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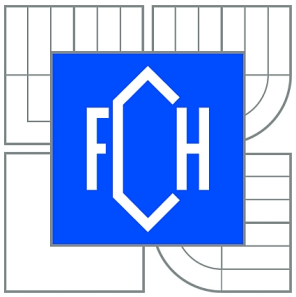

FAKULTA CHEMICKÁ

ÚSTAV CHEMIE POTRAVIN A BIOTECHNOLOGIÍ

FACULTY OF CHEMISTRY

INSTITUTE OF FOOD SCIENCE AND BIOTECHNOLOGY

CONTROLLED BIOTECHNOLOGICAL PRODUCTION OF POLYHYDROXYALKANOATES

ŘÍZENÁ BIOTECHNOLOGICKÁ PRODUKCE POLYHYDROXYALKANOÁTŮ.

DIPLOMOVÁ PRÁCE

MASTER'S THESIS

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BRNO 2012

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Master's thesis Assignment

Number of master's thesis:	**FCH-DIP0608/2011**	Academic year:	**2011/2012**
Institute:	Institute of Food Science and Biotechnology		
Student:	**Bc. Ondřej Šnajdar**		
Study programme:	Chemistry and Technology of Foodstuff (N2901)		
Study field:	Food Science and Biotechnology (2901T010)		
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Title of master's thesis:

Controlled biotechnological production of polyhydroxyalkanoates

Master's thesis assignment:

1. Theoretical overview of studied problematics
2. Preparation and selection of mutant bacterial strains overproducing PHA
3. Biochemical characterization of mutant strains.

Deadline for master's thesis delivery: 11.5.2012

Master's thesis is necessary to deliver to a secreatry of institute in three copies and in an electronic way to a head of master's thesis. This assignment is enclosure of master's thesis.

Bc. Ondřej Šnajdar Student	Ing. Stanislav Obruča, Ph.D. Head of thesis	doc. Ing. Jiřina Omelková, CSc. Head of institute

In Brno, 21.2.2012

prof. Ing. Jaromír Havlica, DrSc.
Dean

ABSTRACT

This diploma thesis deals with production of polyhydroxyalkanoates (PHA) by bacterial strain *Cupriavidus necator* H16. Goal of this work was preparation, selection and characterization of mutant strains overproducing PHA. Theoretical focuses on the most important PHA, bacteria *Cupriavidus necator* and mutagenesis techniques. In practical part mutant strains were prepared through physical and chemical mutagenesis. Mutant strains overproducing PHA were selected by cultivation in mineral medium with oil. For further study, 4 mutant strains overproducing PHA were selected. These mutants were biochemically characterized. Specific activities of several intracellular enzymes including enzymes involved in PHA biosynthesis were measured. Resistance of mutants against oxidative stress was measured as well. Mutant strains overproducing PHA revealed higher enzymatic activities of NADPH producing enzymes. Generally, NADPH is one of the substrates influencing flux of acetyl-CoA throughout the metabolism; higher intracellular concentration of NADPH partially inhibits TCA cycle and activates accumulation of PHA. Therefore, activities of acetoacetyl-CoA reductase and PHB synthase, enzymes directly involved in PHA synthesis were higher as compared to wild strain as well as molecular weight of produced materials. It can be concluded that biotechnologically perspective mutagens capable of PHA overproduction can be prepared by application of chemical and physical mutagens.

Key words

Polyhydroxyalkanoates, polyhydroxybutyrate, random mutagenesis, *Cupriavidus necator* H16.

ABSTRAKT

Předložená diplomová práce se zabývá produkcí polyhydroxyalkanoátů (PHA) bakterií *Cupriavidus necator* H16. Cílem práce byla příprava, selekce a charakterizace mutantních kmenů schopných vyšší produkce PHA. V teoretické části byla zpracována literární rešerše zabývající se nejdůležitějšími typy PHA, bakterií *Cupriavidus necator* a způsoby indukce mutageneze. V experimentální části byly připraveny mutantní kmeny pomocí fyzikální a chemické mutageneze. Mutantní kmeny schopné nadprodukce PHA byly selektovány pomocí kultivace na minerálním médium s olejem. Pro další studium byly vybrány 4 mutantní kmeny schopné nadprodukce PHA. Tyto mutantní kmeny byly dále podrobeny biochemické charakterizaci. Byly naměřeny specifické aktivity vybraných intracelulárních enzymů včetně enzymů podílejících se na biosyntéze PHA. Také byla naměřena resistence mutantů vůči oxidačnímu stresu. Bylo zjištěno, že mutantní kmeny schopné nadprodukce PHA mají vyšší aktivity enzymů produkujících NADPH. NADPH je jeden z klíčových substrátů ovlivňujících směr toku acetyl-CoA metabolizmem. Vyšší intracelulární koncentrace NADPH parciálně inhibuje Krebsův cyklus a aktivuje akumulaci PHA. Aktivity acetoacetyl-CoA reduktázy a PHA syntázy, enzymů zapojených do syntézy PHA, těchto mutantů proto byly také vyšší stejně jako molekulová hmotnost připravených polymerů. Aplikace fyzikálních a chemických mutagenů je způsob, kterým lze připravit biotechnologicky perspektivní mutantní kmeny schopné nadprodukce PHA.

Klíčová slova

Polyhydroxyalkanoáty, polyhydroxybutyrát, náhodná mutageneze, *Cupriavidus necator* H16

ŠNAJDAR, O. *Controlled biotechnological production of polyhydroxyalkanoates.* Brno: Vysoké učení technické v Brně, Fakulta chemická, 2012. 85 s. Vedoucí diplomové práce Ing. Stanislav Obruča, Ph.D..

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

.....
Student's signature

Poděkování:

Chtěl bych poděkovat vedoucímu mojí diplomové práce Ing. Stanislavu Obručovi, Ph.D. za odborné vedení, veškerou pomoc a cenné rady. Chtěl bych také poděkovat doc. RNDr. Ivaně Márové, Csc. za konzultace.

Práce byla podpořena z prostředků projektu "Centrum materiálového výzkumu na FCH VUT v Brně" registrovaného pod číslem CZ.1.05/2.1.00/01.0012/ERDF.

CONTENTS

1 INTRODUCTION

The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials. The presence of these non-degradable residues is affecting potential survival of many species. For this reason, many countries have promoted special programs directed towards the discovery of new commonly used materials that can be readily eliminated from the biosphere. Biomaterials are natural products that are synthesized and catabolized by different organisms and that have found broad biotechnological applications. They can be assimilated by many species (they are biodegradable) and do not cause toxic effects in the host (they are biocompatible) [1].

Polyhydroxyalkanoates (PHAs) are families of biodegradable polymers that have great potential in the future due to their variability in properties; however, the wide-spread substitution of conventional plastics has been limited by high production costs. More efforts need be devoted to making this process economically feasible by increasing our understanding of the PHB accumulation process and improving productivity. Reduction of PHA costs can be accomplished by several methods [2].

One of the important factors in determining the economics of PHAs production on an industrial scale is the high substrate price. Using cheap carbon source as a substrate in PHA biosynthesis is important step in lowering the price of final product.

Reduction of PHA cost can be also accomplished by various strain improvement methods including modification of metabolic pathways such as the TCA cycle or PHB biosynthetic pathway. Modification of strains is accomplished by random or site-directed mutagenesis and it can lead to mutant strains capable of PHA overproduction [3].

Production of PHAs in recombinant organisms like other bacteria, yeast and transgenic plants can be also promising.

Goal of this work was preparation, selection and characterization of mutant strains of *Cupriavidus necator* overproducing PHA.

2 THEORETICAL PART

2.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are family of polyesters of (*R*)-3-hydroxyalkanoic acids, produced by a variety of bacterial species under nutrient-limiting conditions with excess carbon [3]. The 3-hydroxyalkanoic acids are all in *R* position due to stereospecificity of the polymerizing enzyme, PHA synthase [4]. PHAs are biodegradable, insoluble in water, nontoxic, biocompatible, piezoelectric, thermoplastic and elastomeric. These features make them suitable for applications in the packaging industry, medicine, pharmacy, agriculture and food industry [3]. PHAs are stored in cells in a form of inclusions and they serve as a carbon and energy source for bacteria. Typical PHA inclusion is 0,2 – 0,5 μm in diameter and it is localized in cell cytoplasm. The proteins involved in biosynthesis and degradation of PHA are located on the surface of PHA inclusion. Figure 1 shows possible model of *in vivo* PHA inclusion [5].

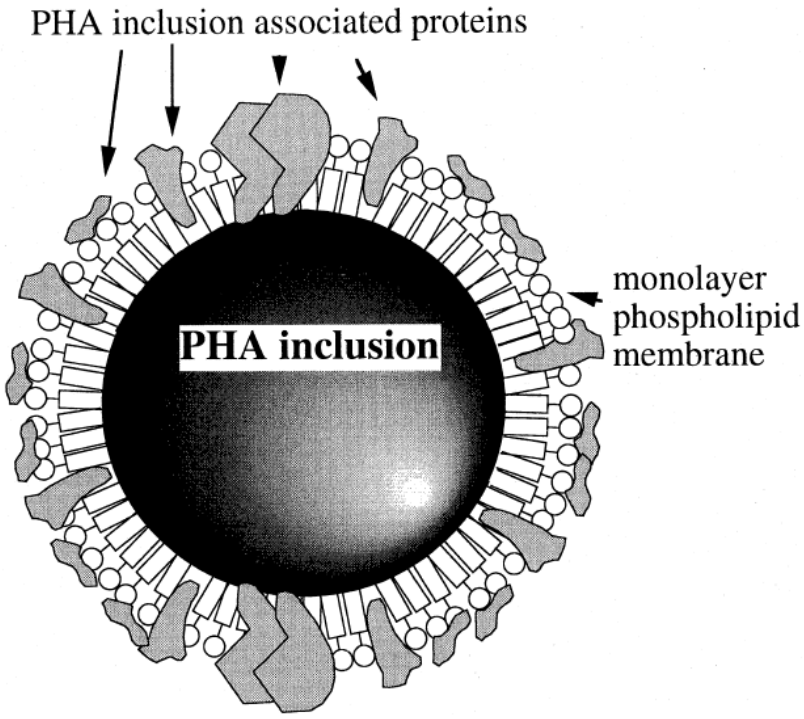


Figure 1: *Model of in vivo PHA inclusion* [5]

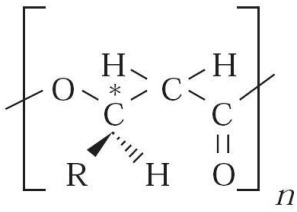
2.1.1 History of PHAs

In 1926 Lemoigne isolated first known PHA poly(3-hydroxybutyrate) (PHB) from bacteria *Bacillus megaterium* [6]. In early 1960s PHB was first commercially used by company Werber at W.R. Grace & Co. (U.S.A). They used PHB to fabricate prosthetic devices. Nevertheless fermentation yields were low and polymer was contaminated with bacterial residues [7]. The petroleum crisis in 1970s boosted research for alternative plastics. Company ICI (UK) was able to produce PHB with *Alcaligenes latus.* PHB content in cells was 70% of its dry cell weight. Produced PHB had poor mechanical properties and it was expensive. Company also produced polymer BIOPOL® that was copolymer of poly(3-hydroxybutyrate and 3-hydroxyvalerate (P(HB-*co*-HV)). BIOPOL® had better mechanical

properties, such as lower crystallinity and more elasticity than PHB [8]. In 1993 ICI transferred its biological division to Zeneca BioProducts. Zeneca Bioproducts continued developing PHAs and then in 1996 they sold license to BIOPOL® to American company Monsanto. In 1998 was license to BIOPOL® sold to company Metabolix Inc. [3]. Company Metabolix Inc. developed transgenic approach to PHAs production. They used large scale fermentations and agricultural biotechnology. Recent interest was in the use of PHA for medical applications. Company Tepha Inc. is currently engaged in development of tissue-engineered products based on PHA [9].

2.1.2 Structure and properties of PHA

PHAs can be classified into two groups depending on the number of carbon atoms in the monomer units. Short-chain-length (SCL) PHAs contain 3 – 5 carbon atoms. Medium-chain-length (MCL) PHAs contain 6 – 14 carbon atoms. General structure of PHA is shown in Figure 2 [8].



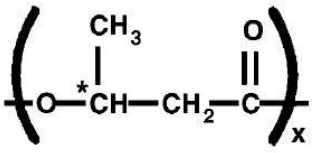
$R = CH_3\text{-}C_2H_5$ Short-chain-length PHAs
$R = C_3H_7\text{-}C_{14}H_{29}$ Medium-chain-length PHAs

Figure 2: *General structure of PHA* [8]

Microorganisms in nature are able to synthetize various types of PHAs depending on the type of carbon source and biochemical pathways available. Homopolymer PHB is the most common; nevertheless, its mechanical properties are not ideal. Copolymers of 3HB with other hydroxyacids have higher molecular weight and better mechanical properties than homopolymer PHB. Random copolymers contain 3HB monomer units as well as other hydroxyalkanoate units with 3 – 14 carbon atoms in random order [10].

2.1.2.1 Poly(3-hydroxybutyrate)

PHB is the most common SCL PHA produced by variety of microorganisms in nature. PHB monomer contains 4 carbon atoms as shown in Figure 3 [11]



***R*-3HB**

X = 120-200: low molecular weight P[3HB]
X = 1,000-20,000: high molecular weight P[3HB]
X ~ 100,000: ultrahigh molecular weight P[3HB]

Figure 3: *General structure of PHB* [11]

PHB isolated from bacteria contains 55 – 80 % of crystallinity. On the other hand natural PHB in bacteria is in amorphous form and exists as water insoluble inclusion [5]. Molecular weight of PHB from wild-strain bacteria is usually in the range $1 \cdot 10^4$ – $3 \cdot 10^6$ g·mol^{-1}. Polydispersity index is around two [10]. The glass transition temperature of PHB is around 4°C while the melting temperature is near 180°C. The density of amorphous PHB is 1,18 g·cm^{-3}. The density of crystalline PHB is 1,26 g·cm^{-3}. Mechanical properties like the Young's modulus (3,5 GPa) and the tensile strength (43 MPa) of PHB are close to those of polypropylene. The extension to break (5%) of PHB is; however, lower than extension to break of polypropylene (400%). Overall PHB is stiffer and more brittle plastic material compared to polypropylene. The brittleness is due to the formation of large crystalline domains in the form of spherulites. [12].

Ultra-high molecular PHB polymer have better mechanical properties then regular PHB polymer. Ultra-high molecular PHB was prepared by transgenic *Escherichia coli* with genes from *Cupriavidus necator*. Weight average molecular weight of ultra-high molecular PHB is $3 \cdot 10^6$ – $1{,}1 \cdot 10^7$ g·mol^{-1} under special fermentation conditions [13].

2.1.2.2 Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

Significant improvement in mechanical properties was achieved by incorporation of 3-hydroxyvalerate (3HV) into 3HB sequence. General structure of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-*co*-HV)) is shown in Figure 4 [11]

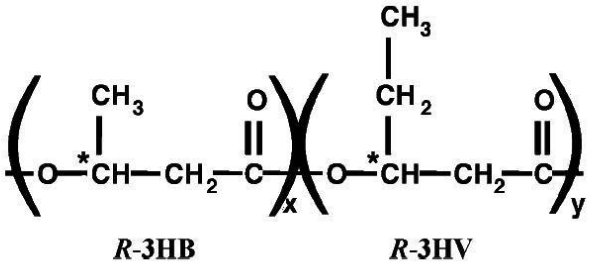


Figure 4: *General structure of P(HB-co-HV)* [11]

This copolymer was prepared by fermentation of *Cupriavidus necator* grown on glucose with addition of propionic acid. Propionic acid serves as precursor carbon source leading to incorporation of 3HV units into polymer. Mechanical properties of copolymer can be altered by controlling 3HV content [14]. The easiest way to control the content of 3HV units in copolymer is by changing the concentration of the carbon source that contributes to the formation of 3HV units [11]. Incorporation of 3HV improves flexibility of material. Young's modulus decreases below 0,7 GPa and tensile strength decreases below 30 MPa. The elongation to break of polymer increases as 3HV content increases. The melt temperature is greatly depressed, down to 130°C, dependent on the 3HV content. The degradation temperature is little affected [8]

2.1.2.3 Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)

Another type of PHA with improved properties is poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB). General structure of P(3HB-*co*-4HB) is shown in Figure 5 [11].

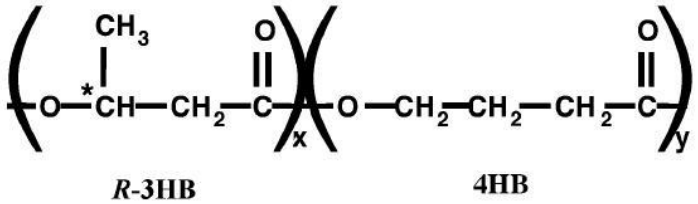


Figure 5: *General structure of P(3HB-co-4HB)* [11]

With increase of 4HB content in copolymer its mechanical properties change from one characterized by high crystallinity to one that is a strong elastomer [15]. P(3HB-*co*-4HB) cannot form isomorphic crystals because of the extent of the structural differences between the monomer units. The glass transition temperature decreases from 5 to -50 °C, and the melting temperature decreases from 180 to 54 °C as the mol% of 4-hydroxybutyrate increases from 0 to 100%. Young's modulus, tensile strength, and % elongation at break (for 94 mol% 4-hydroxybutyrate) are reported as 55 MPa, 39 MPa, and 500%, respectively [8].

2.1.2.4 Other PHA copolymers

Copolymers containing 3HB units and 3-hydroxyalkanoate units with 6 – 14 carbon atoms are quite rare due to substrate specificity of PHB synthase. Their general structure is shown in Figure 6 [11]

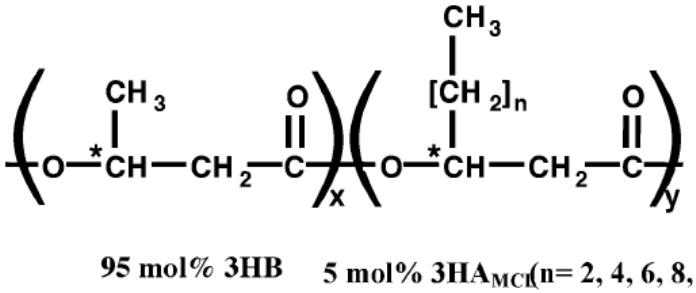


Figure 6: *General structure of copolymer containing 3HB units and MCL hydroxyalkanoates* [11]

MCL PHAs are thermoplastic elastomers with melting points of about 45-60 °C and glass transition temperatures near −40 °C. The biosynthesis of PHA copolymers containing both the SCL and the MCL units are relatively rare. They are produced by some *Pseudomonas* strains [16].

2.1.2.5 Comparison of mechanical properties of PHAs with polypropylene

Comparison of mechanical properties of PHAs with polypropylene (PP) is shown in Table 1 [8].

Table 1: *Comparison of mechanical properties of PHAs properties with PP* [8]

Polymer	Melting point (°C)	Tensile strength (MPa)	Young's modulus (GPa)	Elongation to break (%)
PHB	179	40	3,5	3,0
P(HB-*co*-HV) 90:10	150	25	1,2	20
P(HB-*co*-HV) 80:20	135	20	0,8	100
P4HB	53	104	149	1000
P(3HB-*co*-4HB) 90:10	159	24	-	242
P(3HB-*co*-4HB) 10:90	50	65	100	1080
PP	170	34,5	1,7	400

2.1.3 Biosynthesis of PHA

In 1956 Macrae and Wilkinson discovered that PHA accumulation of bacteria *Bacillus megaterium* increases as carbon to nitrogen ratio in medium increases [17]. Their results suggested that, like polyphosphate and carbohydrate reserves, PHA accumulation occurred in response to an imbalance in growth brought about by nutrient limitations. Bacteria make and store PHA when they lack complete range of nutrients required for cell division but have generous supplies of carbon. The biosynthesis of PHA was initiated by magnesium or sulfate deficiency as well as phosphate and nitrogen limitations. For many bacteria, the polymer, once accumulated, serves as both carbon and energy source during starvation. PHA constitutes an ideal carbon-energy storage material due to its low solubility and high molecular weight [18]. On other hand there are some bacteria like *Alcaligenes latus* and *Azotobacter vinelandii* that accumulate PHA even in the absence of nutrient limitation [19, 20].

PHA synthase, enzyme involved in PHA synthesis shows broad substrate specificity and therefore a wide variety of monomers can be polymerized. Carbon source is the main factor determining PHA composition. Microorganisms can produce PHA from variety of carbon sources from simple carbohydrates to complex materials like sugar beads and used oils [21].

