflexus castenholzii* HLO8 for cloning into a modified pLac22 plasmid (4543 bp) and pBBR1MCS-2 (5144bp), respectively. The genomic DNA for *Chloroflexus aurantiacus* strain OK-70-fl was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures) and *Roseoflexus castenholzii* HLO8 genomic DNA was a gift from Dr. Don Bryant from Penn State University. The primers for *C.aurantiacus* were 5'-GCCGCCAGATCTATGGCGACGAGGGGAGTCCAT-3' (forward) and 5'-GCCGCCAAGCTTTACACCGGATATCGCCCGTCC-3' (reverse). These primers introduced the restriction sites BglII and HindIII that were used to clone the malonyl-CoA reductase sequence (3690 bp) into pLac22. The primers for *R. castenholzii* were 5'-GCCGCCAAGCTTTTACACAGGAAAGATCTTTATGAGCAGCAGTGCAGACT-3' (forward) and 5'-GCCGCCGGATCTTACACCGGTAATCGCATTTCC-3' (reverse). These
primes introduced BamHI and HindIII restriction sites that were used to clone the malonyl-CoA reductase gene (3687bp) into pBBR1MCS-2. Ligation mixture was used to transform E. coli strain DH5α and transformants were selected by plating on LB agar supplemented with 100 µg/ml of ampicillin for pLac22 and LB agar, X-gal, IPTG plates supplemented with 25 µg/ml of kanamycin for pBBR1MCS-2. The new plasmids were named as pNA1 and pNA5 respectively. Plasmid DNA isolated from selected transformants was analyzed by restriction digestion and DNA sequencing.

**Growth of the malonyl-CoA reductase expression strains and preparation of cell extracts:** E. coli strain DH5α was grown in 500 ml LB ampicillin (100 µg/ml) and LB kanamycin (25 µg/ml) broth incubated at 37°C with shaking in a 1 liter baffled Erlenmeyer flask. Cells were grown to an OD₆₀₀ 0.6-0.8. The expression of malonyl-CoA reductase was induced using IPTG with final conc. of 0.5 mM. Cells were then incubated for additional 3-4 hours and harvested by centrifugation at 5000 rpm for 20 min using a Beckman J-25 centrifuge and Beckman JA-10 rotor. Cells were suspended in 3 ml of buffer, 50mM sodium phosphate having 300 mM NaCl, pH 7.0. To inhibit protease activity Pefabloc was added at the final concentration of 0.2 mM. Cells were broken using a French pressure cell (SLM Amico) at 1000 atm pressure. Soluble proteins were separated from inclusion bodies by centrifugation of cell extracts at 30000×g for 30 min using a Beckman JA-17 rotor. The supernatant obtained was the soluble cell extract used for further studies. Control strains DH5α (E. coli DH5α/pLac22-no insert) and (E. coli DH5α/pBBR1MCS-2-no insert) was grown in parallel with expression strains DH5α/pNA1 and DH5α/pNA5 and cell extracts of this strain were similarly prepared.
**Malonyl-CoA reductase assays:** Malonyl-CoA reductase assays were carried out as described previously by Hügler, *et al.* (Hugler, Menendez *et al.* 2002). The only modification was DTT was used instead of DTE. Assay mixtures (1 ml total volume) contained 100 mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 3 mM DTT, 0.3 mM NADPH, 0.3 mM malonyl-CoA, H₂O and cell free extract. The reaction mixture was heated to 37°C for 3 min before starting the reaction. The reaction was started by addition of cell free extract. Two control reactions were performed in parallel. In one control reaction, malonyl-CoA was omitted. In the other control reaction, the cell free extract from control strain was used. The reaction rates were determined by monitoring the decrease in absorbance at 340 nm and using ∆ε₃₄₀=6220 M⁻¹ cm⁻¹ for calculations. The absorbance was measured at 340 nm and activity was calculated based on NADPH consumed.

**GCMS analysis of 3HP:** Chromatographic analysis for *C. aurantiacus* MCR was performed with a gas chromatograph (Model 6890 series, Agilent Technologies, Palo Alto, CA), equipped with a mass detector Model 5973 (Agilent Technologies). Chromatography was conducted with DB-WAX column (30m length, 0.25 ID.) using helium as a carrier gas. The GC-MS was equipped with auto sampler and the 50ºC was initial column temperature which was raised to 250ºC at a rate of 5ºC/min.

*E. coli* strain DH5α harboring pNA1 was grown in 50 ml LB medium as well as NCE minimal medium with different carbon sources and 100 µg/ml ampicilin and 0.5 mM IPTG. Cultures were grown mainly under aerobic conditions, 1% succinate, 0.2% glucose and 0.2% glycerol were used as carbon sources for minimal medium. Cultures of NCE minimal medium with glucose carbon source were also grown under anaerobic conditions. The cultures were incubated at 37°C with shaking. The cells were grown overnight. Malonyl-CoA
reductase enzyme assay reaction mixture was also analyzed by GC-MS for the presence of 3HP as a final product of the reaction. One ml of supernatant or reaction mixture was used for GC-MS analysis. Purified 3HP was used as standard. The methylation of 3HP was carried out as described (Holdeman and Moore 1972), 1 ml of sample; 2 ml of methanol and 0.4 ml of 50% H₂SO₂ were heated at 55ºC for 30 min, then 1ml water and 0.5 ml of chloroform was added. Centrifugation was used to break down the emulsion and the lower chloroform phase was collected which contains methylated 3HP. The chloroform phase was concentrated to 2 µl by flushing nitrogen before loading for GC-MS analysis. Benzoic acid was used as an internal standard. 250 µM of benzoic acid was added to the samples just before methylation.

2.3 RESULTS

Production of Malonyl-CoA reductase: E.coli strain DH5α/pNA1 was constructed to produce recombinant malonyl-CoA reductase protein. Protein production by this strain as well as control strain DH5α/pLac22 was analyzed by SDS-PAGE (Fig. 2). For expression strain, a band with a molecular mass near that predicted for malonyl-CoA reductase (132.62 kDa) was observed on gel while in control strain that band was weak.

Malonyl-CoA reductase enzyme activity: The cell extracts used for SDS-PAGE were assayed for malonyl-CoA reductase activity using the continuous spectrophotometric assay described in the methods section. Cell free extracts (CFE) of E. coli DH5α/pNA1 catalyzed the malonyl-CoA dependant oxidation of NADPH, corresponding to the specific activity of 75 nmol min⁻¹ (mg of protein)⁻¹. When malonyl-CoA was left out of the reaction or CFE from DH5α/pLac22 was used no activity was observed. Similarly cell extracts with
DH5α/pNA5 catalyzed the malonyl-CoA reductase enzymatic reaction giving specific activity of 11.25 nmol min⁻¹ (mg of protein)⁻¹ and no activity was observed when malonyl-CoA was left out or CFE from DH5α/pBBR1MCS-2 was used. This indicates that NADPH was consumed by malonyl-CoA reductase and not another enzyme present in the crude extract.
Production of 3HP: Under the GC-MS conditions used the retention time for 3HP methyl ester was about 15 min. Methylation with benzoic acid was not complete and two peaks were observed one for benzoic acid and other for benzoic acid methyl ester, the retention time for both was found to be 14 min, and 32.8 min respectively. 3HP was measured in the cultures of strain DH5α/pNA1 grown under different conditions (Table 1.). 3HP was not observed in the cultures of strain DH5α/pLac22. Likewise 3HP was detected in the enzyme assay with DH5α/pNA1 CFE, but not in assay with the control strain. The highest conc. of 3HP was detected with LB media. 0.102µM of 3HP was detected with enzyme assay reaction and no
3HP was detected in the control reaction which indicates that the 3HP is produced by the cloned malonyl-CoA reductase gene in vector pNA1.

**Table 1: 3HP concentrations in genetically modified *E. coli* grown under various conditions.**

<table>
<thead>
<tr>
<th>Media, carbon source, DH5α/pNA1</th>
<th>Conc. of 3HP µM/ml</th>
<th>Media, carbon source, DH5α/pLac22</th>
<th>Conc. of 3HP µM/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>2.587</td>
<td>LB</td>
<td>0.0</td>
</tr>
<tr>
<td>Glucose aerobic</td>
<td>0.268</td>
<td>Glucose aerobic</td>
<td>0.0</td>
</tr>
<tr>
<td>Glucose anaerobic</td>
<td>0.0</td>
<td>Glucose anaerobic</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.090</td>
<td>Glycerol</td>
<td>0.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.216</td>
<td>Succinate</td>
<td>0.0</td>
</tr>
<tr>
<td>Enzyme assay reaction mix</td>
<td>0.102</td>
<td>Enzyme assay reaction mix</td>
<td>0.0</td>
</tr>
</tbody>
</table>

2.4 DISCUSSION

The petrochemical industry is one of the world’s most important industries but petroleum reserves are limited. So it is important to develop methods for the conversion of renewable sources from plants and agricultural waste to chemical building blocks same as petrochemical industry. One of these chemicals is 3HP, (Suthers 2001; Vollenweider and Lacroix 2004) which is a one of the top 12 platform chemicals selected by the DoE biomass program. (T. Werpy 2004). It has potential to become a key building block for commodity and specialty chemicals. The derivatives of 3HP include 1, 3-propanediol, malonic acid, acrylic acid, acryl amide, ethyl 3HP and poly (hydroxypropionate) which have many uses in day-to-day life. Also, 3HP can be used as antifreeze, food preservative, and food additive and has low corrosivity (Selfinova 2002)

Several authors have described the production of 3HP during glycerol fermentation giving equimolar concentration of 1, 3-propanediol (1, 3-PDO) and 3HP (van Maris,
In 2001, formation of low concentration of 3-hydroxypropionate from glycerol, via 3-hydroxypropanal was demonstrated with genetically modified *E. coli* expressing glycerol dehydratase from *Klebsiella pneumoniae* and a non specific aldehyde dehydrogenase (ALD4) from *S. cerevisiae* (Suthers 2001).

In this study, *E. coli* was genetically modified but only a single enzyme malonyl-CoA reductase was introduced into *E. coli* DH5α using vector. Malonyl-CoA reductase was expressed and specific activity measured was 75 nmol min⁻¹ (mg of protein)⁻¹ and low levels of 3HP were produced. In this strain acetyl-CoA is converted to malonyl-CoA by biotin dependant acetyl-CoA carboxylase enzyme and then it is transformed to 3HP via MCR.

Malonyl-CoA is an intermediate in fatty acid metabolism in bacteria and is synthesized using acetyl-CoA carboxylase; it was assumed that part of malonyl-CoA will be used by malonyl-CoA reductase. In case of inadequate malonyl-CoA, expression of acetyl-propionyl CoA carboxylase might increase 3HP production. The pathway described in this paper is shown in figure 4. When *E. coli* is cultivated under aerobic conditions pyruvate is converted to Malonyl-CoA which is used by MCR to form 3HP. When *E. coli* is cultivated under anaerobic conditions it is assumed that pyruvate will be converted to formate and acetyl-CoA and then to malonyl-CoA and expression of malonyl-CoA reductase allows the production of 3HP. However, in this study the production of 3HP under anaerobic conditions could not be detected. A possible reason is *E. coli* was grown on minimal media with glucose as a carbon source. The vector pLac22 has the wild type lac promoter which is sensitive to catabolic repression. Thus, repression of MCR from the lac promoter is observed when glucose is the main carbon source because very little c-AMP is present and thus low amounts
of c-AMP-activated CAP protein are available. Hence, under anaerobic conditions expression of malonyl-CoA reductase would be observed during growth on poor carbon sources such as lactose and glycerol which increase the conc. of cAMP (Warren, Walker et al. 2000). Another feature of this study is that a range of carbon sources can be used to produce 3HP unlike in the previous study where only glycerol or glucose could be used. In this study, 3HP was produced with glucose, glycerol, LB and succinate.

Figure 4: Proposed pathways for the production of 3HP A. Under anaerobic conditions, B. under aerobic conditions. 1. Acetyl-CoA carboxylase, 2. Malonyl-CoA reductase.
CHAPTER 3: EVOLUTION OF MALONYL-COA REUCTASE

3.1 INTRODUCTION

The production of 3HP in \textit{E. coli} should be large enough to be commercially viable. To achieve this, different strategies can be used to improve the enzymatic activity of MCR in \textit{E. coli}. One such strategy is the metabolic evolution of \textit{E. coli}. Zhou et al. used the similar method to improve the sugar fermentation to produce D (\textit{-})-lactate by engineering \textit{E. coli} (Zhou, Yomano et al. 2005).

The metabolic evolution for MCR can be accomplished by evolving MCR to regenerate NAD during fermentation (Figure 5) (Hugler, Menendez et al. 2002). Glyceraldehyde 3-phosphate dehydrogenase is a key enzyme in glycolysis that converts D-glyceraldehyde 3-phosphate to 1, 3-bisphosphoglycerate and NAD$^+$ is converted to NADH. When \textit{E. coli} is grown anaerobically this NAD$^+$ must be regenerated to support Glyceraldehyde 3-phosphate dehydrogenase. If the pathways leading to ethanol and D-lactate are blocked under anaerobic conditions NAD$^+$ would become limiting for growth of \textit{E. coli}. NAD regeneration could be accomplished by MCR assuming that NADP$^+$ is used to convert NADH to NAD$^+$ in \textit{E. coli} by transhydrogenase; or if MCR itself is modified to use NADH rather than NADPH.