2.1.3.1 Biosynthesis of PHB

PHB in most bacteria is synthetized by three step reaction. Three enzymes are involved in biosynthesis of PHB in *Cupriavidus necator* (see Figure 7) [8]

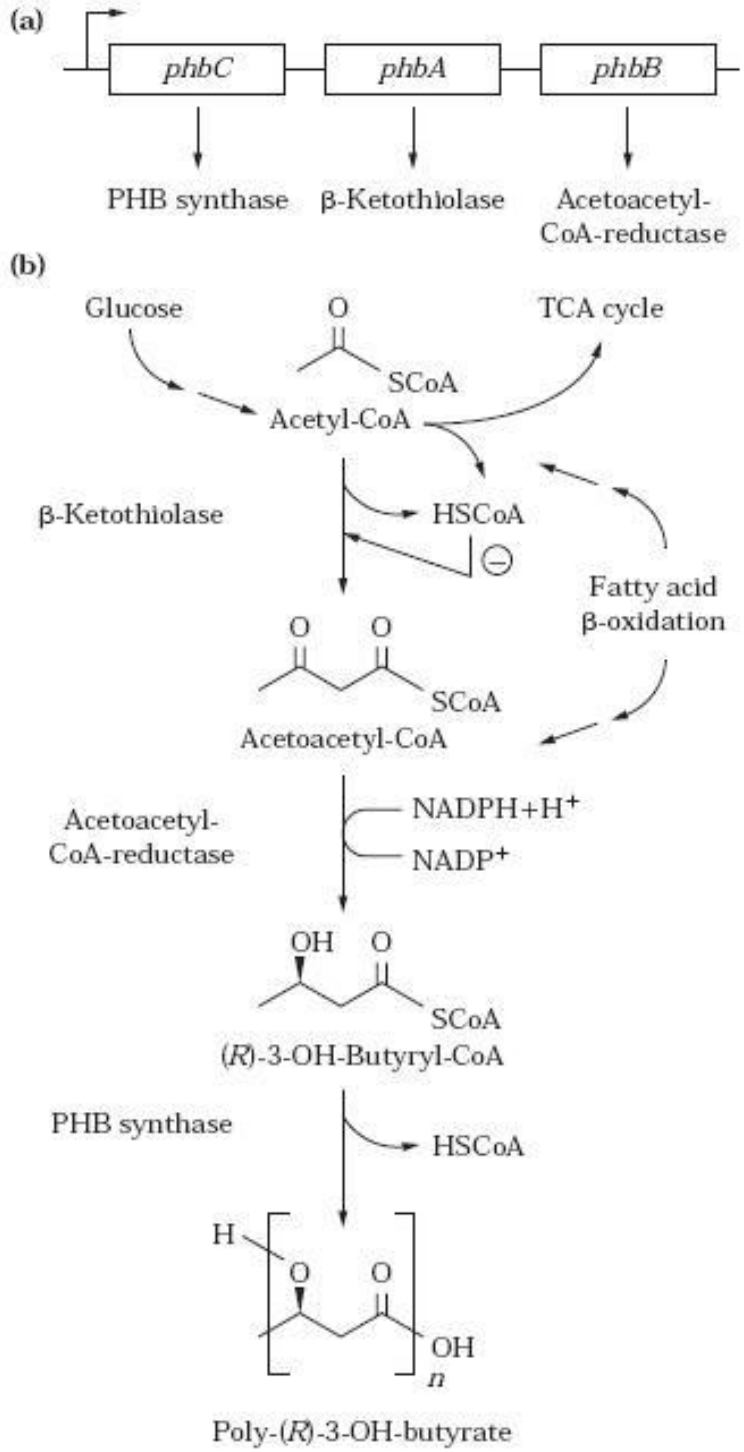


Figure 7: *Biosynthesis of PHA in Cupriavidus necator* [8]

a) The genes *phbA*, *phbB*, and *phbC* of *Alcaligenes eutrophus* are located in one operon and code for the β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase, respectively.
b) biosynthesis of PHB

Biosynthesis of PHB starts with condensation of two acetyl-CoA into acetoacetyl-CoA. Reaction is catalyzed by enzyme β-ketothiolase. Acetoacetyl-CoA is then reduced by NADPH dependent acetoacetyl-CoA reductase into (*R*)-3-hydroxybutyryl-CoA. Reaction is stereoselective and only R form of 3-hydroxybutyryl-CoA is formed. Final step of synthesis is polymerization of 3-hydroxybutyryl-CoA into PHB by PHB synthase [22].

As pointed out above, PHB synthesis is induced by high carbon to nitrogen ratio. Intracellular concentration of acetyl-CoA and free CoA play important role in polymer synthesis. Under balanced growth conditions is acetyl-CoA catabolized in TCA cycle. NADH is generated and used for growth of cells. When growth ceases the NADH concentration increases, which reduces the activity of the TCA cycle enzymes citrate synthase and isocitrate dehydrogenase. TCA cycle is inhibited and acetyl-CoA enters PHB synthetic pathway. Enzyme β-ketothiolase is inhibited by free CoA [8].

Acetyl-CoA can be generated by several metabolic pathways. Most common pathways are catabolism of sugars and β-oxidation of fatty acids [5].

2.1.3.2 Biosynthesis of copolymers

Copolymers are synthesized when suitable substrates are used as a carbon sources. P(HB-*co*-HV) is synthesized when precursors containing three (propionic acid, propanol) or five carbons are used with other carbon source or when a direct precursor of 3-hydroxyvalerate is used as a carbon source (valeric acid). Fermentation of propionate or propanol leads to formation of propyonyl-CoA. Propyonyl-CoA and acetyl-CoA are afterwards condensed with β-ketothiolase into 3-ketovaleryl-CoA. This leads to random incorporation of 3HV units into polymer structure [23].

P(3HB-*co*-4HB) can be synthesized when γ-butyrolacton is used as a precursor of 4HB. When γ-butyrolactone is used as the sole carbon source, 4-hydroxybutyryl-CoA is formed in the cells. Random copolymerization of 4-hydroxybutyryl-CoA and 3-hydroxybutyryl-CoA then leads to random incorporation of 4HB units into polymer structure [24].

2.2 *Cupriavidus necator* H16

The gram-negative bacteria *Cupriavidus necator* H16 was isolated from a spring near Göttingen as a chemolithoautotrophic 'Knallgas' bacterium capable of growing with molecular hydrogen as electron and energy donor. 30°C is optimal temperature for growth [25]. The bacteria accumulates large amount of PHB; thus, *Cupriavidus necator* serves as a model organism for study of PHB metabolism. As was mention earlier PHB is in the form of insoluble inclusion and serves as storage compound for carbon and energy. In 1970s bacteria was considered as a candidate for single cell protein (SCP). The SCP would serve as a feed for animals and as a food for astronauts in space. Large amounts of PHB would prohibit use of bacteria as SCP; therefore several PHB^- mutants were prepared. PHB^-4 is most prominent PHB^- mutant [26].

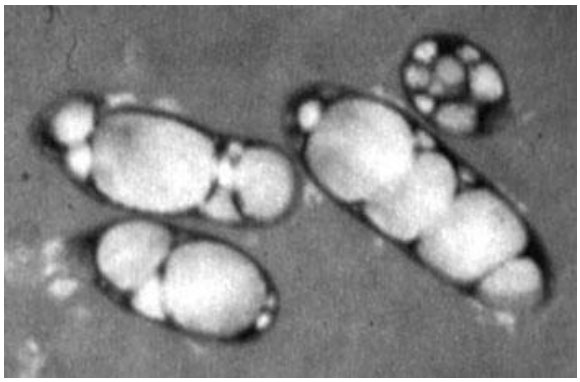


Figure 8: *Bacteria Cupriavidus necator H16* [27].

Genome of *Cupriavidus necator* H16 comprises of three replicons; two chromosomes and one megaplasmid pHG1 [28]. Genes encoding enzymes involved in PHB synthesis are located on megaplasmid PHG1. Recently whole genome of *Cupriavidus necator* H16 was sequenced and all genes were annotated. Chromosome 1 contains almost all essential genes for metabolism and essential cell functions. Chromosome 2 contains genes extending range of substrates and terminal electron acceptors (H_2 oxidation) [29].

Bacteria is able to utilize fructose and gluconic acid. These substrates are metabolized via Entner-Doudoroff pathway. Embden-Meyerhoff-Parnas pathway (glycolysis) is incomplete because bacteria is lacking enzyme fructose-1,6-bisphosphate aldolase [30]. Enzymes alcohol dehydrogenase and lactate dehydrogenase were isolated, purified and partially characterized [31, 32]. Bacteria *Cupriavidus necator* generally grows well on mineral medium but it can grow on complex media as well. Lack of ability to metabolize glucose and lactose is only minor drawback. Number of other substrates, including oils has been used. Bacteria can also grow chemolithoautotrophically by utilization of CO_2 [21].

2.2.1 Enzymes involved in PHB metabolism

As was mention above enzymes β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase are directly involved in PHA synthesis. Enzyme PHB depolymerase is involved in degradation of PHB [33].

Table 2: *Genes relevant for PHB metabolism in Cupriavidus necator H16* [33].

Locus	Gene	Product
PHA synthases		
A1437	phaC1	Poly(3-hydroxybutyrate) synthase
A2003	phaC2	Poly(3-hydroxybutyrate) synthase
β-Ketoacyl-CoA thiolases		
A1438	phaA	Acetyl-CoA acetyltransferase
A1445	bktB	Acetyl-CoA acetyltransferase
A0170		Acetyl-CoA acetyltransferase
A0462		Acetyl-CoA acetyltransferase
A1528		Acetyl-CoA acetyltransferase
A1713		Acetyl-CoA acetyltransferase
A1720		Acetyl-CoA acetyltransferase
A1887		Acetyl-CoA acetyltransferase
B0200	pcaF	Beta-ketoadipyl CoA thiolase

Table 2: *Genes relevant for PHB metabolism in Cupriavidus necator H16* [33]

Locus	Gene	Product
B0381		Acetyl-CoA acetyltransferase
B0662		Acetyl-CoA acetyltransferase
B0668		Acetyl-CoA acetyltransferase
B0759		Acetyl-CoA acetyltransferase
B1369		Acetyl-CoA acetyltransferase
B1771		Acetyl-CoA acetyltransferase
β-Ketoacyl-CoA reductases		
A1439	phaB1	Acetoacetyl-CoA reductase
A2002	phaB2	Acetoacetyl-CoA reductase
A2171	phaB3	Acetoacetyl-CoA reductase
A0743		Short chain dehydrogenase
A0931		Dehydrogenase
A1267		Short chain dehydrogenase
A1287		3-oxoacyl-ACP reduktase
A1325		Short chain dehydrogenase
A1334		D-β-hydroxybutyrate dehydrogenase
A1531		Short chain dehydrogenase
A1814		D-β-hydroxybutyrate dehydrogenase
A2152		Short chain dehydrogenase
A2460	abmB	Putative β-hydroxyacyl-CoA dehyrogenase
A2473		Short chain dehydrogenase
A2567	fabG	3-oxoacyl-ACP reduktase
A3164		Predicted short chain dehydrogenase
A3487		Putative short chain dehydrogenase
B0201		Short chain dehydrogenase
B0361		3-oxoacyl-ACP reduktase
B0385		3-oxoacyl-ACP reduktase
B0394		Dehydrogenase
B0601		Cyclohexanol dehydrogenase
B0651		Short chain dehydrogenase
B0663		Short chain alcohol dehydrogenase
B0666		Short chain dehydrogenase
B0687		Short chain dehydrogenase
B0713		Short chain alcohol dehydrogenase
B1075		3-oxoacyl-ACP reduktase
B1240		Dehydrogenase
B1297		Short chain dehydrogenase
B1334		Short chain dehydrogenase
B1442		Short chain dehydrogenase
B1696		Short chain CoA dehydrogenase
B1834		Alcohol dehydrogenase
B1904		3-oxoacyl-ACP reduktase
B2339		Short chain dehydrogenase
B2510		3-oxoacyl-ACP reduktase

Table 2: *Genes relevant for PHB metabolism in Cupriavidus necator H16* [33]

Locus	Gene	Product
Phasins		
A1381	phaP1	Phasin
PHG202	phaP2	Phasin
A2172	phaP3	Phasin
B2021	phaP4	Phasin
Transcriptional regulator of phasin expresion		
A1440	phaR	Regulator PhaR
PHB depolymerases		
A1150	phaZ1	Intracellular PHB depolymerase
A2862	phaZ2	Intracellular PHB depolymerase
B1014	phaZ3	Intracellular PHB depolymerase
PHG178	phaZ4	Putative PHB depolymerase
B0339	phaZ5	Intracellular PHB depolymerase
B2073	phaZ6	PHB depolymerase
B2401	phaZ7	PHB depolymerase
3HB-oligomer hydrolases		
A2251	phaY1	D-(-)-3HB oligomer hydrolase
A1335	phaY2	D-(-)-3HB oligomer hydrolase

2.2.1.1 β-Ketothiolase

β-Ketothiolase is enzyme catalyzing condensation of two acetyl-CoA into acetoacetyl-CoA. *Cupriavidus necator* H16 contains several β-ketothiolases with different substrate specificity [34]. β-Ketothiolase from phaCAB operon is strictly specific for acetyl-CoA, therefore it cannot be used for copolymer P(HB-*co*-HV) synthesis. β-Ketothiolase from gene bktB has wider substrate specificity, therefore it is very useful in copolymer synthesis [33].

2.2.1.2 Acetoacetyl-CoA reductase

Acetyl-CoA reductase catalyzes reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. There are 39 homologues of this enzyme in *Cupriavidus necator* H16. Large amount of these reductases have unknown function in PHB metabolism [33].

2.2.1.3 PHB synthase

PHB synthase catalyzes synthesis of 3-hydroxybutyryl-CoA to PHB. In *Cupriavidus necator* H16 is enzyme constitutively expressed and occurs as soluble protein in cells without PHB. Protein bonds to PHB granule, when PHB is present in cells. The substrate specificity of this enzyme is quite broad; therefore copolymers can be synthetized [22,33].

2.2.1.4 Phasins

Phasins are small amphiphilic proteins binding to PHB and providing, along with phospholipids, layer at the surface of granule. These proteins occur in all PHB accumulating bacteria. Large amount of phasins are required in cells and they contribute 3 – 5% of all proteins. Role of phasins is to stabilize dispersion of water-insoluble PHB in cytoplasm. Presence of phasins also prevents binding of other hydrophobic proteins to granules [35].

2.2.1.5 PHB depolymerase

PHB depolymerase is water-soluble enzyme able to hydrolyze PHB. There are 9 enzymes in *Cupriavidus necator* H16 that can be involved in PHB depolymerization [33]. Five of these enzymes contain DepA domain (phaZ1 – phaZ5); 2 contain LpqC domain (phaZ6, phaZ7) and 2 are D-(-)-3HB oligomer hydrolases. At least three PHB depolymerases contribute to degradation of PHB in cells [36].

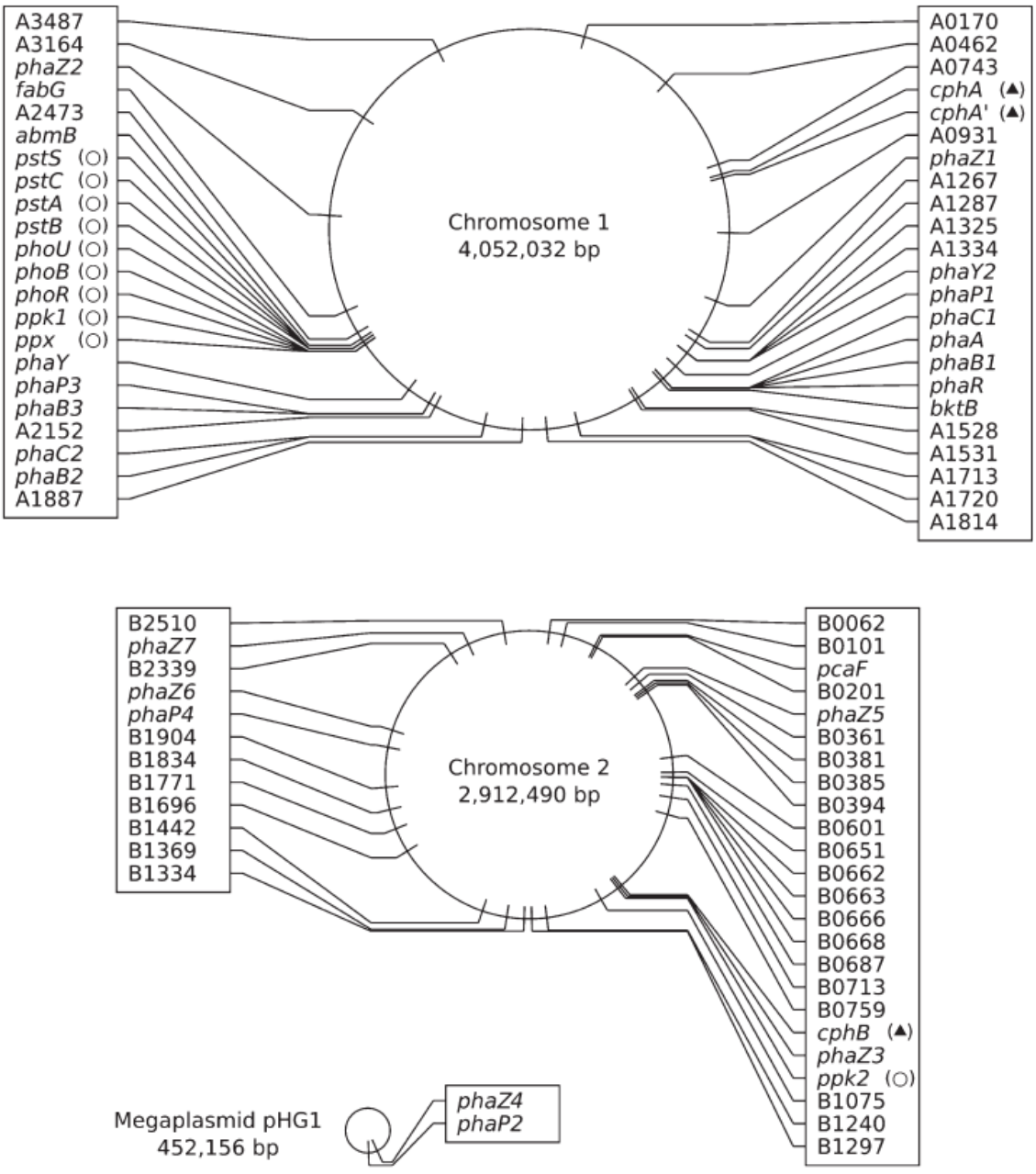


Figure 9: *Distribution of genes involved in PHB metabolism in Cupriavidus necator H16* [33]

2.3 Mechanisms of random mutations

Random mutagenesis is based on application of mutagens – chemical or physical factors that directly attack and randomly change genetic information of selected microorganism. Application of mutagen can lead to death or mutations among survivors. Any agent that damages DNA can be used for mutagenesis [37].

2.3.1 UV mutagenesis

Many types of irradiation have been used to generate mutations. The higher energy rays such as X-ray and gamma ray however require expensive equipment and they are not suitable for routine use in laboratory. These rays also produce gross damages in cell chromosome that is not easily repaired by cell. Ultraviolet ray on the other hand requires inexpensive equipment and it is suitable for routine use in laboratory [37].

Principal effects of UV radiation are introduction of pyrimidine (thymine) dimers in cell (figure 10) [37].

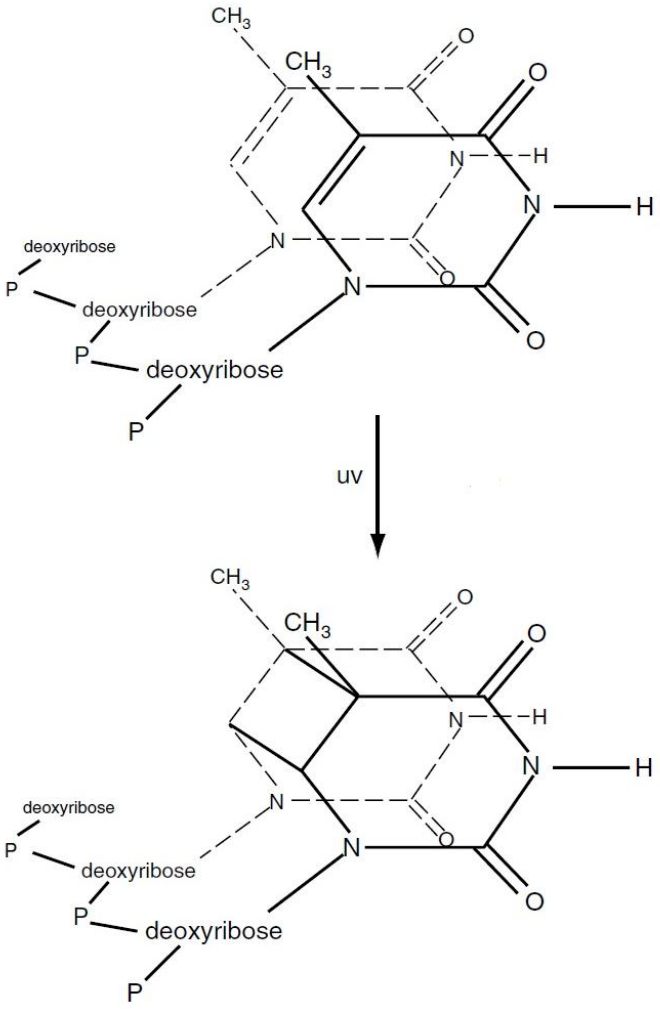


Figure 10: *Structure of thymine dimers* [37]

UV radiation causes covalent link between two pyrimidine residues to be created on the same DNA strand. These pyrimidine dimers cannot be replicated and they are lethal to cell unless it is able to repair the damage. Incorrect reparation of these damages can lead to introduction of mutations in DNA [37].