For directed evolution of MCR, the pathways leading to ethanol and D-lactate were inactivated by knocking out the genes for aldehyde/alcohol dehydrogenase (\textit{adhE}) and D-lactate dehydrogenase (\textit{ldhA}). The mutations in \textit{adhE} and \textit{ldhA} were transferred from respective \textit{E. coli} mutants from \textit{E. coli} Keio collection. The Keio collection is the collection of single gene knock out mutants of all nonessential genes/ORFs including putative ones has
been performed by using linear transformation of PCR products (Baba, Ara et al. 2006). This method is a state-of-art method to create a knock out mutant in one step by using λ red recombinase. (Datsenko and Wanner 2000). The adhE and ldhA mutations from Keio collection were transferred to wild type E. coli MG1655 by P1 transduction and the kanamycin resistance cassette was flipped out using plasmid harboring FLP recombinase.

![Figure 5: Mixed acid fermentation in E. coli.](image)

The modification of MCR to change specificity from NADPH to NADH and/or improvement in the enzyme activity could be achieved by introducing random mutations in the gene sequence of MCR from C. aurantiacus and R. castenholzii. The random mutations
were introduced using the *E. coli* XL1 red mutator strain from Stratagene which is deficient in three of the primary DNA repair pathways: *mutS* (error-prone mismatch repair), *mutD* (deficient in 3’- to 5´-exonuclease of DNA polymerase III) and *mutT* (unable to hydrolyze 8-oxodGTP). After the mutations were introduced into both the plasmids harboring MCR genes, these plasmids were transferred into the *adhE-ldhA* double mutant *E. coli* and grown anaerobically. Any mutation that could restore the growth of double mutant *E. coli* would potentially have the mutated MCR with improved enzyme activity and/or changed specificity from NADPH to NADH.

### 3.2 MATERIALS AND METHODS

**Chemicals and other reagents:** Restriction enzymes were from NEB. Chemicals for buffers and media were from Fisher or Sigma.

**Bacterial strains, Virus and Media:** The *E. coli* strains used in this study are given in the table 2. Bacteriophage P1 was a gift from Dr. Gregory Phillips at Iowa State University. The rich media used were LB (Luria-Bertani) for general growth and SOC for electrotransformation (Miller 1972). Minimal medium was NCE (Miller 1972) supplemented with 1 mM MgSO$_4$ and a carbon source used was 0.2% glucose. Kanamycin is used at the conc. of 25 µg/ml. The top agar used contained (per liter of water) Bacto-tryptone, 10g; yeast extract, 5g; NaCl, 5g; 2 mM CaCl$_2$; 10 mM MgSO$_4$ 0.1% glucose and bacto-agar, 5g. Phage adsorption buffer was MC (10 mM MgSO$_4$ and 5 mM CaCl$_2$). The bacteriophage P1 dilution buffer contained 10 mM tris-HCl; 10 mM MgSO$_4$; 5 mM CaCl$_2$; and 50 mM NaCl. And P1broth is LB containing 0.2% glucose and 5 mM CaCl$_2$.

**General molecular methods:** As described in Chapter 1.
**DNA sequencing and analysis:** As described in Chapter 1.

**Table 2: Bacterial strains and plasmids used for MCR evolution.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F’λ- endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1 ∆(lacZYA-argF) U169 (Φ80dlacZ∆M15)</td>
<td></td>
</tr>
<tr>
<td>XL1-Red</td>
<td>endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT Tn10 (TetR)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>MG1655</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td>JW1228</td>
<td>rnb3 ∆lacZ4787 hsdR514 ∆(araBAD567 (rhaBAD)568 rph-1 adhE::kan</td>
<td>(Baba, Ara et al. 2006)</td>
</tr>
<tr>
<td>JW1375</td>
<td>rnb3 ∆lacZ4787 hsdR514 ∆(araBAD567 (rhaBAD)568 rph-1 ldhA::kan</td>
<td>(Baba, Ara et al. 2006)</td>
</tr>
<tr>
<td>NA19</td>
<td>F’λ- ilvG- rfb-50 rph-1, adhE::FRT</td>
<td>This study</td>
</tr>
<tr>
<td>NA20</td>
<td>F’λ- ilvG- rfb-50 rph-1, adhE::FRT ldhA::FRT</td>
<td>This study</td>
</tr>
<tr>
<td>NA24</td>
<td>NA20/pNA3</td>
<td>This study</td>
</tr>
<tr>
<td>NA27</td>
<td>NA20/pNA5</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Cloning of MCR from C. aurantiacus in pBE522:** The plasmid pBE522 is modified from pET41a to have constitutive T7 promoter and a regulated lac promoter. The MCR gene in pNA1 was amplified using the primers; 5’-

GCCGCCAGATCTATGGCGACGGGGGAGTCCAT-3’ (forward) and 5’-

GCCGCCAAGCTTTCACACGGTAATCGCCCGTCC-3’ (reverse). These primers introduced the restriction sites BgIII and HindIII that were used to clone the malonyl-CoA reductase sequence (3690 bp) into pBE522. Ligation mix was used to transform *E. coli* strain
DH5α and transformants were selected by plating on LB agar supplemented with 25µg/ml of kanamycin. The new plasmid was named as pNA3. Plasmid DNA isolated from selected transformants was analyzed by restriction digestion and DNA sequencing.

**Preparation of Bacteriophage P1:** The P1 phage was prepared by broth method. Host strain (*E. coli* MG1655/JW1228/JW1375) was grown overnight at 37°C. 100µl of cells were added to 10 ml P1 broth and incubated at 37°C for 60 min without shaking. 100µl of P1 virus was added (P1 virus titer used was 3.2×10⁹ pfu/ml) and incubated at 37°C for 2 hours with vigorous shaking until the culture is cleared. The cells were transferred to centrifuge tube and chloroform was added before centrifugation. The supernatant was stored at 4°C with 50µl of chloroform. Titering of the phage was done as described elsewhere. (Goldberg, Bender et al. 1974). MC buffer was used instead of AM buffer. The titer obtained for P1 virus was 3.2×10⁹ pfu/ml. The titer for *adhE* phage lysate was 10⁹ pfu/ml and for *ldhA* phage lysate was 1.3×10⁹ pfu/ml.

**Deletion of *adhE*:** The P1 transduction was performed with *adhE* phage lysate. The host for transduction was *E. coli* MG1655. The transduction procedure similar to the one described by Goldber et al. (Goldberg, Bender et al. 1974) except the temp. used was 37°C instead of 30°C and selective medium plates were LB kanamycin. The deletion of *adhE* was confirmed with PCR amplification. The primers used were 5’-AAAGCGTCAGGCAGTGTTGTA-3’ (primer #1) and 5’- CGGTGCCCTGAATGAACTGC -3’ (primer #2). Primer #2 is the primer that aligns with kanamycin sequence and primer #1 is the primer downstream of *adhE* gene on the *E. coli* K12 genome. The antibiotic resistance was eliminated as described by Wanner et al (Datsenko and Wanner 2000). The *E. coli* strain obtained here was named NA19 (F⁻, λ⁻, *ilvG-, rfb-50, rph-1, adhE::FRT*).
Deletion of \textit{ldhA}: The P1 transduction was performed with \textit{ldhA} phage lysate. The host strain for transduction was NA19. Transduction was performed as described above and \textit{ldhA} deletion was confirmed with PCR. The primers used were 5’-GTTTACGCTTTCCAGCACAA-3’ (primer #1) and 5’-CGGTGCCCTGAATGAACGC-3’ (primer #2). Primer #2 is the primer that aligns with kanamycin sequence and primer #1 is the primer downstream of \textit{ldhA} gene on the \textit{E. coli} K12 genome. The antibiotic resistance was eliminated as mentioned above. The \textit{E. coli} strain obtained here was named NA20 (F’ λ’ ilvG- rfb-50 rph-1, \textit{adhE::FRT ldhA::FRT}).

Mutagenesis in pNA3 and pNA5: The random mutations were introduced into pNA3 and pNA5 by passing through \textit{E. coli} mutator strain XL1-Red (Stratagene) according to manufacturer’s instructions along with five serial transfers to improve the chances of random mutations. The plasmids were then isolated and transformed into NA20.

Growth of double mutant: The transformed double mutant NA20 harboring pNA3 or pNA5 named as NA24 and NA27 respectively were spread on NCE agar plates and incubated in anaerobic chamber at 37° C.

3.3 RESULTS

Cloning of MCR from \textit{C. aurantiacus} in pBE522: As mentioned previously pNA1 contains the wild type lac promoter that is sensitive to catabolic repression and expresses well only with poor carbon source. For this study it was important to have MCR cloned under the promoter that could express well with rich carbon source such as glucose. Also the previous attempts to clone MCR in original pET41a failed due to plasmid rearrangement. Plasmid
pBE522 is modified to have regulated lac promoter and a constitutive T7 promoter and thus the expression of MCR can be initiated with the addition of IPTG.

**Deletion of adhE and ldhA:** Transfer of adhE and ldhA mutation into E. coli replaces the respective genes with kanamycin resistance marker flanked by 35 nucleotides of FRT site. The successful transfer of mutations was confirmed by the PCR reaction. Primer #1 for adhE is 232 bps downstream of the gene and for ldhA it is 250 pbs downstream and expected PCR product for both genes was about 1000 bps.

The plasmid pCP20 is Cm\(^R\) and Amp\(^R\) and has temperature sensitive replication of origin. It has the gene for FLP recombinase that recognizes the FRT sites flanking the kanamycin resistance gene (Cherepanov and Wackernagel 1995). After transformation with pCP20, NA20 was selected for Amp\(^R\) at 30\(^\circ\)C. Few transformants were colony purified at 37\(^\circ\)C and again tested for loss of antibiotic resistance, FRT flanked resistance marker and pCP20 were lost simultaneously and scar fragment was left as shown in figure 6.

**Growth of NA24 and NA27:** E. coli strains NA24 and NA27 harboring pNA3 and pNA5 respectively were grown on NCE-kan agar plates or liquid media supplemented with 0.1% yeast extract, 1mM MgSO\(_4\), 0.2% glucose and 0.5 mM IPTG at 37\(^\circ\)C under anaerobic conditions. But no growth was observed even after 4 weeks of incubation. One reason could be that MCR has not evolved enough to show higher enzyme activity and other reason for the absence of the growth could be that the normal regeneration of NAD\(^+\) by AdhE and ldhA is disturbed and it depends on the E.coli transhydrogenases and failure to convert NADPH to NAD\(^+\) at high rate would result in no growth.
3.4 DISCUSSION

The use of enzymes and whole cells as biocatalysts for commercial purposes is rapidly increasing (Schmid, Dordick et al. 2001; Zaks 2001). Biocatalysts are more desirable because of high selectivity and their environmental friendliness. But naturally occurring enzymes and proteins are sometimes not suitable for the commercial processes because of, low enzyme activity, stability, specificity and other chemical constraints. These proteins evolved as a result of natural selection and their properties are ideally suited for the natural conditions in which they occur. For example the specific activity of MCR from C. aurantiacus is sufficient according to the growth rate of the organism (Hugler, Menendez et al. 2002) although it is not commercially favorable. Recent advances in recombinant DNA technologies, genomics, proteomics, etc has fuelled the development of new biocatalysts and directed evolution has emerged as a powerful tool.

Directed evolution mimics the Darwinian evolution in the test tube and involves the generation and selection of molecular library with sufficient diversity for the desired function. Directed evolution is dated back to 1970’s but really drew the attention when Gold et al came up with the SELEX protocol (Tuerk and Gold 1990). Directed evolution usually involves recombination, mutagenesis and selection. After successful recombination and mutagenesis selection turns out to be a crucial process since it is the window to look for the desired molecule with desired properties.
In case of metabolic evolution of MCR, there is no good test for selection and with current selection method various problems could generate false negatives. One problem could be the regeneration of NAD\(^+\) by *E. coli* transhydrogenase. *E. coli* contains two isoforms of transhydrogenase encoded by *PntAB* and *UdhA* genes. *PntAB* is a membrane bound form and *UdhA* is soluble and energy independent form. Transhydrogenase catalyzes the reaction; 

\[
\text{NADP}^+ + \text{NADH} \leftrightarrow \text{NADPH} + \text{NAD}^+. 
\]