2.3.2 Chemical mutagenesis

There are many different chemical agents interacting with DNA or replication system. These chemical agents produce alternation in DNA sequence. Some chemical mutagens chemically modify a base on DNA so that it resembles a different base. Nitrous acid is chemical mutagen of this kind. Nitrous acid causes oxidative deamination of bases. Amino groups of bases are converted to keto groups and thus cytosine is converted to uracil. Uracil is not part of DNA and cell contains enzyme that will remove it. If it persists it can change C-G pair to U-A pair (ultimately T-A pair) (figure 11). Nitrous acid can react directly with DNA in vitro which is useful technic for mutagenesis of plasmids [37].

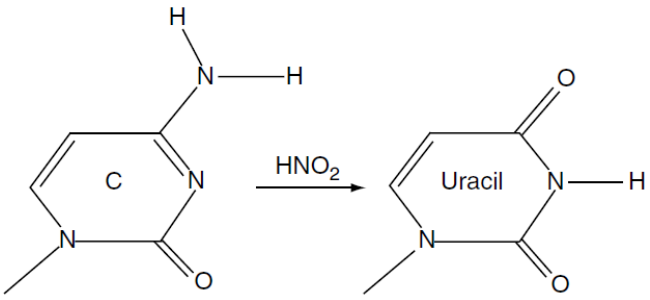


Figure 11: *Nitrous acid causes oxidative deamination of cytosine to uracil* [37]

Another group of chemical mutagens are alkylating agents. Alkylating agents like ethyl methane sulphonate (EMS) and 1-methyl-3-nitro-1-nitroso-guanidine (MNNG) are very powerful mutagens. They introduce alkyl groups on nucleotides at various positions and cause multiple closely linked mutations [37].

The intercalating agents such as acridine orange and ethidium bromide contain flat ring structure that is capable of inserting (intercalating) into the core of double helix between neighboring bases. The consequence of this is addition of single base during DNA replication [37].

Last type of mutagens are bases analogues such as 5-bromouracil. 5-bromouracil can be incorporated into DNA in the place of thymine. 5-bromouracil during replication pairs with guanine rather than adenine. Pair A-T is than changed to C-G [37].

2.3.3 Random mutagenesis of *Cupriavidus necator* for PHA overproduction

Cupriavidus necator was subjected to UV mutagenesis and chemical mutagenesis to increase PHA production in the past. Several different strategies were used to increase PHA production of mutant strains [38,39].

Santanu et al. used UV mutagenesis to prepare mutant strains of *Cupriavidus necator* overproducing PHA on inexpensive carbon sources. His team was able to isolate 5 mutants overproducing PHA on minimal salt medium with fructose as a carbon source. Some of these mutants were able to utilize molasses with high PHA yields [38].

Park and Lee were able to prepare PHA overproducing mutant by chemical mutagenesis. They used *N*-methyl-*N´*-nitrosoguanidine to prepare mutant strains with modification of TCA cycle-related enzymes. Isocitrate dehydrogenase leaky mutant of *Cupriavidus necator* was isolated after mutagenesis. The PHB accumulation was significantly increased due to the increased flow of carbon to the PHB biosynthetic pathway instead of the TCA cycle, due to the activation of PHB biosynthesis-related enzymes induced by the acetyl-CoA accumulated intracellularly [39].

3 MATERIALS AND METHODS

3.1 Bacterial strain, chemicals and instruments

3.1.1 Bacterial strain

Bacterial strain *Cupriavidus necator* H16 was used in all the experiments. The strain was obtained from Czech collection of microorganisms (CCM 3726)

3.1.2 Chemicals

3.1.2.1 Standards for Gas Chromatography

Poly[(R)-3-hydroxybutyric acid], Fluka (USA)
Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (12% 3HV). Fluka (USA)

3.1.2.2 Standards for SDS-PAGE electrophoresis

SERVA recombinant SDS PAGE Protein Marker 10 - 150 kDa PLUS, Serva (USA)
Precision Plus Protein™ All Blue Standards, Bio-Rad (USA)

3.1.2.3 Other chemicals

β-Nicotinamide adenine dinucleotide phosphate, Sigma-Aldrich (USA)
β-Nicotinamide adenine dinucleotide, Sigma-Aldrich (USA)
β -Nicotinamide adenine dinucleotide phosphate reduced, Sigma-Aldrich (USA)
β-Nicotinamide adenine dinucleotide reduced, Sigma-Aldrich (USA)
Coenzyme A, Sigma-Aldrich (USA)
Acetyl coenzyme A, Sigma-Aldrich (USA)
Acetoacetyl coenzyme A, Sigma-Aldrich (USA)
DL-β-hydroxybutyryl coenzyme A, Sigma-Aldrich (USA)
5,5'-dithiobis-(2-nitrobenzoic acid), Sigma-Aldrich (USA)

3.1.2.4 Chemicals for cultivation of microorganisms

Agar Powder, Himedia (India)
Nutrient Broth, Himedia (India)

3.1.3 Laboratory equipment

Gas chromatograph:
GC-FID Hewlett Packard, Series II 5890 (USA)
Column: DB-WAX 30 m by 0,25 mm

Spectrophotometer Nanophotometer, Implen (Germany)
Elisa Reader ELx808, Biotek (USA)
Centrifuge Boeco U-32R, Hettich Zentrifugen (Germany)
Analytical scale, Boeco (Germany)
Laminary box Aura Mini, Bio Air Instruments (USA)
Thermostat IP 60, LTE Scientific, Ltd. (Germany)
Orbital shaker incubator, Heidolph Unimax 1010, Labicom s.r.o. (Czech Republic)
Thermostat, LS-35 (Czech Republic)
Vortex, TK3S, Kartell spa (USA)

Ultra sonicator, Sonopuls HD 3100, Bandelin (Germany)
Electrophoretic cell, Mini-Protean Tetra electrophoresis system, Bio-rad (USA)
Common laboratory glass and equipment

3.1.4 Media for cultivation

Following media were used in all the experiments. 20 $g \cdot l^{-1}$ of agar was added to media for preparation of solid media.

3.1.4.1 Nutrition broth medium

Beef extract	10 g
Peptone	10 g
NaCl	5 g
Distilled water	1000 ml

3.1.4.2 Mineral medium

Oil	20 g
$(NH_4)_2SO_4$	3 g
Na_2PO_4	11,1 g
KH_2PO_4	1,02 g
Micro element solution*	1 ml
Distill water	1000 ml

*Micro element solution

$FeCl_3$	9,7 g
$CaCl_3$	7,8 g
$CuSO_4$	0,156 g
$CoCl_2$	0,119 g
$NiCl_2$	0,118 g
$CrCl_2$	0,062 g
Distill water	1 000 ml

3.1.4.3 Oil medium

This medium was the same as mineral medium with the oil as a carbon source except 2 $g \cdot l^{-1}$ of lecithin and 20 $g \cdot l^{-1}$ of agar was added to the medium prior to sterilization and medium was emulsified with sonicator for 1 minute

3.2 Preparation and selection of mutant strains overproducing PHA

3.2.1 Culture preservation and inoculum preparation

The wild strain and subsequent mutants were grown on Petri dish on Nutrition broth medium with agar (2%) in thermostat at 30°C. Strains were stored at 8°C for up to 30 days. Strains were transferred onto fresh Petri dishes every 30 days.

Inoculum was prepared by transferring culture from Petri dish with bacteriologic loop 3 times to 100 ml Erlenmeyer flask containing 50 ml of Nutrition broth medium. Culture was then maintained at 30°C and agitated 150 rpm. The 24 hour old culture was used for inoculation of Production medium.

3.2.2 Mutant strains preparation

UV mutagenesis and chemical mutagenesis were two methods used for mutant strains preparation. UV light was used in UV mutagenesis. Ethyl methane sulfonate (EMS) and methyl methane sulfonate (MMS) were used in chemical mutagenesis. Following agar plates were used for mutant selection: Nutrition broth medium, mineral medium with fructose as a carbon source and oil medium.

3.2.2.1 UV mutagenesis

The wild strain of *Cupriavidus necator* H16 was cultivated in Nutrition broth medium for 24 hours (30°C, 150 rpm). Approximately 10 ml of cell suspension was transferred to sterile plate and placed under UV light (15 W at 253 nm). Cell suspension was irradiated with UV light for 30 minutes. The 250 ml Erlenmeyer flask containing 100 ml of mineral medium was inoculated with this cell suspension and it was cultivated for 48 hours (30°C, 150 rpm). After 48 hours of cultivation cells were sterilely centrifuged (8000 rpm, 5 minutes) and supernatant was discarded. Cells were washed with sterile water and centrifuged again (8000 rpm, 5 minutes). Supernatant was discarded and cells were suspended in 10 ml of sterile water. Cell suspension was afterwards transferred to mineral medium without any carbon source. In the first experiment, the culture was agitated at 150 rpm and maintained at 30°C for 24 hours with samples taken every 3 hours. In the second experiment, the culture was maintained at 30°C and agitated at 150 rpm for 576 hours with samples taken approximately every 24 hours.

Dry cell weight (DCW) (3.2.4), Viable cell count (3.2.5) and PHA concentration (3.2.6) were measured in all the samples.

Mutants were selected in at the 24^{th} of cultivation in the first experiment and in at the 576^{th} hour of cultivation in the second experiment. Culture was suitably diluted and mutants were selected on Nutrition broth agar plate.

3.2.2.2 Chemical mutagenesis

The wild strain of *Cupriavidus necator* H16 was cultivated in Nutrition broth medium for 24 hours (30°C, 150 rpm). 50 ml of mineral medium in 100 ml Erlenmeyer flask with 1 % propanol and following mutagens: EMS 1 ml/l, EMS 0,1 ml/l, MMS 1 ml/l, MMS 0,1 ml/l was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm and maintained at 30°C for 48 hours. After 48 hours of cultivation, the cell suspension was suitably diluted and the mutants were selected on following plates: Nutrition broth, mineral medium with fructose as a carbon source, oil medium.

3.2.3 Mutant Selection

To determine whether selected mutants are able of overproduction of PHA following analysis were performed and results were compared to wild strain and strains able of PHA overproduction were selected for further experiments.

3.2.3.1 Cultivation on mineral medium

The mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 72 hours and then samples were collected. DCW (3.2.4) was measured in all the samples and PHA concentration (3.2.6) was estimated in the samples which grew similarly to control.

3.2.3.2 Cultivation on mineral medium with yeast extract

The mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium with addition of yeast extract (1 $g \cdot l^{-1}$) in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 72 hours and then samples were collected. DCW (3.2.4) and PHA concentration (3.2.6) was measured in all the samples.

3.2.3.3 Cultivation on mineral medium with propanol

The mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C. 1 % (v/v) propanol was added to the culture at the 24^{th} hour of cultivation. Mutants were cultivated for 72 hours and then samples were collected. DCW (3.2.4) and PHA concentration (3.2.6) was measured in all samples.

3.2.4 Dry cell weight determination

Dry cell weight (DCW) was determined measuring absorbance of cell suspension at 630 nm after suitable dilution against water as a blank. Dry cell weight of samples was calculated from standard curve.

3.2.4.1 Standard curve determination

Standard curve was constructed from relationship between absorbance at 630 nm and DCW. The suspension original dry weight was determined gravimetrically. 10 ml of cell suspension was centrifuged (8000 rpm, 5 minutes). Supernatant was discarded and cells were washed with 5% (w/w) Triton X-100. Cells were centrifuged again (8000 rpm, 5 minutes) and supernatant was discarded. Cells were washed with distilled water and centrifuged (8000 rpm, 5 minutes). Supernatant was discarded and cells were suspended in 1 ml of distilled water. Cell suspension was quantitatively transferred to pre weight aluminum weighing pan. The cell suspension was dried in oven at 105°C to constant weight. Aluminum weighting pan was weighted after cooling in desiccator. DCW was determined from differences in weight of aluminum weighting pan with and without cell suspension. Cell suspension of known weight was suitable diluted and standard curve was constructed.

3.2.5 Viable cell count

Spread plate method was used to determine number of viable bacteria in sample. Cell suspension was suitably diluted and 100 µl of diluted cell suspension was placed on sterile agar plates with solid medium. The culture was then spread with sterile spreader. Plates were cultivated at 30°C in thermostat for 48 hours. Plates with 30 to 300 colonies were selected and number of colony-forming units (CFU) was counted. Number of viable cells in original suspension was calculated.

3.2.6 PHA determination by gas chromatography with FID detection

This method is based on acidic extraction, hydrolysis, and methylation of PHA to 3−hydroxy fatty acid methyl esters. These esters are afterwards separated and analyzed by GC.

3.2.6.1 Standard curve determination

Standard curve was constructed from relationship between peak area and PHA concentration. PHA samples with concentration from 2 to 10 g/l of PHA in chloroform were prepared. 1 ml of sample was mixed with 0,8 ml of solution containing 15% H_2SO_4 (w/w) in methanol and benzoic acid as internal standard. The mixture was heated at 98°C for 2 hours, and then it was cooled to room temperature. For extraction 0,5 ml of 0,05 M NaOH was added to the mixture. After phases separation the chloroform layer was transferred to clean vial and analyzed with GC. Temperature programing was used for effective separation of methyl esters on the column: Initial column temperature was 80°C for 4 minutes then temperature gradient 8°C/min was applied for 6 minutes. Injector temperature was 230°C and detector temperature was 275°C. Nitrogen was used as carrier gas. Flame ionization detector (FID) was used for detection.

3.2.6.2 PHA determination in cells

For PHA amount determination in cells the cells suspension was centrifuged (8000 rpm, 5 minutes) and supernatant was discarded. The cells were washed with 5 % (w/w) Triton X−100. Cells were centrifuged again (8000 rpm, 5 minutes) and supernatant was discarded. Cells were washed with distilled water and centrifuged (8000 rpm, 5 minutes). Supernatant was discarded and cells were suspended in 1 ml of distilled water. The cell suspension was then transfer to eppendorf tube and centrifuged (12 000 rpm, 5 minutes). Supernatant was discarded and cell pellets were dried in thermostat at 70°C to constant weight.

Approximately 10 mg of dried cell pellets were accurately weighted for analysis. 1 ml of chloroform was added to the sample and it was mixed with 0,8 ml of solution containing 15% H_2SO_4 (w/w) in methanol and benzoic acid as internal standard. The mixture was heated at 98°C for 2 hours, and then it was cooled to room temperature. For extraction 0,5 ml of 0,05 M NaOH was added to the mixture. After phases separation the chloroform layer was transferred to clean vial and analyzed with GC. Temperature programing was used for effective separation of methyl esters on the column: Initial column temperature was 80°C for 4 minutes then temperature gradient 8°C/min was applied for 6 minutes. Injector temperature was 230°C and detector temperature was 275°C. Nitrogen was used as carrier gas. Flame ionization detector (FID) was used for detection.

The PHA concentration in cells was calculated from standard curve.

3.3 Biochemical characterization of mutants

In order to better understand PHA overproducing mutant activity of extracellular secretion and activity of several intracellular enzymes was measured.

3.3.1 Total proteins concentration determination by Hartree-Lowry assay

This assay combines the reaction of copper ions with peptide bond under alkaline conditions with oxidation of aromatic protein residues. There are 3 reagents used in this assay. First 2 reagents are biuret reagents. Third reagent is Folin-Ciocalteu phenol reagent. Two distinct reactions lead to final color of sample: reaction of protein with copper in alkaline conditions and reduction of the phosphomolybdic-phosphotungstic reagent by the copper-treated protein.

Reagent A consists of 2 g sodium potassium tartrate · 4 H_2O, 100 g sodium carbonate, 500 ml 1M NaOH, H_2O to one liter.

Reagent B consists of 2 g sodium potassium tartrate · 4 H_2O, 1 g copper sulfate (CuSO4 · 5H20), 90 ml H_2O, 10 ml 1M NaOH.

Reagent C consists of 1 volume of Folin-Ciocalteau reagent diluted with 15 volumes of water.

3.3.1.1 Standard curve determination

Standard curve was constructed from relationship between absorbance at 750 nm and protein concentration. Protein samples with concentration from 40 to 400 $mg \cdot l^{-1}$ of protein were prepared from bovine serum albumin. 333 µl of protein sample and 300 µl of reagent A were mixed in test tube. The tube was incubated 10 minutes in a 50°C bath and then it was cooled to room temperature. 33 µl of reagent B was added to the tube and it was incubated 10 minutes at the room temperature. 1 ml of reagent C was added to the tube and it was incubated 10 minutes in 50°C bath and then it was cooled to room temperature. The absorbance at 750 nm against blank was measured and standard curve was constructed.

3.3.1.2 Sample determination

For protein determination 333 µl of dilute protein sample and 300 µl of reagent A were mixed in test tube. The tube was incubated 10 minutes in a 50°C bath and then it was cooled to room temperature. 33 µl of reagent B was added to the tube and it was incubated 10 minutes at the room temperature. 1 ml of reagent C was added to the tube and it was incubated 10 minutes in 50°C bath and then it was cooled to room temperature. The absorbance at 750 nm against blank was measured. The protein concentration was calculated form standard curve.

3.3.2 Extracellular secretion

In order to prepare extracellular secretion samples suitable for assay following cultivation was performed. Mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 72 hours. 10 ml of samples were taken in 72^{nd} hour of cultivation. Cell suspension was centrifuged (8000 rpm, 5 minutes), extracellular secretion was collected and cells were discarded.

Activity of extracellular secretion of mutants was determined according to Weber [40].

3.3.3 Intracellular enzymes

In order to prepare intracellular enzyme extract suitable for enzymatic assays following cultivation was performed. Mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. 300 ml of culture was prepared from each mutant. The culture was agitated at 150 rpm at 30°C for 48 hours. Whole cell suspension was collected after 48 hours of cultivation.

The suspension of bacterial cells was treated with ultrasonic sound that results in disruption of cells. 300 ml of cell suspension was centrifuged (5000 rpm, 10 minutes). Supernatant was discarded and cells were washed with 5% Triton X-100. Cells were centrifuged again (8000 rpm, 5 minutes) and supernatant was discarded. Cells were washed with distilled water and centrifuged (8000 rpm, 5 minutes). Supernatant was discarded and cells were suspended in 20 ml of 20 mM Tris-HCl buffer pH 8,3. The suspension was

sonicated with 80 % amplitude up to 35 kJ. Cell suspension was cooled with ice during sonication due to amount of heat released. Cell debris were removed by centrifugation (12 000 rpm, 10 minutes) and supernatant was used in enzymatic experiments.

3.3.3.1 NADH (NADPH) standard curve determination

Standard curve was constructed from relationship between absorbance at 340 nm and NADH (NADPH) concentration. NADH (NADPH) samples with concentration from 0,05 to 0,5 $mmol \cdot l^{-1}$ were prepared. Absorbance at 340 nm of all samples was measured against water as a blank and standard curve was constructed.

3.3.3.2 CoA standard curve determination

Thiol group of CoA reacts quantitatively with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and forms a yellow product. Amount of product can be quantified measuring its absorbance at 405 nm. Standard curve was constructed from relationship between absorbance at 405 nm and CoA concentration. Samples with CoA concentration from 0,1 to 0,5 $mmol \cdot l^{-1}$ in buffer containing 2 $mmol \cdot l^{-1}$ of DTNB were prepared. Absorbance at 405 nm of all samples was measured against particular buffer as a blank and standard curve was constructed.

3.3.3.3 Alcohol dehydrogenase EC 1.1.1.1

Alcohol dehydrogenase (ADH) is an enzyme catalyzing oxidation of alcohol using NAD or NADP as electron acceptor. The reaction is reversible and substrates can be a variety of primary or secondary alcohols [32].

The activity of alcohol dehydrogenase was measured by following assay: 25 µl of intracellular extract was mixed with 25 µl of 10 $mmol \cdot l^{-1}$ NAD (NADP) and 200 µl of 20 $mmol \cdot l^{-1}$ Tris-HCl buffer pH 8,3 containing 25 $mmol \cdot l^{-1}$ of alcohol and 10 µl of micro element solution. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADH per minute. Several experiments were performed with ADH:

Both cofactors (NAD, NADP) were tested in assay and effects on enzyme activity were compared. Furhermore, following alcohols were tested as substrates for alcohol dehydrogenase: Methanol, ethanol, propanol, butanol, pentanol, isoamyl alcohol, butan-1,4-diol.