The regeneration of these pyridine nucleotide coenzymes is essential since they are electron carriers in the redox reactions.
Recent progress in biotechnology has lead to the development of tools that are useful in commercial biocatalysis and metabolic engineering since many reactions of commercial interest are NAD (P) dependant redox reactions and the cost of pyridine nucleotide coenzymes needed to provide to maintain the reaction stoichiometry is very high. In 1997 Galkin et al solved the problem of coenzyme pool regeneration by coupling the expression of different enzymes to produce optically active amino acids (Galkin, Kulakova et al. 1997). Kataoka et al (M. Kataoka 1999) and Weckbecker et al (Weckbecker A 2004) used glucose dehydrogenase to regenerate coenzymes in the production of (R)-4-chloro-3-hydroxybutanoate and chiral alcohols respectively, but using GDH has its own disadvantages like formation of unwanted byproduct like gluconolactone. Boonstra et al (Boonstra, Rathbone et al. 2000) coexpressed the soluble pyridine nucleotide transhydrogenase (STH) from Pseudomonas fluorescens but the disadvantage of this enzyme is that it is inhibited by NADP\(^+\) and activated by NADPH (French, Boonstra et al. 1997). So in case of MCR using this enzyme for coexpression may lead to its inhibition since NADP\(^+\) is produced in the reaction. Lastly, Weckbecker et al (Weckbecker and Hummel 2004) used E. coli PntAB in the production of chiral alcohols. The PntA and PntB genes were coexpressed with NADP\(^+\) dependant alcohol dehydrogenase from Lactobacillus kefir and NAD\(^+\) dependant formate dehydrogenase from Candida boidinii and E. coli transhydrogenase regenerates NAD\(^+\) and NADPH. In similar manner the regeneration of NAD\(^+\)/NADPH could be attempted by co-expression of pyridine nucleotide dehydrogenase into double mutant E. coli NA24 harboring C. aurantiacus MCR and NA27 harboring R. castenholzii MCR. If the random mutations in both MCR genes have increased the activity then NADP\(^+\) generated in the conversion of malonyl-CoA to 3HP would help to revive the growth of double mutant E. coli (Figure 7).
Another way to solve the problem of coenzyme regeneration is to change the specificity of MCR genes from NADPH to NADH. This kind of specificity switch has been done predictably in many enzymes for example Perham et al changed NADP⁺ binding glutathione reductase to NAD⁺ binding enzyme and NAD⁺ binding pyruvate dehydrogenase to NADP⁺ binding enzyme while retaining the substrate specificity in both the enzymes (Scrutton, Berry et al. 1990; Bocanegra, Scrutton et al. 1993). The NAD/NADP binding motif called as Rossmann fold is paired βαβ folds. This structure is conserved in proteins that involve oxidation or reduction of NAD (P)/NAD (P) H. The proteins having NAD/NADP binding
site involve dehydrogenases and reductases. Protein sequence alignment of both MCRs shows partial similarity with the proteins from the short chain oxidoreuctase (SCOR) enzyme family also known as short chain dehydrogenases/reductases (SDR). Amino acids between 1 and 250 and 550 to 800 show similarity to the SCOR family, which is in agreement with the MCR enzyme profile that it is a bifunctional enzyme harboring the activities of alcohol and aldehyde dehydrogenase. And amino acid sequence shows the fingerprint TGxxxGIG β1α2 turn sequence at the position starting 3 and 584 also shows the catalytic sequence YxxxK with a variable linker of 120-190 amino acids (William L. Duax 2003). The crystal structure of the MCR is not known and this could possibly cause a problem in protein engineering to switch the enzyme specificity.

Directed evolution of the enzymes is achieved by creating molecular diversity by random mutagenesis and/or recombination. This can be also be achieved by modern molecular techniques (Kaur and Sharma 2006). Most basic methods are error prone PCR (Pritchard, Corne et al. 2005) and alcohol mediated error prone PCR but these techniques have limited applications since they are useful when limited number of cycles have to be applied and introduce only point mutation. This limits the evolution when block mutations are required for the evolution. This problem was solved by DNA shuffling which can be used much more effectively to create the molecular diversity. There are plenty of examples of DNA shuffling used to improve the protein catalytic efficiency solubility and stability. DNA shuffling is usually involves the number of genes which has similar activity; these genes are mutated and recombined to create several generations of libraries from which the gene with desired properties is selected (Castle, Siehl et al. 2004; Kaur and Sharma 2006). Various protocols are available for DNA shuffling but synthetic shuffling would be more effective in
case of MCR (Ness, Kim et al. 2002). Synthetic shuffling explores sequence space more extensively, in other shuffling protocols recombination is guided by sequence identity of the parent gene but with synthetic shuffling that problem is eliminated. This method is flexible and also codon usage can be altered.

Nakamura et al constructed the strain to produce high levels of 1, 3-propanediol in *E. coli* where gene for glyceraldehyde 3-P dehydrogenase was down regulated and dihydroxy acetone phosphate was directed to the production of 1, 3-propanediol as well as D- glucose transport by phosphotransferase system was eliminated (Nakamura and Whited 2003). Similarly 3HP production can also be improved by engineering central metabolic pathways to direct the metabolic flux in the production of desired metabolite which eventually leads to the production of 3HP. In case of 3HP, malonyl-CoA is converted to 3HP. Thus malonyl-CoA production has to be improved.

Glucose 6-P is converted to phosphoenol pyruvate (PEP) which is then converted to pyruvate. The forward reaction to pyruvate is carried out by pyruvate kinase (*pykF* and *pykA*) and reverse reaction to phosphoenol pyruvate is carried by phosphoenol pyruvate synthase (*pps*). Then pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (*aceEF, lpd*) and acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (*acc*) which is then used for the fatty acid synthesis or converted to 3HP. Pyruvate is also converted to lactate and acetate and acetyl-CoA is also converted to ethanol by fermentation. Another pathway converts phosphoenol pyruvate (PEP) to succinate.

During the production of flavonoids in *E. coli*, 3 molecules of malonyl-CoA are converted to (2S)-flavanone which is a first flavonoid molecule that serves as the precursor of downstream flavonoids. To increase the production of flavonoids acetyl-CoA carboxylase
(acc) from *P. luminescens* and acetate assimilation pathways were over expressed in *E. coli* (Leonard, Lim et al. 2007) based on a rational that the intracellular conc. of malonyl-CoA is low so the production of flavonoids can be increased if carbon flux is directed towards the production of malonyl-CoA. In similar manner, the overproduction of acetyl-CoA could be helpful in increasing the 3HP production (Figure 7). Previous studies have shown that overproduction of acc from *E. coli* slows down the growth of the bacteria still retains the colony forming ability (Davis, Solbiati et al. 2000; Leonard, Lim et al. 2007). Hence, acc has to be from some other organism. In *E.coli*, acetate is naturally converted to acetyl-CoA through two pathways; one pathway uses acetate kinase (*ack*AB) and phosphate acyltransferase (*pta*) to interconvert acetate and acetyl-CoA and the second pathway uses acetyl-CoA synthetase (*acs*). These two pathways would increase the acetyl-CoA production.

Figure 8: Increase in intracellular concentrations of acetyl-CoA and malonyl-CoA may result in increased production of 3HP.
in the cell subsequently increasing the conversion rate of acetyl-CoA to malonyl-CoA (Figure 7) although it was shown that over production of _acs_ was more effective than _ackAB_ and _pta_ (Leonard, Lim et al. 2007).

In another study the production of pyruvate was increased in _E. coli_ by blocking the pathways leading away from pyruvate (Causey, Shanmugam et al. 2004). Pyruvate is converted to lactate and other primary fermentation pathways like formate and acetate. Phosphoenol pyruvate is also converted to succinate. All these pathways along with the disruption of the TCA cycle and oxidative phosphorylation were blocked in the strain that was engineered to produce higher amounts of pyruvate. It has been demonstrated previously that glycolytic flux is limited by ATP utilization during the oxidative metabolism of glucose (Koebmann, Westerhoff et al. 2002). To reduce the potential drain of substrate into the cell mass, a mutation was introduced into the gene encoding two subunits of (F<sub>1</sub>F<sub>0</sub>)H<sup>+</sup>-ATP synthase (atpIBEFHAGDC) that disrupts the oxidative phosphorylation while retains the hydrolytic activity of F<sub>1</sub>-ATPase. Also up to 50% of substrate carbon can be lost as CO₂ due to the high efficiency of the TCA cycle and the electron transport system. CO₂ and NADH production can be reduced by mutating gene encoding 2-ketoglutarate dehydrogenase (sucAB) (Causey, Zhou et al. 2003). These modifications result in the increased glucose utilization and the production of pyruvate.

To increase the production of 3HP in _E. coli_, similar modifications (Figure 9) can be made along with the over production of pyruvate dehydrogenase complex and acetyl-CoA

carboxylase. Over production of pyruvate dehydrogenase complex is necessary to increase the conversion rate of pyruvate to acetyl-CoA and to avoid the excretion of pyruvate by *E. coli* (Causey, Shanmugam et al. 2004). Disruption of oxidative phosphorylation could cause a problem since acetyl-CoA carboxylase uses one molecule of ATP to convert one molecule of acetyl-CoA to malonyl-CoA and its over production would require a higher amount of ATP than usually required by the cell. On the other hand increased glycolytic flux and
substrate level phosphorylation could partially compensate for the increased requirement for the ATP.
CHAPTER 4: PRODUCTION OF 3HP FROM SYNGAS

4.1 INTRODUCTION

Traditionally, when biomass is used for the production of biofuels or chemicals first the sugars are separated by pretreatment of enzyme/acid hydrolysis and only the sugars that can be fermented by bacteria are used. In this case, the biomass is not used completely and pretreatment is expensive. Gasification of biomass to produce syngas allows conversion of feedstock to usable carbon compounds that may approach to 100%. The CO and H₂ present in syngas are substrates for microbial metabolism, which can be converted to various interesting products. It is expected that syngas fermentation will play a role in the conversion of biomass, wastes and residues that form poor substrates for direct fermentation. The challenge is to develop a biocatalyst which can grow on syngas and produce the desired product.

The area of biocatalysis has great promise for commodity and specialty chemical synthesis. Biocatalysis has been used for a long time in food industry for the production of various types of cheese and alcohol. With the significant development in the field of biotechnology the use of biocatalysis in the industries has grown significantly over the years (Zaks 2001). Biocatalysts can catalyze virtually every chemical reaction in nature, and with the development in the technology new chemicals that are costly to synthesize using organic chemistry are being made with biocatalysis. Recently interest in 3HP for the production of Bioplastic and polymers has increased. The petrochemical route for the synthesis of 3HP is not cost effective. A possible 3HP production is the biocatalytic route. In the report “Top Value Added Chemicals From Biomass volume 1: Results of Screening for Potential
Candidates from Sugars and Synthesis Gas” DOE identified 12 building blocks that subsequently can be converted to number of high value chemicals and materials (T. Werpy 2004), 3HP is one of the top 12 platform chemicals. The major considerations in the production of this platform chemical are the productivity, pathway engineering, nutrient requirement and final titer.

The pathway engineering and improvement of productivity are described in the previous chapter. This chapter deals with the pathway engineering in _R rubrum_ with emphasis on cost reduction of nutrients. _R. rubrum_ can use carbon monoxide as a sole carbon and energy source producing H₂ and CO₂ (Kerby, Ludden et al. 1995). The CO is produced in the form of synthesis gas by pyrolysis of biomass such as distillers dried grains (DDG) or from other cheap sources. If the chemical equilibrium is attained then the gas composition is limited to CO, CO₂, H₂ and CH₄. Genetically modified _R. rubrum_ which is capable of production of 3HP could be grown on the synthesis gas, for production of H₂ gas and 3HP will be produced.

### 4.2 MATERIALS AND METHODS

**Chemicals and reagents:** Malonyl-CoA and NADPH were from Sigma. IPTG was from Anatrace and DTT was from ICN. Restriction enzymes, _Taq_ polymerase, _Pfu_ polymerase, T4 ligase were from New England Biolabs.

**Bacterial Strains, plasmids media and buffer:** The bacterial strains and plasmids used in this study are given in Table 3. The LB (Luria-Bertani) medium was the rich medium used (Miller, 1972) for growing _E. coli_ strains and SMN (Galkin, Kulakova et al. 1997) was used for growing _R. rubrum_. Kanamycin was used at 25 µg/ml and IPTG was used at 0.5mM
conc. Sonication buffer used for cell lysis was comprised of 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 3 mM DTT and 0.1% Triton X-100.

**General molecular methods:** As described in Chapter 1.

**DNA sequencing and analysis:** As described in Chapter 1.

**Cloning malonyl-CoA reductase gene in *R. rubrum***: PCR was used to amplify the malonyl-CoA reductase gene from the plasmid DNA of pNA1 for cloning into pBBR1MCS-2 (5144bp) under T7 promoter. The primers were synthesized specifically for malonyl-CoA reductase from *C. aurantiacus*. The primers for *C. aurantiacus* were 5’-GCCGCCGAGCTCGTGAATTGTGAGCGGATAAC-3’ (forward) and 5’-GCCGCCATGACATTACACGGTAATCGCCCGTCC-3’ (reverse). These primers introduced the restriction sites SacI and NsiI that were used to clone the malonyl-CoA reductase sequence (3690 bp) into pBBR1MCS-2. Ligation mixture was used to transform *E. coli* strain DH5α and transformants were selected by plating on LB agar supplemented with LB agar, X-gal, IPTG plates supplemented with 25 µg/ml of kanamycin, new plasmid was named as pNA12. Plasmid DNA isolated from selected transformants was analyzed by restriction digestion and DNA sequencing.