Activity of ADH of mutants was measured with propanol and butan-1,4-diol as substrates and NAD as a cofactor.

3.3.3.4 NAD dependent isocitrate dehydrogenase EC 1.1.1.41

NAD dependent isocitrate dehydrogenase is enzyme of TCA cycle. This enzyme catalyzes oxidation of isocitrate to α-ketoglutarate using NAD as cofactor [41].

The activity of NAD isocitrate dehydrogenase was measured by following assay: 25 µl of intracellular extract was mixed with 25 µl of 10 $mmol \cdot l^{-1}$ NAD and 200 µl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 50 $mmol \cdot l^{-1}$ of isocitrate, 10 $mmol \cdot l^{-1}$ $MgSO_4$ and 100 $mmol \cdot l^{-1}$ of KCl. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADH per minute.

3.3.3.5 NADP dependent isocitrate dehydrogenase EC 1.1.1.42

NADP dependent isocitrate dehydrogenase is enzyme that allows cells to generate NADPH. NADPH is important reducing agent in cell reduction of reactive oxygen species. NADP dependent isocitrate dehydrogenase catalyzes oxidation of isocitrate to α-ketoglutarate and reduction of NADP to NADPH in process [42].

The activity of NADP isocitrate dehydrogenase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 10 $mmol \cdot l^{-1}$ NADP and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 50 $mmol \cdot l^{-1}$ of isocitrate, 10 $mmol \cdot l^{-1}$ of $MgSO_4$ and 100 $mmol \cdot l^{-1}$ of KCl. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADPH per minute.

3.3.3.6 Malate dehydrogenase EC 1.1.1.37

Malate dehydrogenase is enzyme of TCA cycle. Enzyme catalyzes oxidation of malate to oxaloacetate with NAD as an electron acceptor [43].

The activity of malate dehydrogenase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 10 $mmol \cdot l^{-1}$ NAD and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 50 $mmol \cdot l^{-1}$ of malate, 10 $mmol \cdot l^{-1}$ of $MgSO_4$ and 100 $mmol \cdot l^{-1}$ of KCl. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADH per minute.

3.3.3.7 Malic enzyme EC 1.1.1.40

Malic enzyme is another enzyme allowing cells to generate NADPH. Malic enzyme catalyzes oxidation of malate to pyruvate. CO_2 is released and NADP is reduced to NADPH in process [42].

The activity of malic enzyme was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 10 $mmol \cdot l^{-1}$ NADP and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 50 $mmol \cdot l^{-1}$ of malate, 10 $mmol \cdot l^{-1}$ of $MgSO_4$ and 100 $mmol \cdot l^{-1}$ of KCl. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes..

3.3.3.8 Glucose 6-phosphate dehydrogenase EC 1.1.1.49

Glucose 6-phosphate dehydrogenase is the first enzyme of pentose phosphate pathway that generates pentoses and NADPH. NADPH is important reducing agent in cell as was mention above. Glucose 6-phosphate dehydrogenase catalyzes oxidation of glucose 6-phosphate to 6-phosphoglucono-δ-lactone with NADP as electron acceptor [42].

The activity of glucose 6-phosphate dehydrogenase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 10 $mmol \cdot l^{-1}$ NADP and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 5 $mmol \cdot l^{-1}$ of glucose 6-phosphate and 30 $mmol \cdot l^{-1}$ of MgCl. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADPH per minute.

3.3.3.9 Glutamate dehydrogenase EC 1.4.1.4

Glutamate dehydrogenase is enzyme catalyzing oxidation of glutamate to α-ketoglutarate using NAD(P) as electron acceptor. The reaction is reversible [44].

The activity of glutamate dehydrogenase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 10 $mmol \cdot l^{-1}$ NADP and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 5 $mmol \cdot l^{-1}$ of L-glutamic acid. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of NADPH per minute.

3.3.3.10 Glutathion reductase EC 1.8.1.7

Glutathion serves as an important antioxidant in cell. Its role is to prevent damage caused by reactive oxygen species and free radicals. Glutathion reductase catalyzes reduction of oxidized glutathione to reduced glutathione with NADPH serving as electron donor [45].

The activity of glutathione reductase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 4 $mol \cdot l^{-1}$ NADPH and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 5 $mmol \cdot l^{-1}$ of oxidize form of glutathione. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes decomposition of nmol of NADPH per minute.

3.3.3.11 Malate synthase EC 2.3.3.9

Malate synthase is enzyme of glyoxalate cycle. Enzyme catalyzes aldol condensation of glyoxylate with acetyl-CoA during which malate is formed [46].

The activity of malate synthase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 2 $mmol \cdot l^{-1}$ Acetyl-CoA, 25 μl of 50 $mmol \cdot l^{-1}$ glyoxalate and 175 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 1 $mmol \cdot l^{-1}$ of DNTB. Absorbance of sample at 405 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of CoA per minute.

3.3.3.12 β-Ketothiolase EC 2.3.1.9

β-Ketothiolase is the first enzyme involved in PHA synthesis. This enzyme catalyzes biosynthesis of two acetyl-CoA into acetoacetyl-CoA [33]. CoA is released during synthesis. Released CoA reacts quantitatively with DNTB into yellow product that absorbs at 405 nm.

The activity of β-Ketothiolase was measured by following assay: 25 μl of intracellular extract was mixed with 25 μl of 2 $mmol \cdot l^{-1}$ acetyl-CoA and 200 μl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 1 $mmol \cdot l^{-1}$ of DNTB. Absorbance of sample at 405 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of CoA per minute.

3.3.3.13 Acetoacetyl-CoA reductase EC 1.1.1.36

Acetoacetyl-CoA reductase is second enzyme involved in PHA synthesis. This enzyme catalyzes reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA [33]. NADPH is oxidized into NADP during reaction.

The activity of acetoacetyl-CoA was measured by following assay: 25 µl of intracellular extract was mixed with 25 µl of 2 $mmol \cdot l^{-1}$ acetyl-CoA, 25 µl of 4 $mmol \cdot l^{-1}$ NADPH and 175 µl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4. Absorbance of sample at 340 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes reduction of nmol of NADPH per minute.

3.3.3.14 PHB synthase EC 2.3.1.B2

PHB synthase is last enzyme involved in PHA synthesis. Enzyme catalyzes synthesis of PHB [33]. CoA is released during synthesis. Released CoA reacts quantitatively with DNTB into yellow product that absorbs at 405 nm.

The activity of PHB synthesis was measured by following assay: 25 µl of intracellular extract was mixed with 25 µl of 2 $mmol \cdot l^{-1}$ 3-hydroxybutyryl-CoA and 200 µl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4 containing 1 $mmol \cdot l^{-1}$ of DNTB. Absorbance of sample at 405 nm was measured every 30 seconds up to 10 minutes. One unit of enzyme activity is defined as amount of enzyme that catalyzes formation of nmol of CoA per minute.

3.3.4 Resistance against oxidative stress

The mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 48 hours and then 10 ml samples were collected. Samples were diluted with 40 ml of sterile water. Viable cell count (3.2.5) of samples was measured. After that oxidative stress was applied; cells were suspended in 50 $mmol \cdot l^{-1}$ hydrogen peroxide. Samples were collected at the 5^{th} and the 30^{th} minutes of oxidative stress exposition. Viable cell count (3.2.5) of samples was measured.

3.3.5 Polymer analysis by GPC

The mutants were cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 72 hours. Whole cell suspension was collected after 72 hours of cultivation. The cells suspension was centrifuged (8000 rpm, 5 minutes) and supernatant was discarded. The cells were washed with 5 % (w/w) Triton X−100. Cells were centrifuged again (8000 rpm, 5 minutes) and supernatant was discarded. Cells were washed with distilled water and centrifuged (8000 rpm, 5 minutes). Supernatant was discarded and cell pellets were dry in thermostat at 70°C. Cells were afterwards suspended in chloroform. PHA from the cells was extracted with chloroform at 60°C overnight. Cell residues were filtrated out and PHA was precipitated from chloroform solution with addition 5x volume of mixture methanol/water (7:3). Solvents were removed in rotary vacuum evaporator and polymer was re-dissolved in chloroform. Polymer was analyzed by gel permeation chromatography.

3.4 Extracellular secretion characterization and purification

In order to prepare extracellular secretion samples suitable for assay following cultivation was performed: *Cupriavidus necator* was cultivated in Nutrition medium for 24 hours (30°C, 150 rpm). 100 ml (300 – 400 ml total) of mineral medium in 250 ml Erlenmeyer flask was inoculated with 5 ml of inoculum. The culture was agitated at 150 rpm at 30°C for 24 hours. Cell suspension was centrifuged (8000 rpm, 5 minutes), extracellular secretion was collected and cells were discarded. The amount of extracellular secretion of mutants was determined according to Weber [40].

3.4.1 Extracellular secretion characterization

Several experiments were performed to characterize extracellular secretion.

3.4.1.1 pH activity optimum

Extracellular secretion was prepared as described previously (3.4). Several buffers were used in assay and activity of extracellular secretion was measured.

3.4.1.2 Ionic strength activity optimum

Extracellular secretion was prepared as described previously (3.4). Several buffers with ionic strength from 0,5 to 5 $mol \cdot l^{-1}$ were used in assay and activity of extracellular secretion was measured.

3.4.2 Purification methods

Extracellular secretion contains number of protein and other substances. All the substances in secretion are also much diluted. Series of experiment were performed to purify and concentrate the extracellular secretion.

3.4.2.1 Acetone precipitation

2 ml of extracellular secretion was mixed with 8 ml of cold acetone (-20°C) in centrifugation tube. Tubes were vortexed and incubated for half hour at -20°C. The tubes were centrifuged (12000 rpm, 15 minutes). Supernatant was discarded and acetone was evaporated at room temperature for 30 minutes. Precipitate was afterwards suspended in 50 µl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4.

3.4.2.2 Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed by following assay: 400 ml of extracellular secretion was precipitated with solid ammonium sulfate on a magnetic stirrer to 60, 70 and 80% of saturation. 10 ml samples were taken at 60 and 70% of saturation. Whole amount was taken at 80% of saturation and it was transferred to 10 ml centrifugation tubes. Samples were centrifuged (12000 rpm, 15 minutes). Supernatant was discarded and precipitate was suspended in 250 µl of 50 $mmol \cdot l^{-1}$ phosphate buffer pH 7,4.

3.4.2.3 Dialysis

20 ml of protein solution after ammonium sulfate precipitation was transferred to dialysis bag. Bag was put into reservoir of distilled water (3 l) and was dialyzed overnight at 4°C.

3.4.2.4 Ultrafiltration

300 ml of extracellular secretion was filtered on 10 kDa membrane. Final volume after ultrafiltration was 50 ml.

3.5 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used for separation of proteins in extracellular secretion and in intracellular extract. During SDS-PAGE are proteins separated according to their size in polyacrylamide gel. SDS helps to shape all proteins into linear form. Staining is used for visualization of separated proteins.

3.5.1.1 Solutions

Table 3: *Solutions for SDS-PAGE electrophoresis*

Solution	Chemicals
Acrylamide solution	Rotiphorese gel 30 (30 % acrylamide/ bisacrylamide, mixing ratio 37,5:1.)
Resolving buffer	1,5 mol·l^{-1} Tris-HCl pH 8,8
Stacking buffer	0,5 mol·l^{-1} Tris-HCl pH 6,8
Electrode buffer	25 mmol·l^{-1} Tris/glycine-SDS pH 8,3
Sample buffer	3,55 ml of deionized water; 1,25 ml of 0,5 mol·l^{-1} Tris-HCl pH 6,8; 2,5 ml of glycerol; 2 ml of 10% (w/v) SDS; 0,2 ml of 0,5% (w/v) Bromophenol Blue
SDS solution	10% solution of SDS
Initialization reagent	10% ammonium persulfate solution (APS)

3.5.1.2 Polyacrylamide gel preparation

Resolving gel was prepared by mixing acrylamide solution with water, resolving buffer and SDS solution. Solution was mixed and then TEMED and APS were added and solution was gently stirred. Solution was immediately poured between glasses. Small amount of butanol was poured on top of gel and gel was left to polymerize for 1 hour.

Butanol was dried after gel polymerized and gel was washed with distilled water and dried. Stacking gel was prepared by mixing acrylamide solution with water, resolving buffer and SDS solution. Solution was mixed and then TEMED and APS were added and solution was gently stirred. Solution was poured on top of resolving gel and comb was added. Gel was left to polymerize for 1 hour.

Table 4: *Polyacrylamide gel preparation*

Solution	14% resolving gel (ml)	8% stacking gel (ml)
Acrylamide solution	4,7	2,7
Deionized water	2,7	4,7
Resolving gel	2,5	-
Stacking gel	-	2,5
SDS solution	0,1	0,1
TEMED	0,005	0,01
APS	0,05	0,05

3.5.1.3 Sample loading and electrophoresis

Protein samples were mixed with sample buffer in 1:1 ratio. Samples were afterwards heated at 95°C for 4 minutes. From 10 to 20 µl of sample was loaded to wells. Protein standard was loaded as well.

Electrophoresis was performed at following conditions: voltage 120 V, current 400 mA, time 120 minutes. Gel was removed from glass and washed with distilled water when electrophoresis was finished. Staining was afterwards used for visualization of separated proteins.

3.5.1.4 Staining with Coomassie Brilliant Blue R-250

Gel was fixed for 1 hour with fixing solution (10% of acetic acid, 40% of methanol, 50% of water). Gel was afterwards stained with Coomassie Brilliant Blue R-250 solution (0,25 % of Coomassie Brilliant Blue, 50 % of methanol, 10 % of acetic acid, 40 % of water) overnight. Gel was then destained with destaining solution (10% of acetic acid, 40% of methanol, 50% of water).

3.5.1.5 Staining with silver

For staining proteins following solutions were prepared:

Table 5: *Solutions for staining with silver*

Solution	Chemicals
Fixing solution	120 ml of acetic acid, 500 ml of methanol, 380 ml of distilled water, 500 µl of formaldehyde (35%)
Washing solution	200 ml of ethanol, 800 ml of distilled water
Sensibilizing solution	200 mg of sodium thiosulfate, 1000 ml of distilled water
Staining solution	2 g of silver nitrate, 760 µl of formaldehyde (35%), 1000 ml of distilled water
Developing solution	60 g of sodium carbonate, 4 mg of sodium thiosulfate, 500 µl of formaldehyde (35%), 1000 ml of distilled water
Stop solution	120 ml of acetic acid, 500 ml of methanol, 380 ml of distilled water

Gel was developed by following procedure: Gel was fixed 2 hours in fixing solution. Gel was then washed with washing solution for 20 minutes (during this time was washing solution changed 3 times). Gel was dipped to sensibilizing solution for 2 minutes then was gel washed 2 times with deionized water. Gel was stained with cold (4°C) staining solution for 20 minutes. Gel was washed with deionized water and approximately 100 ml of developing solution was poured on gel. After suitable development process was stopped with stop solution.

Stained gels were photographed or scanned with scanner. Gels were analyzed with program Scion Image.

4 RESULTS AND DISCUSION

4.1 Preparation and selection of mutant strains overproducing PHA

Random mutagenesis is a biotechnological approach of construction of mutants that may have superior biotechnological (or other) properties to wild strain. This method is based on application of mutagens – chemical or physical factors that directly attack and randomly change genetic information of selected (micro)organism. During typical experiment, thousands of mutants are constructed; however, only trace amounts of them are capable of growing and revealing other desired properties. Hence, mutant selection plays a crucial role in if random mutagenesis is used. This work employed several mutation as well as mutant selection strategies (see Figure 12)

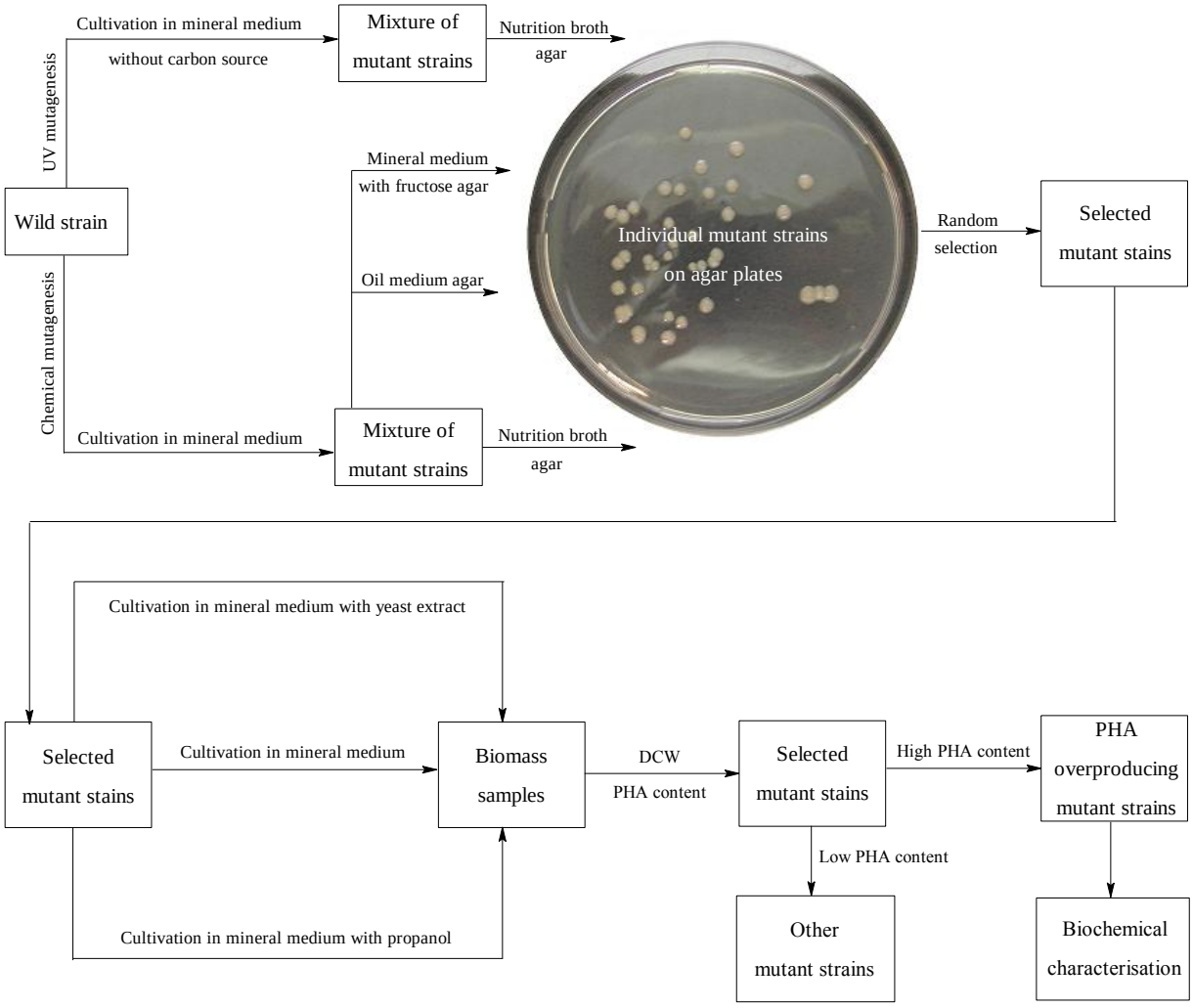


Figure 12: *Overview of preparation and selection of mutant strains overproducing PHA*

4.1.1 Mutant strains preparation

4.1.1.1 UV mutagenesis and cultivation of mutant strains in mineral medium without carbon source

Goal of this experiment was to prepare mutant strains of bacteria *Cupriavidus necator* overproducing PHA by UV mutagenesis. After mutagenesis mutants as well as wild strain were transferred to medium without any carbon source and they were cultivated for 24 or 576 hours. The idea behind this cultivation was that mutants overproducing PHA will be able to survive in medium without any carbon source longer than mutants with lower PHA content. This cultivation can serve as method of selection of mutant strains overproducing PHA. Wild strain of *Cupriavidus necator* H16 was cultivated and mutants were selected as described previously (3.2.2.1).

Table 6: *Viable cell count (CFU) and PHB content of mutant strains and wild strain during cultivation in mineral medium without carbon source for 24 hours.*

Time (h)	Mutants		Wild	
	CFU (10^{10}·ml^{-1})	PHB (%)	CFU (10^{10}·ml^{-1})	PHB (%)
0	0,34 ± 0,04	47,29 ± 0,02	0,85 ± 0,05	32,54 ± 0,01
3	0,95 ± 0,80	41,34 ± 0,10	0,80 ± 0,07	40,06 ± 0,06
6	9,07 ± 3,69	29,24 ± 0,01	1680 ± 100	25,11 ± 0,02
9	3500 ± 0	27,60 ± 0,01	4200 ± 0	20,97 ± 0,00
24	7,97 ± 0,74	18,92 ± 0,03	433 ± 54,4	15,76 ± 0,02

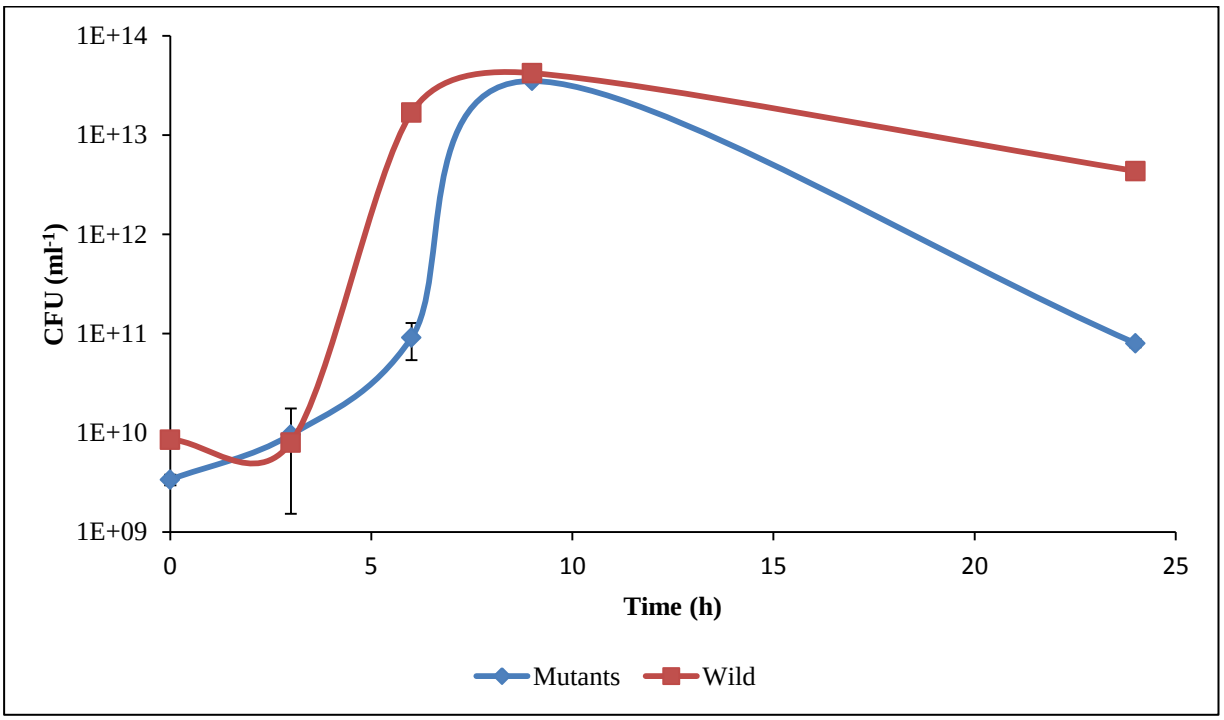


Figure 13: *Viable cell count (CFU) of mutant strains and wild strain during cultivation in mineral medium without carbon source.*

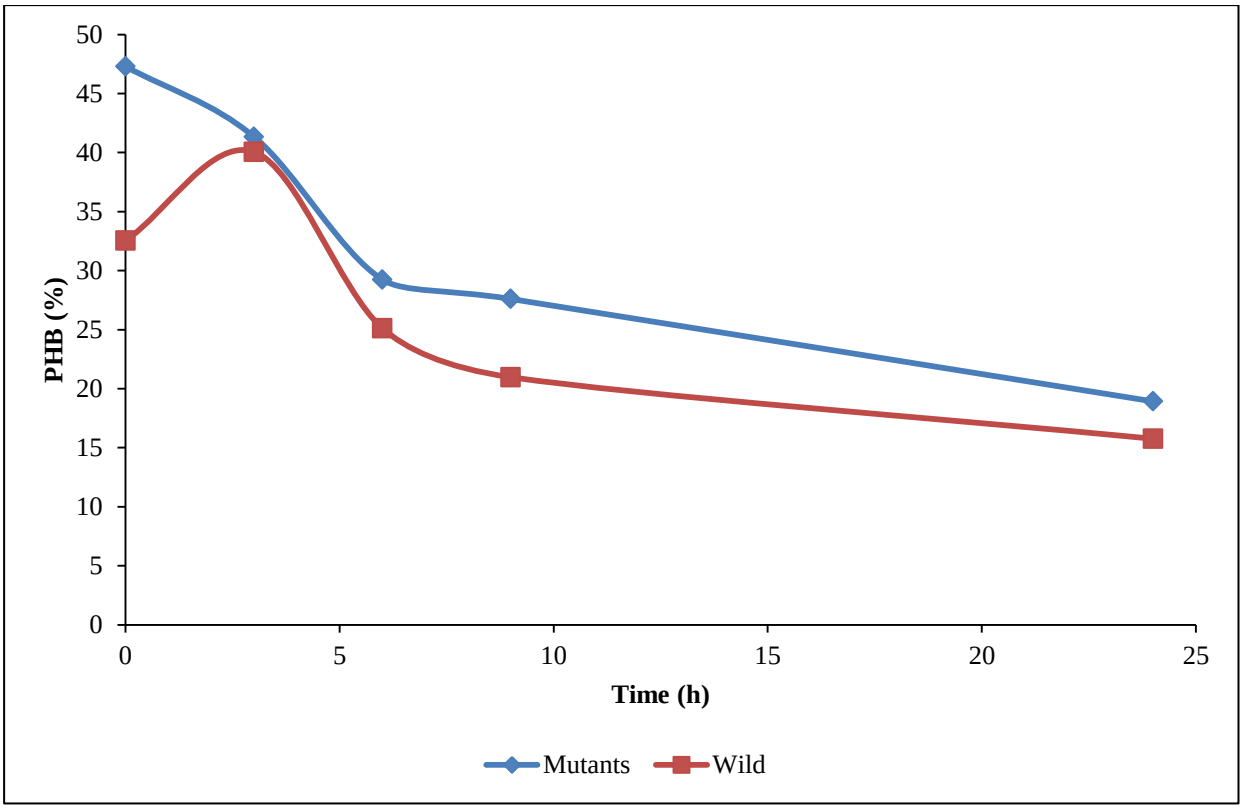


Figure 14: *PHB content in cells of mutant strains and wild strain during cultivation in mineral medium without carbon source.*

It can be seen from the experiment results that the number of viable cells of mutant strains and wild strain in medium without carbon source rose up to the 9th hour of cultivation. Number of viable cells of mutant and wild strains slightly declined after 9th hour of cultivation.

At first bacteria continued to grow in medium without carbon source on a residue of carbon source left in the medium during transfer of bacteria. Bacteria slowly began to die of when residue of carbon source is all metabolized.

From PHB percentage in cells it can be seen that PHB cell content of wild and mutant strains declines with time. Bacteria during cultivation in medium without carbon source used PHB as a backup carbon source and amount of PHB in cells declined for this reason.

It can be seen that the number of viable bacteria in medium without carbon source is at the 24th hour of cultivation still large. Therefore, longer cultivation in medium without carbon source was performed to study bacteria further.

Table 7: *Viable cell count (CFU) and dry cell weight of mutant strains and wild strain during cultivation in mineral medium without carbon source for 576 hours.*

Time	Mutants		Wild	
(h)	CFU (ml^{-1})	DCW ($g \cdot l^{-1}$)	CFU (ml^{-1})	DCW ($g \cdot l^{-1}$)
0	$(4{,}65 \pm 1{,}25) \cdot 10^{8}$	$4{,}96 \pm 0{,}12$	$(2{,}25 \pm 0{,}15) \cdot 10^{8}$	$4{,}55 \pm 0{,}39$
12	$(9{,}80 \pm 0{,}57) \cdot 10^{12}$	$4{,}62 \pm 0{,}10$	$(8{,}10 \pm 0{,}80) \cdot 10^{10}$	$3{,}79 \pm 0{,}08$
24	$(9{,}42 \pm 0{,}64) \cdot 10^{11}$	$3{,}86 \pm 0{,}04$	$(3{,}21 \pm 0{,}51) \cdot 10^{13}$	$3{,}61 \pm 0{,}08$
36	$(2{,}13 \pm 0{,}39) \cdot 10^{13}$	$3{,}05 \pm 0{,}09$	$(7{,}80 \pm 2{,}73) \cdot 10^{13}$	$2{,}83 \pm 0{,}03$
48	$(1{,}90 \pm 0{,}24) \cdot 10^{13}$	$2{,}51 \pm 0{,}11$	$(1{,}25 \pm 0{,}13) \cdot 10^{13}$	$2{,}77 \pm 0{,}07$
60	$(3{,}00 \pm 0{,}27) \cdot 10^{13}$	$3{,}30 \pm 0{,}09$	$(2{,}67 \pm 0{,}25) \cdot 10^{13}$	$2{,}78 \pm 0{,}04$
72	$(7{,}22 \pm 0{,}90) \cdot 10^{11}$	$3{,}32 \pm 0{,}04$	$(5{,}34 \pm 0{,}40) \cdot 10^{11}$	$2{,}85 \pm 0{,}05$
96	$(1{,}22 \pm 0{,}14) \cdot 10^{12}$	$2{,}56 \pm 0{,}05$	$(2{,}99 \pm 0{,}38) \cdot 10^{11}$	$2{,}1 \pm 0{,}07$
172	$(2{,}14 \pm 0{,}20) \cdot 10^{12}$	$2{,}35 \pm 0{,}02$	$(5{,}05 \pm 0{,}18) \cdot 10^{11}$	$1{,}87 \pm 0{,}03$
196	$(1{,}52 \pm 0{,}06) \cdot 10^{12}$	$1{,}87 \pm 0{,}04$	$(1{,}28 \pm 0{,}05) \cdot 10^{12}$	$1{,}52 \pm 0{,}01$
210	$(1{,}81 \pm 0{,}13) \cdot 10^{14}$	$1{,}75 \pm 0{,}03$	$(9{,}05 \pm 1{,}42) \cdot 10^{14}$	$1{,}46 \pm 0{,}01$
288	$(4{,}50 \pm 2{,}94) \cdot 10^{11}$	$1{,}59 \pm 0{,}01$	$(3{,}33 \pm 0{,}47) \cdot 10^{11}$	$1{,}28 \pm 0{,}01$
360	$(1{,}30 \pm 0{,}21) \cdot 10^{8}$	$1{,}76 \pm 0{,}04$	$(1{,}27 \pm 0{,}40) \cdot 10^{8}$	$1{,}37 \pm 0{,}03$
384	$(1{,}80 \pm 0{,}22) \cdot 10^{8}$	$1{,}76 \pm 0{,}02$	$(2{,}47 \pm 0{,}26) \cdot 10^{8}$	$1{,}38 \pm 0{,}01$
432	$(5{,}45 \pm 1{,}10) \cdot 10^{8}$	$1{,}81 \pm 0{,}01$	$(5{,}53 \pm 1{,}03) \cdot 10^{8}$	$1{,}37 \pm 0{,}02$
528	$(7{,}92 \pm 2{,}41) \cdot 10^{7}$	$1{,}82 \pm 0{,}04$	$(3{,}06 \pm 0{,}10) \cdot 10^{7}$	$1{,}36 \pm 0{,}06$
576	$(6{,}06 \pm 2{,}74) \cdot 10^{7}$	$1{,}59 \pm 0{,}02$	$(5{,}35 \pm 0{,}88) \cdot 10^{7}$	$1{,}20 \pm 0{,}00$

Table 8: *PHB content in cells of mutant strains and wild strain during cultivation in mineral medium without carbon source for 576 hour.*

Time (h)	Mutants		Wild	
	PHB ($g \cdot l^{-1}$)	PHB (%)	PHB ($g \cdot l^{-1}$)	PHB (%)
0	$3{,}63 \pm 0{,}09$	$73{,}22 \pm 0{,}05$	$3{,}71 \pm 0{,}32$	$81{,}67 \pm 0{,}00$
12	$3{,}48 \pm 0{,}07$	$75{,}21 \pm 0{,}11$	$2{,}67 \pm 0{,}06$	$70{,}52 \pm 0{,}00$
24	$2{,}13 \pm 0{,}02$	$55{,}16 \pm 0{,}11$	$2{,}11 \pm 0{,}05$	$58{,}49 \pm 0{,}10$
36	$1{,}56 \pm 0{,}05$	$51{,}17 \pm 0{,}01$	$1{,}80 \pm 0{,}02$	$63{,}38 \pm 0{,}33$
48	$1{,}24 \pm 0{,}06$	$49{,}52 \pm 0{,}03$	$1{,}25 \pm 0{,}03$	$45{,}13 \pm 0{,}00$
60	$1{,}39 \pm 0{,}04$	$42{,}00 \pm 0{,}00$	$1{,}15 \pm 0{,}02$	$41{,}16 \pm 0{,}00$
72	$1{,}23 \pm 0{,}02$	$37{,}01 \pm 0{,}29$	$1{,}39 \pm 0{,}03$	$48{,}74 \pm 0{,}24$
96	$1{,}02 \pm 0{,}02$	$39{,}81 \pm 0{,}01$	$0{,}77 \pm 0{,}03$	$36{,}44 \pm 0{,}16$
172	$0{,}74 \pm 0{,}01$	$31{,}43 \pm 0{,}01$	$0{,}45 \pm 0{,}01$	$23{,}83 \pm 0{,}01$
196	$0{,}49 \pm 0{,}01$	$26{,}43 \pm 0{,}09$	$0{,}37 \pm 0{,}00$	$24{,}46 \pm 0{,}03$
210	$0{,}45 \pm 0{,}01$	$25{,}74 \pm 0{,}05$	$0{,}33 \pm 0{,}00$	$22{,}82 \pm 0{,}03$
288	$0{,}31 \pm 0{,}00$	$19{,}19 \pm 0{,}03$	$0{,}23 \pm 0{,}00$	$18{,}03 \pm 0{,}04$
360	$0{,}28 \pm 0{,}01$	$15{,}61 \pm 0{,}05$	$0{,}19 \pm 0{,}00$	$13{,}84 \pm 0{,}06$
384	$0{,}26 \pm 0{,}00$	$14{,}63 \pm 0{,}03$	$0{,}22 \pm 0{,}00$	$15{,}81 \pm 0{,}05$
432	$0{,}27 \pm 0{,}00$	$14{,}81 \pm 0{,}03$	$0{,}16 \pm 0{,}00$	$11{,}53 \pm 0{,}01$
528	$0{,}26 \pm 0{,}01$	$14{,}16 \pm 0{,}04$	$0{,}07 \pm 0{,}00$	$5{,}26 \pm 0{,}04$
576	$0{,}25 \pm 0{,}00$	$15{,}47 \pm 0{,}09$	$0{,}11 \pm 0{,}00$	$9{,}20 \pm 0{,}12$

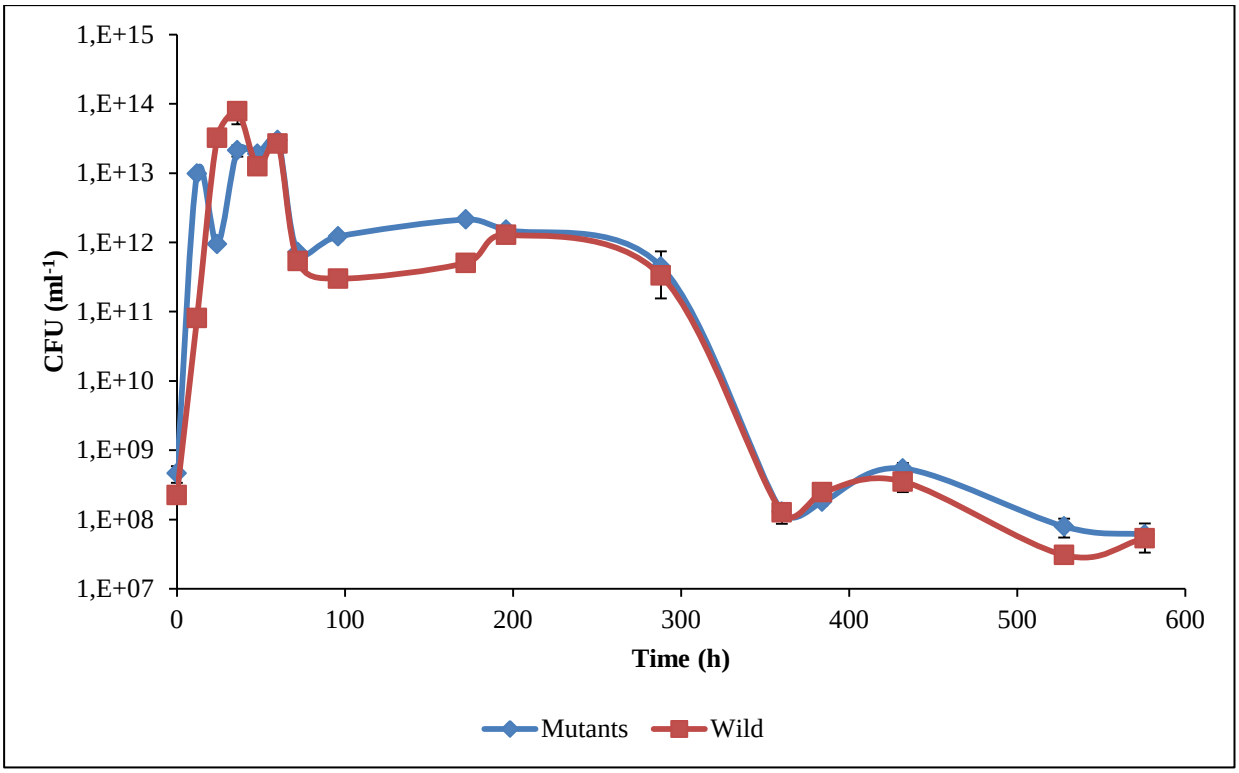


Figure 15: *Viable cell count (CFU) of mutant strains and wild strain during cultivation in mineral medium without carbon source for 576 hours.*

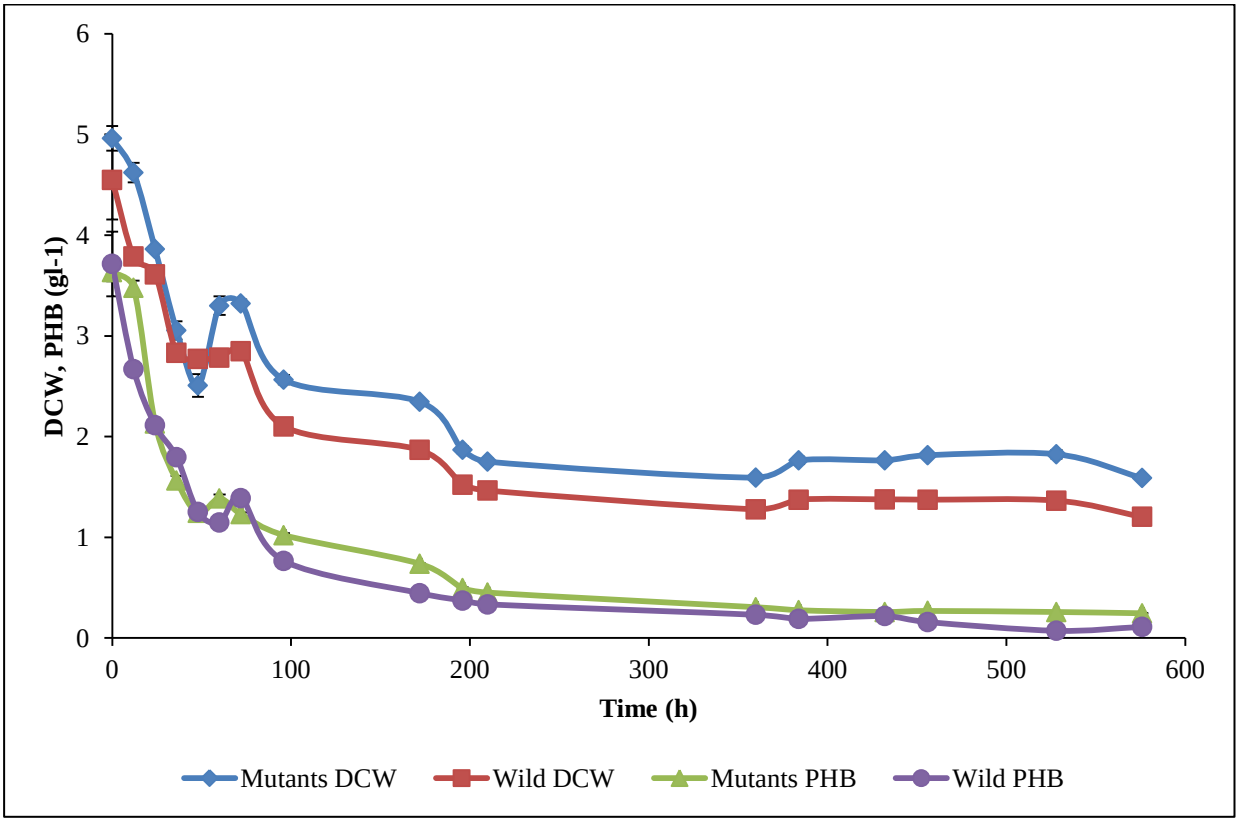


Figure 16: *Dry cell weight and PHB content in cells of mutant strains and wild strain during cultivation in mineral medium without carbon source.*