**Cloning T7 RNA polymerase**: T7 RNA polymerase was cloned into the *R. rubrum* suicide vector pUX19. First Glucan 1, 4-alpha-glucosidase gene from *R. rubrum* was cloned into pUX19 that will undergo homologous recombination with *R. rubrum* chromosome to insert T7 RNA polymerase into the *R. rubrum* genome. PCR was used to amplify the 1, 4-alpha-glucosidase gene from the genomic DNA of *R. rubrum* for cloning into pUX19 (3868bp). The primers were synthesized specifically for Glucan 1, 4-alpha-glucosidase. The
Table 3: Bacterial strains and plasmids for production of 3HP from syngas.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 hsdR17 relA1 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF) U169 (Φ80lacZΔM15)</td>
<td>(Simon 1983)</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>recA1 pro thi; has the tra genes from plasmid RP4 integrated in the chromosome</td>
<td></td>
</tr>
<tr>
<td>R. rubrum</td>
<td>Wild type</td>
<td>From Dr. DiSpirito</td>
</tr>
<tr>
<td>S17-1/pNA5</td>
<td>S17-1 harboring R. castenholzii MCR; Kan resistance</td>
<td>This Study</td>
</tr>
<tr>
<td>S17-1/pBBR1MCS-2</td>
<td>S17-1 harboring pBBR1MCS-2; Kan resistance</td>
<td>This study</td>
</tr>
<tr>
<td>R. rubrum/pNA5</td>
<td>R. rubrum expressing MCR from R. castenholzii; Kan, Nalidixic acid resistance</td>
<td>This study(Baba, Ara et al. 2006)</td>
</tr>
<tr>
<td>R. rubrum/pBBR1MCS-2</td>
<td>Control strain Kan, Nalidixic acid resistance, Kan resistance</td>
<td>This study</td>
</tr>
</tbody>
</table>

Cloning T7 RNA polymerase

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE21 DE3</td>
<td>F- ompT hsdSB (rB-, mB-) gal dcm (DE3)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>NA25</td>
<td>E. coli DH5α/pNA4</td>
<td>This study</td>
</tr>
<tr>
<td>NA33</td>
<td>E. coli DH5α/pNA11</td>
<td>This study</td>
</tr>
<tr>
<td>NA34</td>
<td>E. coli DH5α/pNA12</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBBR1MCS-2</td>
<td>Derivative of pBBR1MCS; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Kovach, Elzer et al. 1995)</td>
</tr>
<tr>
<td>pUX19</td>
<td>Suicide vector for R. rubrum, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Zhang, Pohlmann et al. 2001)</td>
</tr>
<tr>
<td>pNA5</td>
<td>pBBR1MCS-2/MCR from R. castenholzii</td>
<td>This study</td>
</tr>
<tr>
<td>pNA4</td>
<td>pUX19/ Glucan 1,4-alpha-glucosidase from R. rubrum; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNA9</td>
<td>pNA4/Tetracycline gene from pLac22; Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNA10</td>
<td>T7 promoter deletion from pNA9.</td>
<td>This study</td>
</tr>
<tr>
<td>pNA11</td>
<td>pNA10/T7 polymerase from BE21 DE3</td>
<td>This study</td>
</tr>
<tr>
<td>pNA12</td>
<td>pBBR1MCS-2/MCR from C. aurantiacus (under T7 promoter)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Primers used were 5’- GCCGCCCTTCGAGTGAAGACTGGCTGACCCACGCA-3’ (forward)

and 5’- GCCGCCTCTAGACTATTGGAGAATGCGGCCCAG-3’ (reverse). These primers introduced the restriction sites XbaI and XhoI that were used to clone the Glucan 1, 4-alpha-
glucosidase sequence (1317 bp) into pUX19. The new plasmid obtained was named as pNA4. The clone was confirmed by colony PCR and sequencing.

PCR was used to amplify the tetracycline gene from the plasmid DNA of pLac22 for cloning into pNA4. The primers were synthesized specifically for tetracyclin. The primers used were 5'- ATGAAATCTAACAATGCGCTC -3' (forward) and 5'- TCAGGTGTGAGGTGGCCCG - 3' (reverse). These primers were cloned into the restriction site BseRI in the Glucan-1, 4-alpha-glucosidase after klenow treatment. The new plasmid obtained was named as pNA9. The clone and orientation was confirmed by colony PCR and sequencing.

Plasmid pUX19 has the T7 promoter that has to be removed before the T7 RNA polymerase could be cloned. PCR was used to amplify the 1516 bp fragment of pUX19 starting at the 1st base pair downstream of T7 promoter. The primers used were 5'- GCCGCCTCTAGAACAATTCACTGGCCGTCGTTT -3' (forward) and 5'- GCCGCCAAGCTTTATGCATTTCTTTCCAGACTTTGT-3' (reverse). The fragment was cloned in XbaI and BsrDI restriction sites. The deletion of T7 promoter was confirmed by colony PCR and sequencing. The new plasmid obtained was named as pNA10.

PCR was used to amplify the T7 RNA polymerase gene from the genomic DNA of E. coli BL21 DE3 for cloning into pNA10. The primers were synthesized specifically for tetracyclin. The primers used were

5’GCCGCCGCTAGCGACGTCCTGCAGGAGCTCAGATCTAGAAAGATCTTTATGAA CACGATTAACATCGCT 3’ (forward) and

5’GCCGCCGATGCAGCATCTTACGGAAGCTCAGAAATTTTGTCCAGAAGTGAA -3’ (reverse).
These primers introduced the restriction sites NheI and SphI in the sequence and the DNA fragment was cloned into the tetracycline gene. The new plasmid obtained was named as pNA11. The clone and orientation was confirmed by colony PCR and sequencing.

**Conjugation:** For conjugation experiments, *R. rubrum* and *Escherichia coli* S17-1 containing the pNA5 and pBBR1MCS-2 plasmids were grown in SMN medium and Luria-Bertani medium, respectively. *E. coli* culture was diluted in 1:100 proportion and incubated at 30°C for 2.5 hours. 2 ml of culture, 500 μl of the donor culture, and 1.5 ml of the recipient culture were mixed gently in a microcentrifuge tube. The cells were centrifuged, the supernatant was decanted, and the pellet was resuspended in about 50 μl of the medium remaining in the tube. The cells were spread on a sterile membrane filter (25 mm, 0.45-p.m pore size) on an SMN plate and incubated at 30°C for 24 h. The cells were then resuspended by vortexing in 1 ml of SMN medium, diluted in SMN medium, and plated onto SMN plates containing 50 μg/ml of kanamycin and 20 μg/ml nalidixic acid.