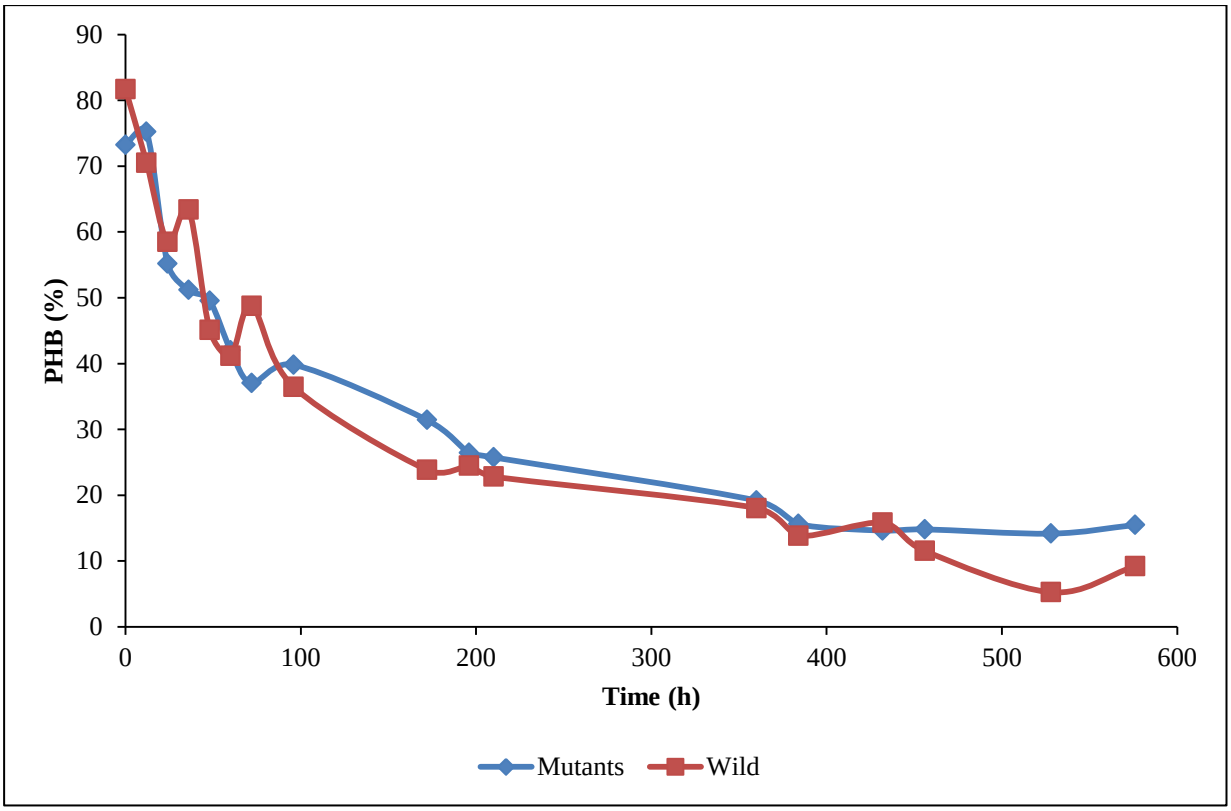


Figure 17: *PHB content in cells of mutant strains and wild strain during cultivation in mineral medium without carbon source*

Number of viable cells of mutant strains and wild strain rose rapidly to the 36th hour of cultivation. This was a short exponential grow phase. The wild and mutant strains then had short stationary phase up to 60th hour of cultivation. Number of viable cells then slowly descended until the end of cultivation. Number of living cells at the 576th hour of cultivation was approximately same as in the beginning of cultivation. Similarly to previous experiment, bacteria first continue to grow on the carbon source residue and when carbon residue is metabolized then bacteria began to die of.

Dry cell weight of mutant strains and wild strain descended rapidly during the first 36 hours of cultivation. The descent after 36th hour of cultivation was not so rapid. The same is true for PHB content in cells. PHB content in cells decreased rapidly during the first 36 hours of cultivation for mutant strains and wild strain. The descent after the 36th hour of cultivation was not so rapid. Bacteria use PHB as a backup carbon source and for this reason PHB percentage in cells declines.

From both experiments it can be seen that there are not significant differences between wild strain and mixture culture of mutant strains. It can be seen that mutant strains behave similarly as a wild strain; therefore, the exposition of mutants to condition of starvation is probably not very effective strategy for selection of highly productive PHA mutants.

Furthermore, the results also showed that due to capability of PHA accumulation bacteria *Cupriavidus necator* can survive long period of starvation. Several mutants were selected from these experiments. Mutants were selected at the 24th or 576th hour of cultivation on Nutrition broth agar after suitable dilution of bacterial suspension. Mutants UA1, UA2, UA3, UA4 and UA5 were selected from the first experiment. Mutants UB1, UB2, UB3, UB4 and UB5 were selected from the second experiment.

4.1.1.2 Chemical mutagenesis

The aim of this experiment was to prepare mutant strains of bacteria *Cupriavidus necator* H16 overproducing PHA by chemical mutagenesis. Two mutagens were used in these experiments: EMS and MMS. Wild strain of *Cupriavidus necator* was cultivated and mutants were selected as described previously (3.2.2.2).

Several experiments involving chemical mutagenesis were performed in order to select mutants overproducing PHA.

In the first series of experiments the wild strain was mutated with EMS (1 $mg \cdot l^{-1}$ and 0,1 $mg \cdot l^{-1}$) and MMS (1 $mg \cdot l^{-1}$ and 0,1 $mg \cdot l^{-1}$). Mutant strains were afterward selected on Nutrition broth agar after suitable dilution of bacterial suspension. Surprisingly, no viable mutant strains were selected from flasks containing MMS as a mutagen. It is likely that selected concentrations of the mutagens were too toxic. Mutant NA1 was selected from flasks containing EMS with concentration 1 $mg \cdot l^{-1}$. Mutants NB1, NB2, NB3, NB4 and NB5 were selected from flasks containing EMS with concentration 0,1 $mg \cdot l^{-1}$.

In the second series of experiments the wild strain was mutated only with EMS (1 $mg \cdot l^{-1}$ and 0,1 $mg \cdot l^{-1}$) and the mutant strains were selected on mineral medium agar with fructose as a carbon source. No viable mutants were selected from flask containing EMS with concentration 1 $mg \cdot l^{-1}$. Mutants FA1, FA2, FA3, FA4, FA5 and FB1 were selected from flasks containing EMS with concentration 0,1 $mg \cdot l^{-1}$.

In the last series of experiment wild strain was mutated with EMS (1 $mg \cdot l^{-1}$ and 0,1 $mg \cdot l^{-1}$) and mutant strains were selected on oil medium agars. Mutants OA2, OA4, OD1, OD2, OD3, OD4 and OD5 were selected from flask containing EMS with concentration 1 $mg \cdot l^{-1}$. Mutants OB2, OC1, OC2, OE1, OE2, OE3, OE4 and OE5 were selected from flasks containing EMS with concentration 0,1 $mg \cdot l^{-1}$.

4.1.2 Screening of mutant strains

Several cultivation experiments were performed to determine whether selected mutant strains were capable of PHA overproduction.

4.1.2.1 Cultivation in mineral media with oil as a carbon substrate

This cultivation was performed with all mutant strains that were selected with UV mutagenesis or chemical mutagenesis. The experiment was performed as described previously (3.2.3.1). Amount of PHA in cells of mutants that didn't exhibited sufficient growth was not measured.

Table 9: *Dry cell weight and PHB content in cells of mutant strains prepared with UV mutagenesis at the 72nd hour of cultivation in mineral medium.*

Mutant	DCW (g·l^{-1})	PHB (g·l^{-1})	PHB (%)
Wild	11,18 ± 0,73	8,05 ± 0,75	72,05 ± 4,75
UA1	0,5 ± 0,04	0,01 ± 0,01	2,84 ± 2,09
UA2	0,57 ± 0,01	0,08 ± 0,00	14,36 ± 0,00
UA3	5,34 ± 0,55	1,65 ± 0,21	30,96 ± 2,31
UA4	0,67 ± 0,03	0,01 ± 0,01	1,78 ± 1,43
UA5	10,11 ± 0,82	7,03 ± 0,65	69,6 ± 3,16
UB1	0,25 ± 0,02	-	-
UB2	0,37 ± 0,02	-	-
UB3	0,25 ± 0,04	-	-
UB4	0,24 ± 0,01	-	-
UB5	0,34 ± 0,03	-	-

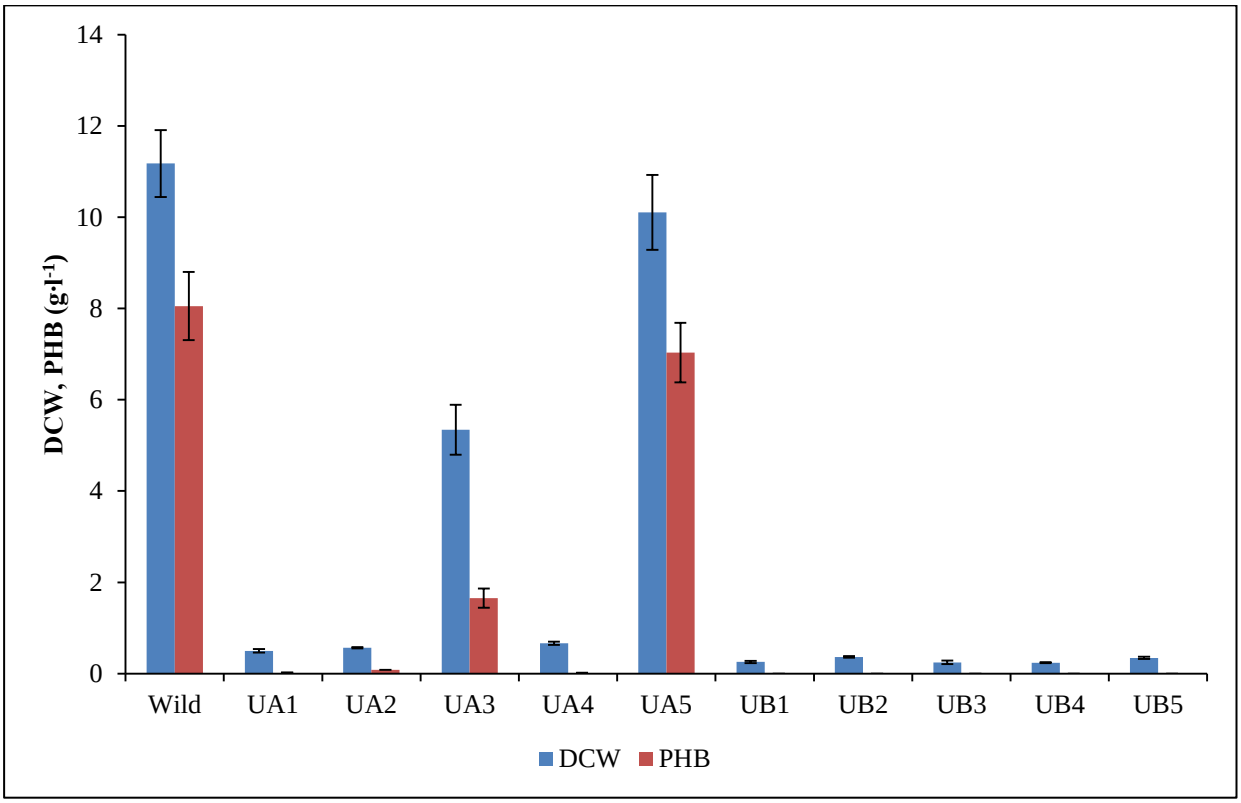


Figure 18: *Dry cell weight and PHB content in cells on mutant strains prepared with UV mutagenesis at the 72nd hour of cultivation in mineral medium.*

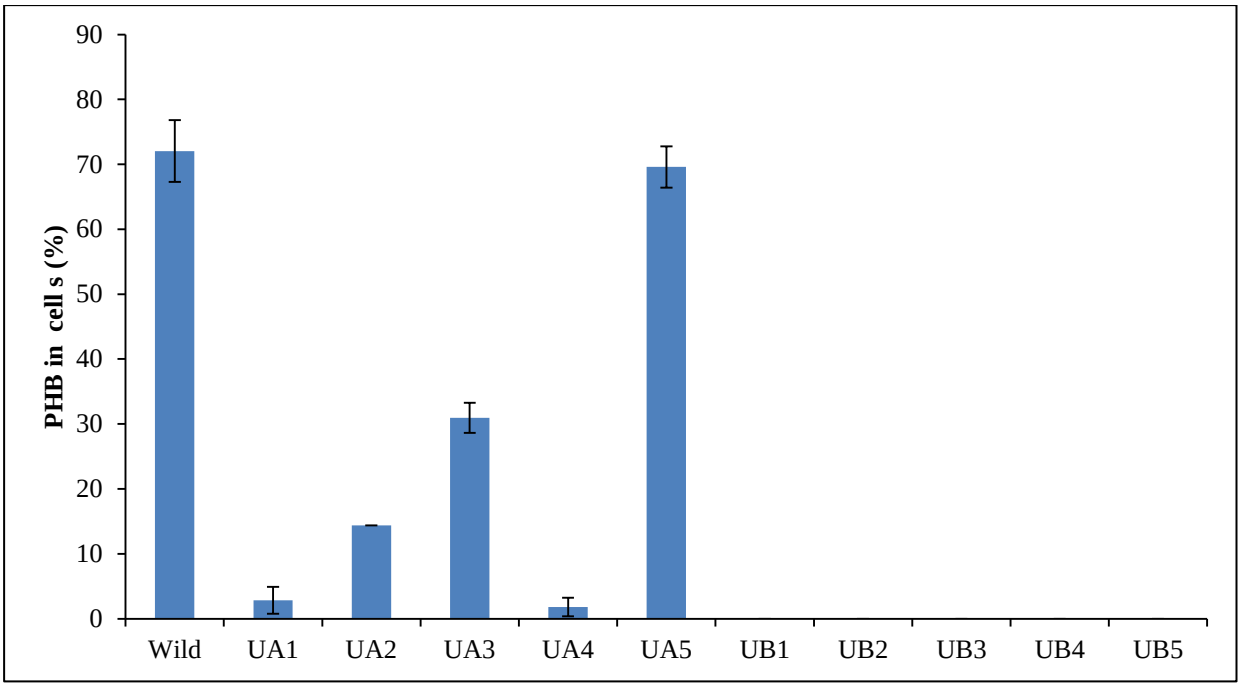


Figure 19: *PHB content in cells of mutant strains prepared with UV mutagenesis at the 72nd hour of cultivation in mineral medium.*

Dry cell weigh of most mutant strains prepared through UV mutagenesis and selected on Nutrition broth at the 72nd hour of cultivation was much smaller than dry cell weight of wild strain. Dry cell weight of mutant UA5 at the 72nd hour of cultivation was comparable to wild strain.

PHB cell content of mutant UA5 at the 72nd hour of cultivation was also comparable with PHB amount in cells of wild strain. It can be concluded that none of mutants prepared by UV-induced mutagenesis could be considered being PHA overproducer as compare to wild strain.

Table 10: *Dry cell weight of mutant strains prepared by chemical mutagenesis and selected on Nutrition broth agar at the 72nd hour of cultivation in mineral medium.*

Mutant	DCW ($g \cdot l^{-1}$)
Wild	8,85 ± 0,06
NA1	0,21 ± 0,00
NB1	2,14 ± 0,06
NB2	1,57 ± 0,01
NB3	1,46 ± 0,06
NB4	1,44 ± 0,01
NB5	1,53 + 0,07

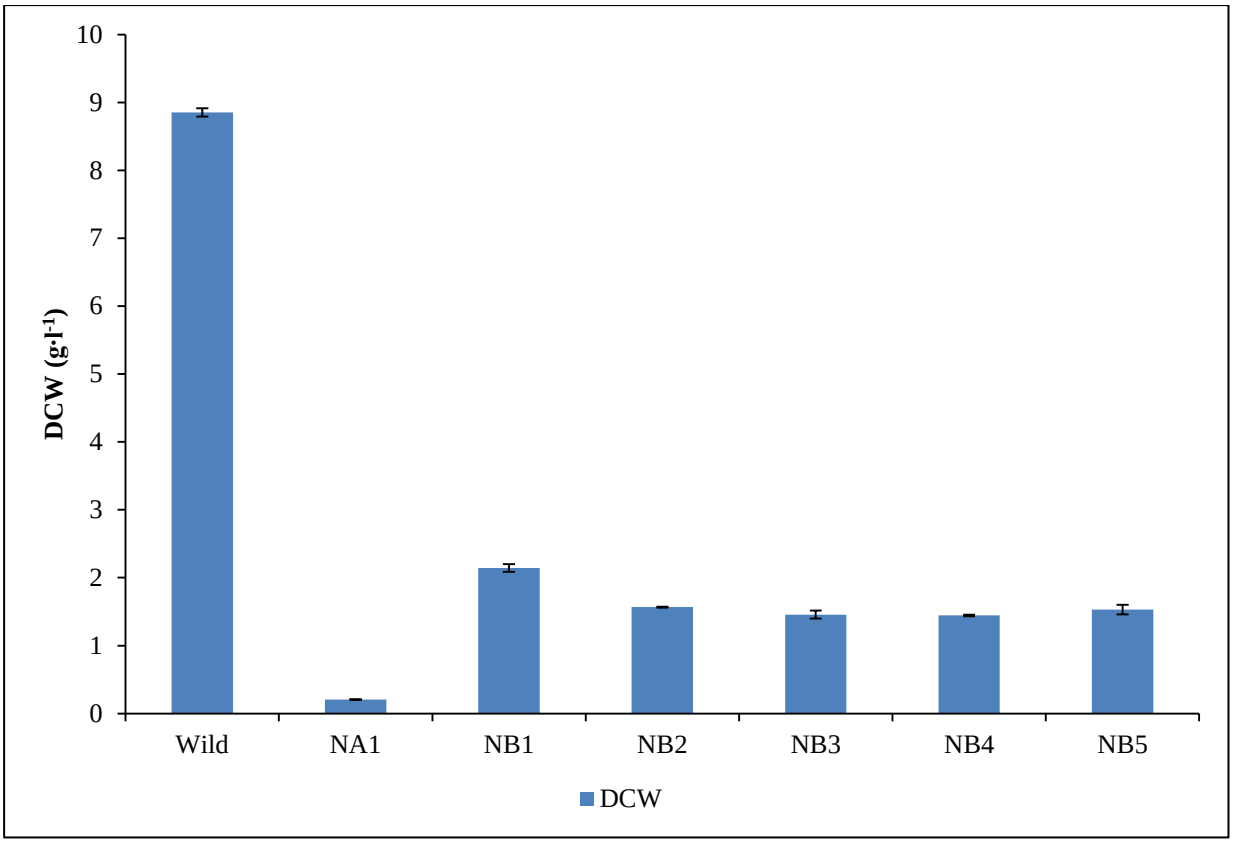


Figure 20: *Dry cell weight of mutant strains prepared by chemical mutagenesis and selected on Nutrition broth agar at the 72nd hour of cultivation in mineral medium.*

Dry cell weight of all mutants prepared through chemical mutagenesis and selected on Nutrition broth agar at the 72nd hour of cultivation was much smaller compared to dry cell weight of wild strain at the 72nd hour of cultivation; therefore, PHA content in cells of these mutants was not measured. It can be concluded that none mutant strains prepared by chemical mutagenesis and selected on Nutrition broth medium could be considered being PHA overproducer as compare to the wild strain.

Table 11: *Dry cell weight and PHB content in cells of mutant strains prepared by chemical mutagenesis and selected on mineral medium agar with fructose as carbon source at the 72nd hour of cultivation in mineral medium.*

Mutant	DCW (g·l^{-1})	PHB (g·l^{-1})	PHB (%)
Wild	7,86 ± 0,39	4,90 ± 0,06	62,31 ± 0,73
FA1	0,21 ± 0,01	-	-
FA2	0,17 ± 0,04	-	-
FA3	0,23 ± 0,02	-	-
FA4	0,26 ± 0,01	-	-
FA5	0,11 ± 0,01	-	-
FB1	8,43 ± 0,61	5,00 ± 0,03	62,58 ± 0,36

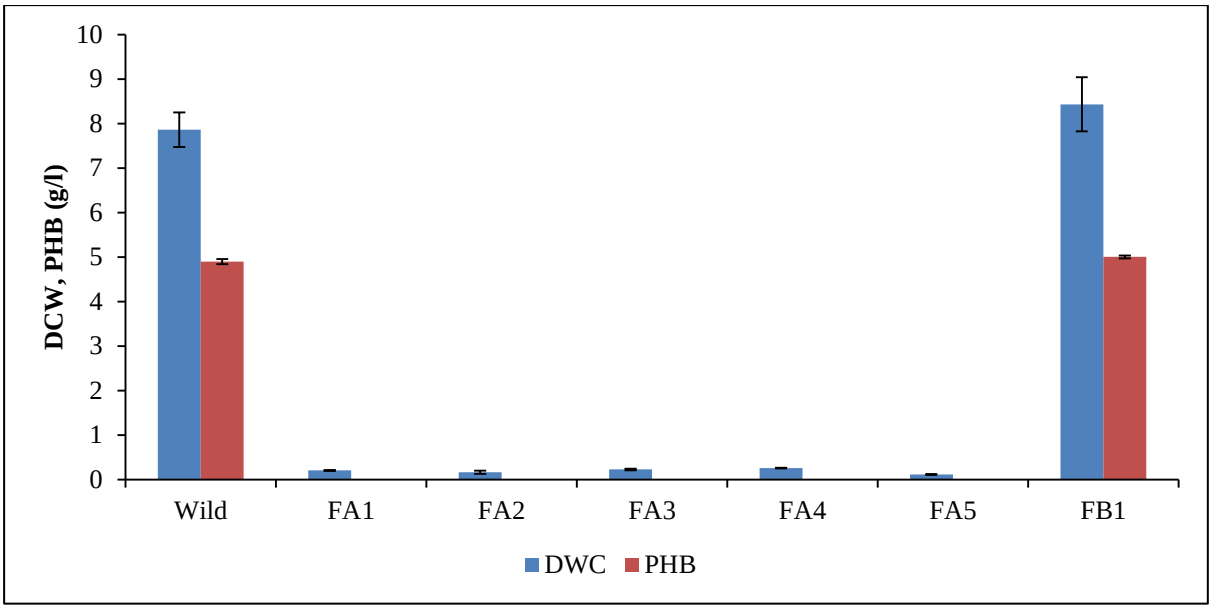


Figure 21: *Dry cell weight and PHB content in cells of mutant strains prepared by chemical mutagenesis and selected on mineral medium agar with fructose as carbon source at the 72nd hour of cultivation in mineral medium.*

Dry cell weight of most mutants prepared through chemical mutagenesis and selected on mineral medium with fructose as a carbon source at the 72nd hour of cultivation was much smaller compared to dry cell weight of wild strain at the 72nd hour of cultivation. Dry cell weight of mutant FB1 at the 72nd hour of cultivation was comparable to dry cell weigh of wild strain at the 72nd hour of cultivation. PHB amount in cells of mutant FB1 at the 72nd hour of cultivation was comparable to PHB amount in cells of wild strain.

Table 12: *Dry cell weight and PHB content in cells of mutant strains prepared by chemical mutagenesis and selected on oil medium agar at the 72nd hour of cultivation in mineral medium.*

Mutant	DCW ($g \cdot l^{-1}$)	PHB ($g \cdot l^{-1}$)	PHB (%)
Wild	7,86 ± 0,39	4,90 ± 0,25	62,31 ± 0,73
OA2	7,58 ± 0,95	5,89 ± 0,77	77,72 ± 2,81
OA4	7,87 ± 0,57	4,56 ± 0,33	57,91 ± 0,01
OD1	9,35 ± 0,94	7,11 ± 0,80	76,03 ± 3,98
OD2	9,67 ± 1,87	7,25 ± 1,69	75,05 ± 9,80
OD3	8,43 ± 0,60	4,79 ± 0,96	56,82 ± 10,61
OD4	8,31 ± 0,41	5,44 ± 0,27	65,45 ± 0,19
OD5	8,64 ± 1,35	5,92 ± 1,03	68,48 ± 5,21
OB2	8,48 ± 0,29	5,53 ± 0,19	65,22 ± 0,10
OC1	7,49 ± 0,62	4,85 ± 0,40	64,76 ± 0,04
OC2	8,75 ± 0,48	5,54 ± 0,30	63,37 ± 0,18
OE1	8,62 ± 0,64	7,57 ± 0,96	87,85 ± 9,05
OE2	7,99 ± 0,78	6,61 ± 0,88	82,72 ± 7,50
OE3	8,34 ± 1,08	6,59 ± 0,92	79,03 ± 4,17
OE4	5,49 ± 0,64	3,43 ± 0,66	62,47 ± 9,65
OE5	8,62 ± 0,29	6,81 ± 0,34	78,95 ± 2,89

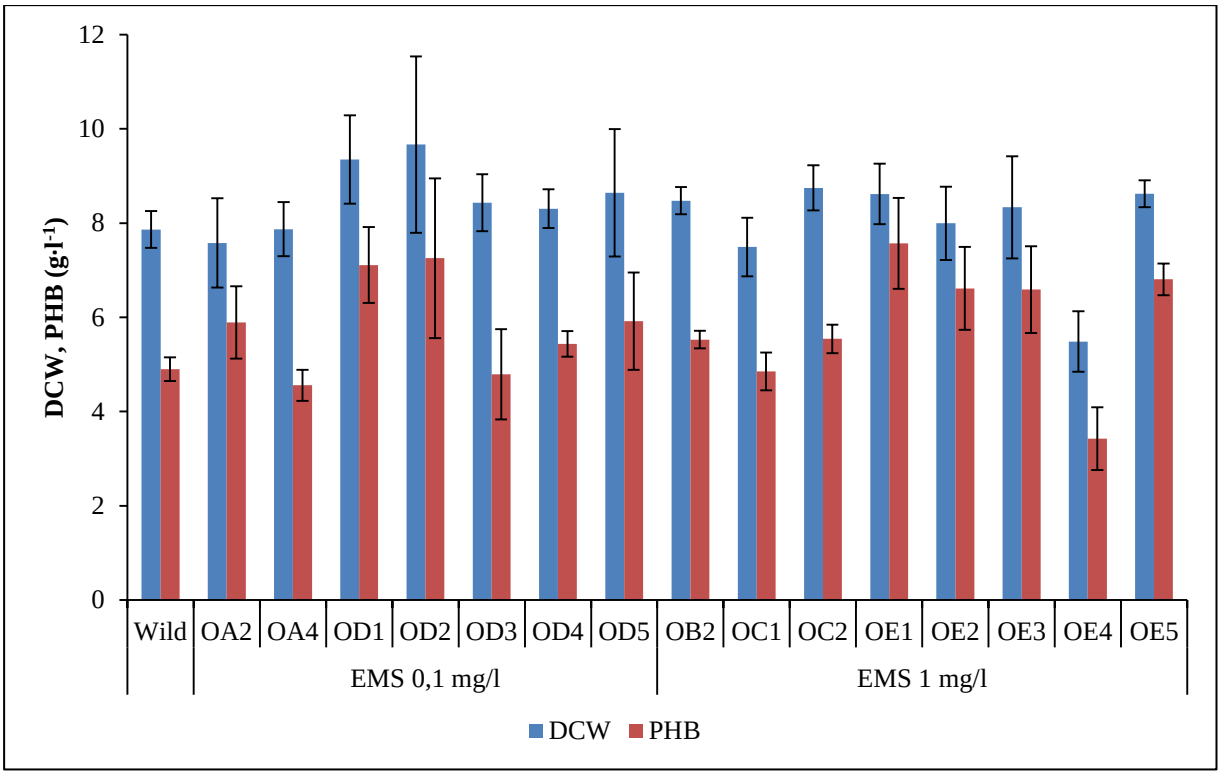


Figure 22: *Dry cell weight and PHB content in mutant strains (chemical mutagenesis, selection on oil medium agar) after 72 hour cultivation in mineral medium.*

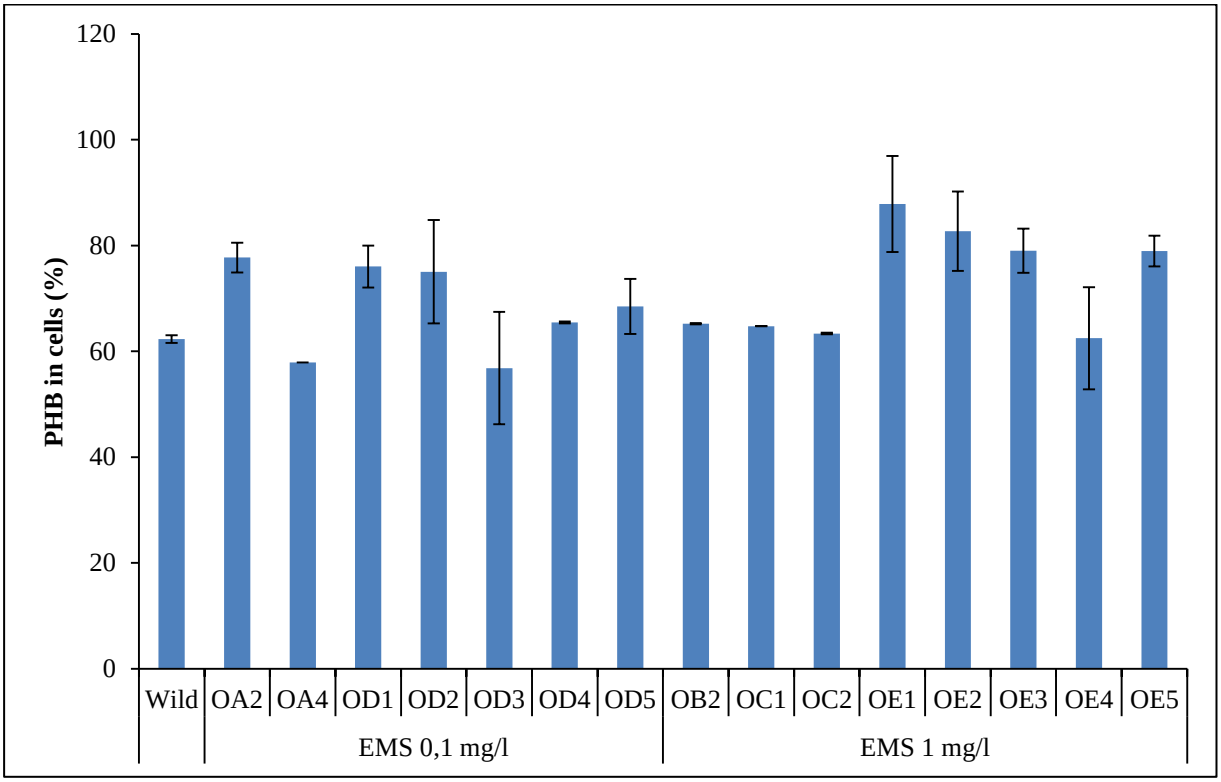


Figure 23: *PHB content in mutant strains (chemical mutagenesis, selection on oil medium agar) after 72 hour cultivation in mineral medium.*

Mutant strains in this experiment were prepared through chemical mutagenesis with EMS (concentration 0,1 and 1 $mg \cdot l^{-1}$) and they were selected on oil medium agar. Dry cell weight of mutant strains OA2, OA4, OD3, OD4, OD5, OB2, OC1, OC2, OE1, OE2, OE3 and OE5 at the 72^{nd} hour of cultivation was comparable to dry cell weight of wild strain at the 72^{nd} hour of cultivation. Dry cell weight of mutant strains OD1 and OD2 at the 72^{nd} hour of cultivation was higher than dry cell weight of wild strain at the 72^{nd} hour of cultivation. Dry cell weight of mutant strain OE4 at the 72^{nd} hour of cultivation was lower than dry cell weight of wild strain at the 72^{nd} hour of cultivation.

PHB content in cells of mutants OA4, OD3, OD4, OD5, OB2, OC1, OC2 and OE4 at the 72^{nd} hour of cultivation was comparable to PHB content in cells of wild strain at the 72^{nd} hour of cultivation. PHB content in cells of mutant strains OA2, OD1, OD2, OE1, OE2, OE3 and OE5 at the 72^{nd} hour of cultivation was higher than PHB content in cells of wild strain. No mutant strain exhibited lower PHB content in cells than wild strain.

Generally, agar plates used for mutant strain selection were determining whether selected mutants will be able to compete with wild strain during cultivation in mineral medium with oil as a carbon source.

Nutrition broth agar plate didn't prove to be good for selection of mutant strains. Only one mutant out of 16 mutants exhibited growth comparable to wild strain. Growth of other 15 mutant strains was much lower than wild strain when cultivated in the oil-base medium. Wild strain is able to synthesize all important growth factor from simple carbon source. Nutrition broth is complex medium that allows growth of mutant strains that have lost ability to synthesize all growth factors from simple carbon source. These mutant strains can supplement growth factors from Nutrition broth media but they cannot grow in mineral medium without growth factors and with simple carbon source. Other possible reason for unsuccessful mutants selected on Nutrition broth agar is that mutant strains lost ability to metabolize oil. Part of metabolism involved in catabolism of oil was probably damaged.

Mineral medium agar plates with fructose as a carbon source were used for mutant strains selection in the next series of experiments. Only one mutant out of 6 mutants exhibited growth comparable to wild strain. Growth of other mutant strains was lower than wild strain. Mineral medium is a simple medium without growth factors. Mutant strains selected from this medium didn't lose ability to synthesize all growth factors. On the other hand it is likely that most of mutant strains lost ability to metabolize oil.

Selection of mutant strains on mineral medium with fructose wasn't successful. Thus, in the next series of experiments mineral medium agar plates with oil as carbon source (Oil medium) were used for mutant selection. The preparation of solid media containing oil as a carbon source involved necessary emulsification of oil, moreover, the emulsion must have been stable even during and after sterilization. To reach this goal, we employed probe ultrasonic homogenizator which created emulsion of desired properties. To our knowledge, there are no other reports on preparation of agar plates containing emulsified oil as a sole carbon substrate. In total, 15 mutants were selected with this method. Only one mutant out of 15 mutants exhibited lower growth than wild strain. Two mutant strains exhibited distinctively higher growth than wild strain and 12 mutants exhibited comparable growth to wild strain. PHB amount in cells in percentages of 7 mutants was distinctively higher than wild strain. Selection of mutant strains on Oil medium proved to be the most successful method of mutant strains selection. Choice of medium favored selection of mutant strains able to grow in simple mineral medium and they were able to metabolize oil as well.

4.1.2.2 Cultivation on mineral media with yeast extract

Growth of some mutants on mineral medium with oil was not sufficient. Cultivation on mineral medium with addition of yeast extract was performed to see if addition of complex carbon and growth factors source to medium could enhance growth of these mutants. Experiment was performed as described previously (3.2.3.2)

Table 13: *Dry cell weight of mutant strains at the 72nd hour of cultivation in mineral medium with yeast extract.*

Mutant	DCW ($g \cdot l^{-1}$)
Wild	6,89 ± 0,14
UB1	0,06 ± 0,01
UB2	0,08 ± 0,00
UB3	0,03 ± 0,01
UB4	0,05 ± 0,01
UB5	0,12 ± 0,01
NA1	0,61 ± 0,01
NB1	0,25 ± 0,01
NB2	0,09 ± 0,00
NB3	0,12 ± 0,01
NB4	0,07 ± 0,01
NB5	0,09 ± 0,01

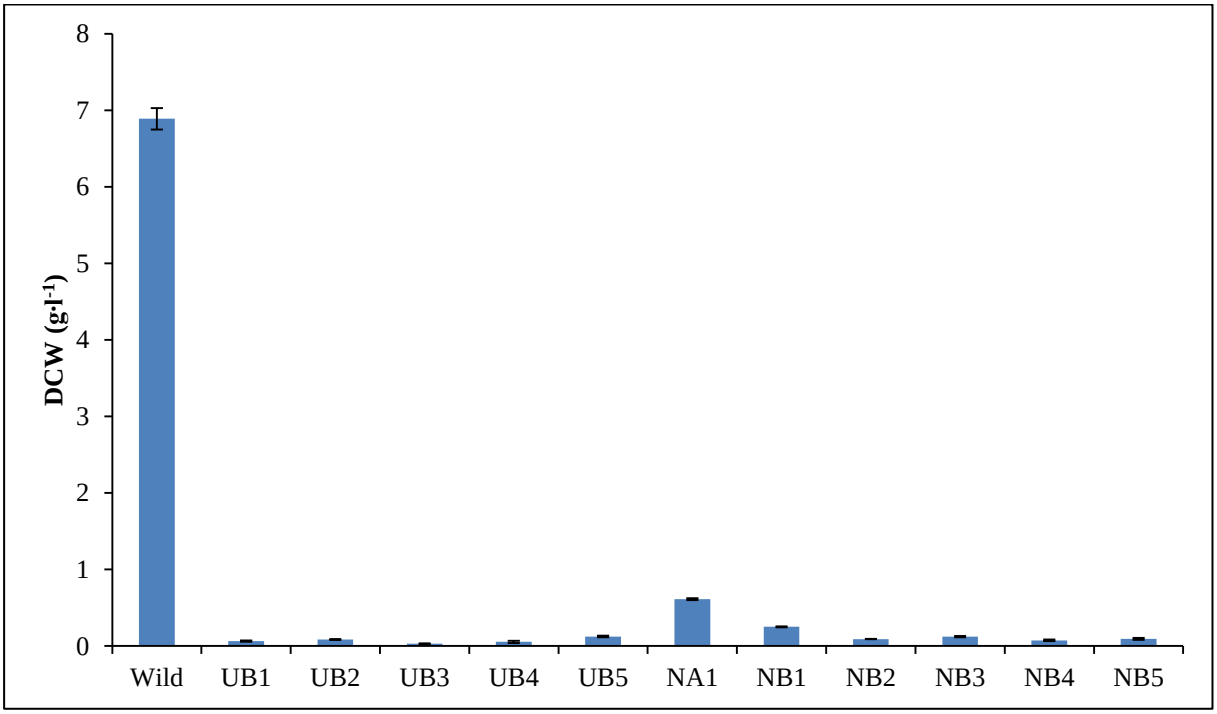


Figure 24: *Dry cell weight of mutant strains at the 72nd hour of cultivation in mineral medium with yeast extract.*

Dry cell weight of all mutant strains in mineral medium with oil as a carbon source and with addition of yeast extract at the 72nd hour of cultivation was much lower than dry cell weight of wild strain. PHB content in cells was not measured due to insufficient growth of mutant strains. Addition of yeast extract to mineral media with oil and yeast extract didn't lead to enhancement of growth of the mutant strains.

4.1.2.3 Cultivation in mineral media with propanol

As was described previously [21], propanol addition to medium at the 24th hour of cultivation causes formation of copolymer P(3HB-*co*-3HV) in cells. This effect is desirable, because the copolymer possesses better mechanical properties as compare to PHB homopolymer. Hence, goal of this experiment was to see how much copolymer mutant strains form and what is the composition of it. Several mutant strains with sufficient growth were selected for this experiment. Experiment was performed as described previously (3.2.3.3).