**Growth of the malonyl-CoA reductase expression strains and preparation of cell extracts:** *R. rubrum* strains were grown in 25ml SMN kanamycin (25 μg/ml) and naladixic acid (25μg/ml) broth and incubated at 30°C with shaking in a 125 ml baffled Erlenmeyer flask. Cells were grown 3-4 days to an OD$_{600}$ 0.6-0.8. 5 ml of cells were harvested by centrifugation. Cells were suspended in 750 μl of sonication buffer. To inhibit the protease activity, Pefabloc was added at the final concentration of 0.2 mM. Cells were incubated at -80°C for 5 min and then incubated in ice water until thawed, freeze and thaw cycle was repeated 3 times. Cells were then treated with lysozyme at the final conc. of 1mg/ml for 1 hour at 30°C and broken with sonication. Sonication was performed on ice for 5 cycles with Artek sysmic dismembrator model 150 at 60 kHz for 10-15s with 1 minute interval. Soluble
proteins were separated from inclusion bodies by centrifugation of cell extracts at 25000g for 15 min at 4°C using bench top centrifuge. The supernatant obtained was the soluble cell extract used for further studies. Control strain *R. rubrum*/pBBR1MCS-2-no insert was grown in parallel with expression strain *R. rubrum*/pNA5 and cell extracts of this strain were similarly prepared.

**Malonyl-CoA reductase assays:** Malonyl-CoA reductase assays were carried out as described previously by Hügler, *et al.* (Hugler, Menendez *et al.* 2002). The only modification was DTT was used instead of DTE. Assay mixtures (1ml total volume) contained 100mM Tris-HCl (pH 7.8), 2 mM MgCl₂, 3 mM DTT, 0.3 mM NADPH, 0.3 mM malonyl-CoA, double deionized H₂O and cell free extract. The reaction mixture was heated to 37°C for 3 min before starting the reaction. The reaction was started by addition of cell free extract. Two control reactions were performed in parallel. In one control reaction malonyl-CoA was left out. And in other control reaction the cell free extract from control strain was used. The reaction rates were determined by monitoring the decrease in absorbance at 340 nm and using \( \Delta \varepsilon_{340} = 6220 \text{ M}^{-1} \text{ cm}^{-1} \) for calculations. The absorbance was measured at 340nm and activity was calculated based on NADPH disappeared.

**4.3 RESULTS**

**Cloning and Expression of Malonyl-CoA Reductase in *R. rubrum*:** Cloning MCR from *R. castenholzii* or *C. aurantiacus* into *R. rubrum* expression vector pBBR1MCS-2 was difficult due to the plasmid rearrangement. The clone of *R. castenholzii* MCR under lac promoter was obtained after several attempts and after screening about 25 colonies.
The cell extracts were assayed for malonyl-CoA reductase activity using the continuous spectrophotometric assay described in methods. Cell extracts of *R. rubrum/pNA5* catalyzed the malonyl-CoA dependant oxidation of NADPH, corresponding to the specific activity of 94 nmol min\(^{-1}\) (mg of protein)\(^{-1}\). When malonyl-CoA was left out of the reaction or CFE from *R. rubrum/pBBR1MCS-2* was used no activity was observed. This indicates that NADPH was consumed by malonyl-CoA reductase and not any other enzyme present in the crude extract. Previously, it is shown that for cell extracts with *E. coli DH5α/pNA5* catalyzed the malonyl-CoA reductase enzymatic reaction giving specific activity of 11.25 nmol min\(^{-1}\) (mg of protein)\(^{-1}\) and no activity was observed when malonyl-CoA was left out or CFE from DH5α/pBBR1MCS-2 was used. This difference between the enzyme activities could be due to the codon usage bias in *E. coli*. *R. castenholzii* and *R. rubrum* both have high GC content of about 65%. Thus, MCR from *R. castenholzii* may have expressed better in *R. rubrum*.

**Cloning T7 polymerase:** In order to express the gene cloned under the T7 promoter, viral T7 RNA polymerase should be expressed in the bacterial strain. No direct technique is present to insert a new gene into the chromosome in one step. To achieve this modification of *R. rubrum* chromosome, first a gene was chosen to disrupt that would not affect the growth of this bacteria and *R. rubrum* genome on NCBI was screened. Glucan 1, 4-alpha-glucosidase was selected for cloning under *lac* promoter in pUX19, the function of the enzyme is to hydrolyze terminal 1, 4-linked alpha-D-glucose residues successively from non-reducing ends of the chains with release of beta-D-glucose. The tetracycline gene from pLac22 was selected to clone into the Glucan 1, 4-alpha-glucosidase. Tetracycline was cloned to make the selection procedure easier and restriction sites on tetracycline could be
used to clone T7 RNA polymerase. Plasmid pUX19 has the T7 promoter downstream of glucan 1, 4-alpha-glucosidase. Attempts to clone T7 polymerase in presence of T7 promoter failed probably due to over production of T7 polymerase that made the cells sick and plasmid was lost. Only one colony was found to have plasmid but it was not healthy and plasmid was rearranging; hence, T7 promoter was removed prior to cloning T7 RNA polymerase (Figure 10).

Conjugation of pNA11 with *R. rubrum* has yet to be done to insert T7 RNA polymerase into the *R. rubrum* chromosome. Then pNA12 will be transferred into new *R. rubrum* strain and expression level will be checked.

### 4.4 DISCUSSION

*R. rubrum* was chosen for this study is because of its ability to use CO as sole carbon and energy source. This CO can come from syngas produced from biomass. Biomass used for pyrolysis is usually municipal organic waste or byproducts of agriculture industry which has no further use and hence very cheap and also the process is very efficient. One of the important considerations in the development of a biocatalyst is the cost of nutrient, use of expensive nutrients like yeast extract increases the final cost of the product. Use of cheap biomass for syngas fermentation would reduce the nutrient cost. BioEngineering Resources, Inc. has successfully developed the technology that uses syngas fermentation to produce ethanol from genetically engineered *Clostridium ljungdahlii* and electricity and ash for fertilizers as by products (Gaddy 2002). The growth of *R. rubrum* on synthesis gas has been reported by Young et al for the production of polyhydroxy alkonate PHA and H₂ gas (Young S. Do 2007).
For successful commercial employment of the biocatalyst the productivity should be high. A DOE report has suggested that for 3HP to be commercially viable, its production should be at least 2.5g/L hr. In the first chapter, it has been shown that the production of 3HP was very low under lac promoter. T7 promoter is very strong promoter so cloning and expressing MCR under T7 promoter may add to the production of 3HP.
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transformant cells coexpressing the aldehyde reductase and glucose dehydrogenase genes." Applied Microbiology and Biotechnology 51(4): 486-490.


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BIOGRAPHIC SKETCH

Ms Netra Rajguru Agarkar was born in Deorukh (Maharashtra), India on 26th August 1977. She did her Bachelor of Science (in Chemistry with honors) from Sangameshwar College, Solapur, India and Master of Science (Biochemistry) from Shivaji University, Kolhapur, India. Then she joined Iowa State University, USA. She visited Japan Marine Sciences and Technology Center while working on Deep sea bacteria project. As a part of her master’s research she worked on the project of the production of 3-hydroxypropionate from biomass, this project was partly funded by US department of energy.