Table 14: *Dry cell weight, PHA content in cells and PHA composition of mutant strains at the 72nd hour of cultivation on mineral medium with propanol.*

Mutant	DCW (g·l^{-1})	PHA (g·l^{-1})	PHA (%)	3HB (%)	3HV (%)
Wild	6,19 ± 0,16	4,51 ± 0,25	72,77 ± 3,54	89,38 ± 5,44	10,62 ± 0,59
OA4	6,45 ± 0,50	4,33 ± 0,34	67,17 ± 0,86	91,65 ± 1,43	8,35 ± 0,13
OD1	6,05 ± 0,72	4,46 ± 0,54	73,72 ± 1,48	88,72 ± 2,22	11,28 ± 0,30
OD2	5,84 ± 0,69	4,08 ± 0,57	69,90 ± 5,32	88,15 ± 8,25	11,85 ± 1,14
OD3	6,26 ± 0,94	4,50 ± 0,68	71,90 ± 0,37	88,64 ± 0,55	11,36 ± 0,11
OD4	5,33 ± 0,17	3,86 ± 0,15	72,40 ± 1,52	89,39 ± 2,08	10,61 ± 0,74
OD5	4,78 ± 0,06	3,76 ± 0,05	78,67 ± 0,10	89,35 ± 0,15	10,65 ± 0,03
FB1	6,51 ± 0,97	4,55 ± 0,70	69,99 ± 2,67	90,26 ± 3,58	9,74 ± 1,54
OB2	6,05 ± 0,41	4,10 ± 0,32	67,86 ± 2,84	87,98 ± 4,36	12,02 ± 0,96
OC1	5,70 ± 0,90	4,23 ± 0,72	74,11 ± 4,60	87,85 ± 6,86	12,15 ± 0,89
OC2	5,78 ± 1,15	3,81 ± 0,76	65,90 ± 1,48	89,17 ± 2,34	10,83 ± 0,51
OE1	4,38 ± 0,13	3,18 ± 0,09	72,74 ± 0,19	89,86 ± 0,29	10,14 ± 0,03
OE2	5,77 ± 0,99	4,42 ± 0,77	76,59 ± 2,35	87,91 ± 3,26	12,09 ± 0,83
OE3	5,32 ± 0,80	4,07 ± 0,77	76,47 ± 8,76	89,05 ± 12,98	10,95 ± 1,42
OE4	6,08 ± 0,88	4,16 ± 0,60	68,40 ± 1,14	88,79 ± 1,80	11,21 ± 0,22
OE5	5,60 ± 0,76	4,13 ± 0,60	73,84 ± 3,61	89,82 ± 5,52	10,18 ± 0,51

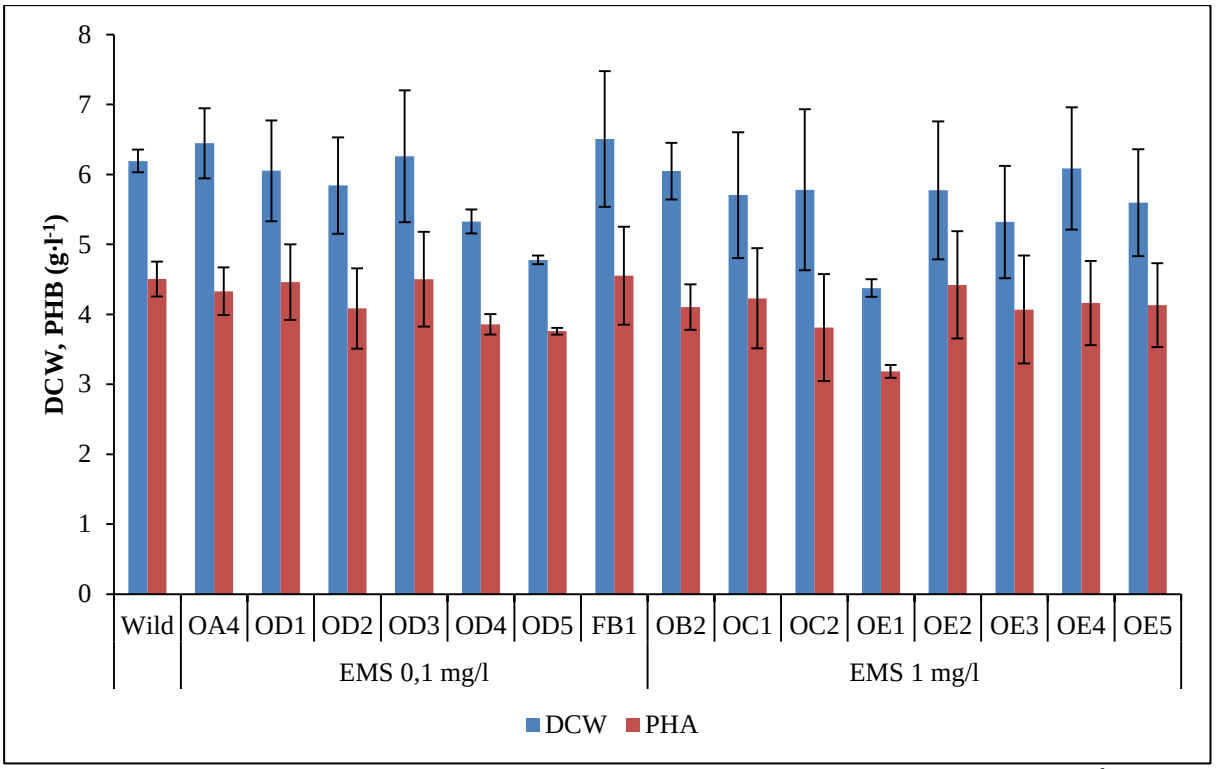


Figure 25: *Dry cell weight and PHA content in cells of mutant strains at the 72nd hour of cultivation in mineral medium with propanol.*

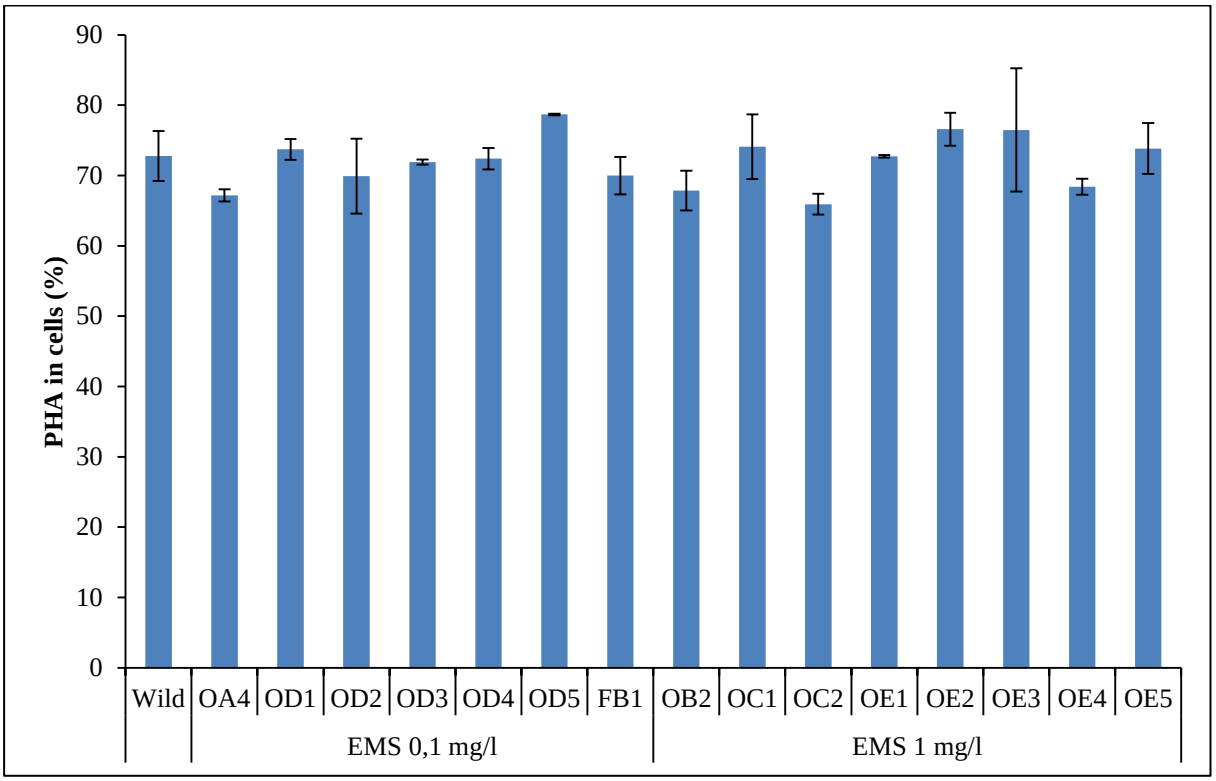


Figure 26: *PHA content in cells of mutant strains at the 72nd hour of cultivation in mineral medium with propanol.*

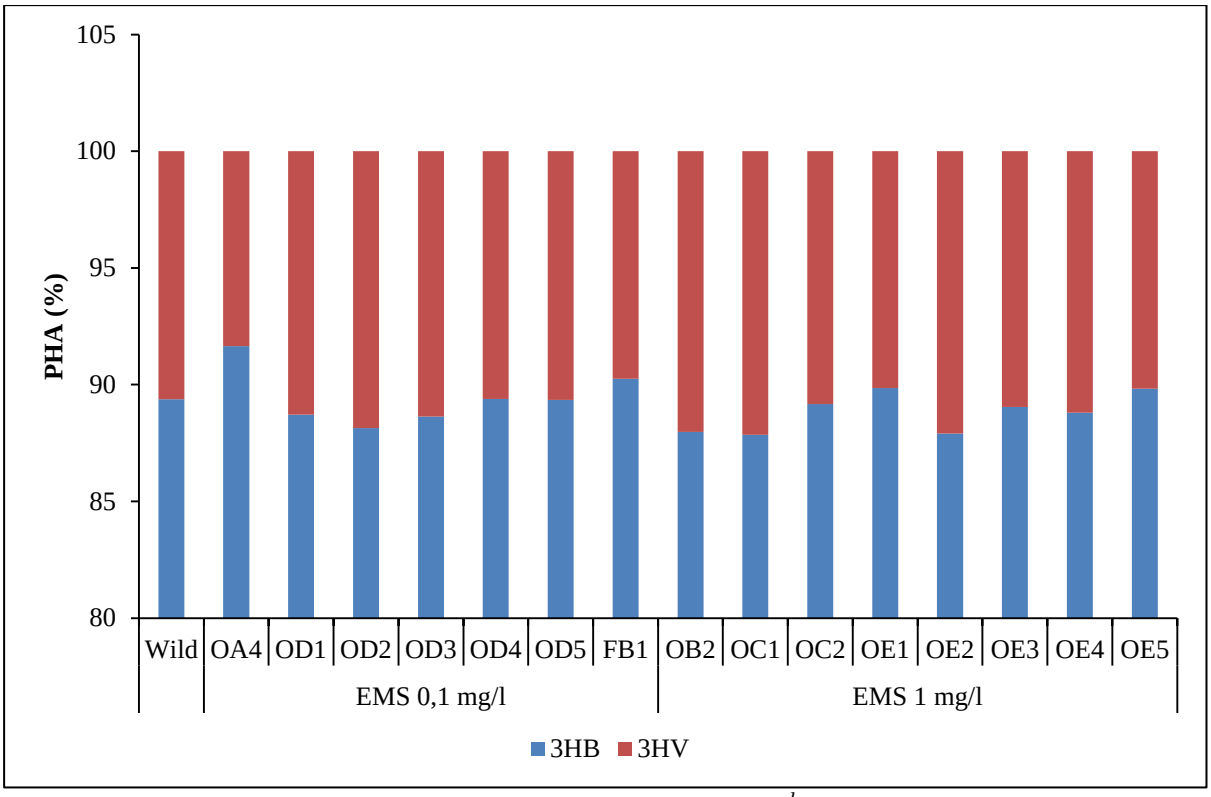


Figure 27: *PHA composition of mutant strains at the 72nd hour of cultivation in mineral medium with propanol.*

Dry cell weight of most mutant strains in mineral medium with propanol at the 72nd hour of cultivation was comparable to dry cell weight of wild strain at the 72nd hour of cultivation. Dry cell weight of mutant stains OD4, OD5, OE1 and OE3 at the 72nd hour of cultivation was significantly smaller than dry cell weight of wild strain. Dry cell weight of none selected mutant at the 72nd hour of cultivation was higher than dry cell weight of wild strain at the 72nd hour of cultivation.

PHA content in cells of mutant strains OD1, OD2, OD3, OD4, FB1, OC1, OE1, OE3 and OE5 at the 72nd hour of cultivation was comparable to PHA content in cells of wild strain at the 72nd hour of cultivation. PHA content in cells of mutant strains OA4, OB2 and OC2 at the 72nd hour of cultivation was lower than PHA content in cells of wild strain at the 72nd hour of cultivation. PHA content in cells of mutant strains OD5 and OE2 at the 72nd hour of cultivation was higher than PHA content in cells of wild strain at the 72nd hour of cultivation.

3HV content in polymer of mutant strains OD4, OC2, OE1, OE3 and OE4 at the 72nd hour of cultivation was comparable to 3HV content in polymer of wild strain at the 72nd hour of cultivation. 3HV content in polymer of mutant strains OD1, OD2, OD3, OB2, OC1 and OE2 at the 72nd hour of cultivation was higher than 3HV content in polymer of wild strain at the 72nd hour of cultivation. 3HV content in polymer of mutant strains OA4, FB1 and OE5 at the 72nd hour of cultivation was lower than 3HV content in polymer of wild strain at the 72nd hour of cultivation.

Mutant strains cultivated in mineral medium with propanol didn't exhibited sufficient growth compared to growth of these mutants on mineral medium without propanol.

4.1.2.4 Statistical analysis of mutant strains

In order to select the most successful mutant strains, statistical analysis was performed. Data used in this analysis were selected from cultivation of mutant strains in mineral medium (3.2.3.1) and cultivation of mutant strains in mineral medium with propanol (3.2.3.3). Student's unpaired t-test was used to compare results of mutant strains with results of wild strain. DCW, PHB and PHV content in cells of individual mutants was tested. Null hypothesis was set as dry cell weight, PHB content in cells or PHV content in cells of individual mutant being statistically same as corresponding value of wild strain. Probability of null hypothesis was calculated with function TTEST in Microsoft Excel for dry cell weight, PHB content in cells and PHV content in cells of each mutant strain. If probability of null hypothesis was lower than 0,05 null hypothesis was rejected. Mutant strains with null hypothesis rejected for most values and with DWC, PHB and PHV content values higher than wild strain were selected for further study.

Table 15: *Statistical probability of null hypothesis* for dry cell weight, PHB amount in cells and PHV amount in cells of mutant strains.*

Mutant	Mineral medium		Mineral medium with propanol		
	DCW ($g \cdot l^{-1}$)	PHB (%)	DCW ($g \cdot l^{-1}$)	PHB (%)	PHV (%)
Wild	0	0	0	0	0
OA2	0,547	0,108	-	-	-
OA4	0,983	0,002	0,332	0,161	0,001
OD1	0,015	0,008	0,724	0,866	0,032
OD2	0,084	0,109	0,447	0,352	0,270
OD3	0,313	0,437	0,895	0,539	0,072
OD4	0,083	0,004	0,000	0,878	0,920
OD5	0,260	0,131	0,000	0,068	0,028
FB1	0,100	0,652	0,558	0,403	0,371
OB2	0,007	0,005	0,501	0,065	0,446
OC1	0,266	0,010	0,345	0,982	0,010
OC2	0,016	0,085	0,518	0,038	0,114
OE1	0,048	0,016	0,000	0,874	0,122
OE2	0,738	0,018	0,392	0,339	0,030
OE3	0,386	0,005	0,155	0,574	0,201
OE4	0,000	0,979	0,848	0,102	0,739
OE5	0,002	0,001	0,196	0,649	0,300

*Null hypothesis: Values for dry cell weight, PHB amount in cells and PHV amount in cells of mutant strain are statistically same as corresponding values of wild strain
Blue: Null hypothesis rejected with values statistically significantly higher than wild strain
Red: Null hypothesis rejected with values statistically significantly lower than wild strain.

Mutants OD1, OB2, OE1 and OE5 exhibited statistically higher growth and higher PHB content than wild strain during cultivation on mineral medium. Mutant OC2 exhibited statistically higher growth than wild strain and mutant strains OA4, OD4, OC1, OE2 and OE3 exhibited higher PHB content in cells during cultivation in mineral medium.

Mutants OD1, OC1 and OE3 exhibited higher 3HV content that wild strain during cultivation in mineral medium with propanol. No mutant exhibited higher growth than wild strain during cultivation in mineral medium with propanol.

Based on these results, we selected mutants OC1, OD1, OE1 and OE5 for further experiments. Mutants OD1, OE1 and OE5 showed significantly higher PHB production and growth properties as compared to wild strain. The mutant OC1 was selected for its improved ability to incorporate relatively high amount of 3HV into PHA structure. Study of activity of intracellular enzymes of these mutants was performed.

4.2 Biochemical characterization of mutant strains

Several experiments were performed in order to identify why the mutants were able to reach higher PHA yields as compare to wild strain. Activity of extracellular secretion and activity of intracellular enzymes of mutant strains were measured. Resistance of mutant strains against oxidative stress was measured as well. SDS-PAGE electrophoresis of intracellular extract was performed. Mutant's polymer was analyzed by GPC.

4.2.1 Activity of extracellular secretion

Activity of extracellular secretion was measured as described previously (3.3.2). Mutant strains were cultivated in mineral medium (3.2.3.1) and mineral medium with propanol (3.2.3.3).

Table 16: *Total secretion concentration, activity and specific activity of mutant strains at the 72nd hour of cultivation in mineral medium*

Mutant	Total concetration ($mg \cdot l^{-1}$)	Activity ($U \cdot ml^{-1}$)	Specific activity ($U \cdot mg^{-1}$)
Wild	0,357 ± 0,023	9,26 ± 1,19	25,94 ± 3,74
OC1	0,410 ± 0,029	0,96 ± 1,54	2,35 ± 3,76
OD1	0,274 ± 0,019	6,30 ± 0,77	23,01 ± 3,24
OE1	0,286 ± 0,012	15,43 ± 4,59	53,91 ± 16,19
OE5	0,281 ± 0,024	7,84 ± 0,18	27,88 ± 2,44

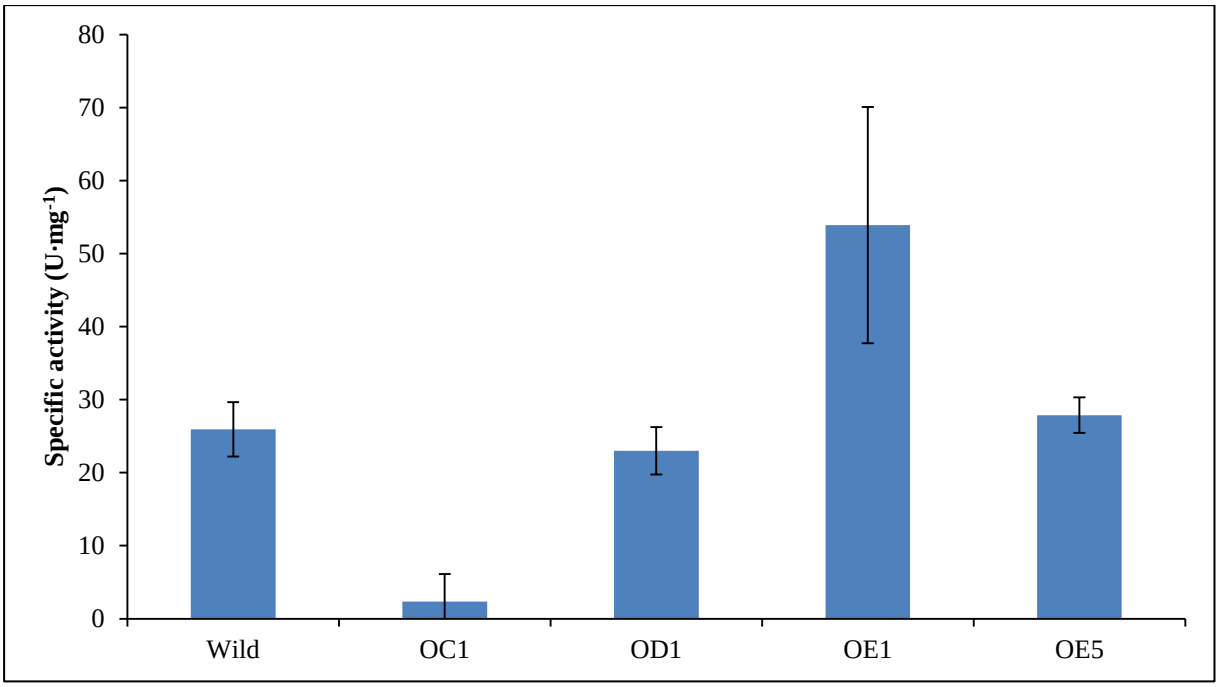


Figure 28: *Specific activity of extracellular secretion of mutant strains at the 72nd hour of cultivation in mineral medium*

Specific activity of extracellular secretion of mutant strains OD1 and OE5 at the 72nd hour of cultivation was comparable to specific activity of extracellular secretion of wild strain at the 72nd hour of cultivation. Specific activity of extracellular secretion of mutant strain OC1 at the 72nd hour of cultivation was lower than specific activity of extracellular secretion of wild strain. Specific activity of extracellular secretion of mutant strain OE1 at the 72nd hour of cultivation was higher than specific activity of extracellular secretion of wild strain.

From the comparison of specific activity of extracellular secretion of mutant strains at the 72nd hour of cultivation with PHB content in cells of these mutants at the 72nd hour of cultivation it can be seen that total specific activity of extracellular secretion is not dependent on PHB content.

Table 17: *Total secretion concentration, activity and specific activity of mutant strains at the 72nd hour of cultivation in mineral medium with propanol*

Mutant	Total concentration ($mg \cdot l^{-1}$)	Activity ($U \cdot ml^{-1}$)	Specific activity ($U \cdot mg^{-1}$)
Wild	0,243 ± 0,022	17,84 ± 3,23	73,32 ± 14,86
OC1	0,294 ± 0,034	7,25 ± 0,93	24,69 ± 4,26
OD1	0,334 ± 0,002	9,83 ± 1,90	29,44 ± 5,69
OE1	0,230 ± 0,022	21,91 ± 1,75	95,32 ± 11,86
OE5	0,341 ± 0,070	12,44 ± 1,45	36,49 ± 8,66

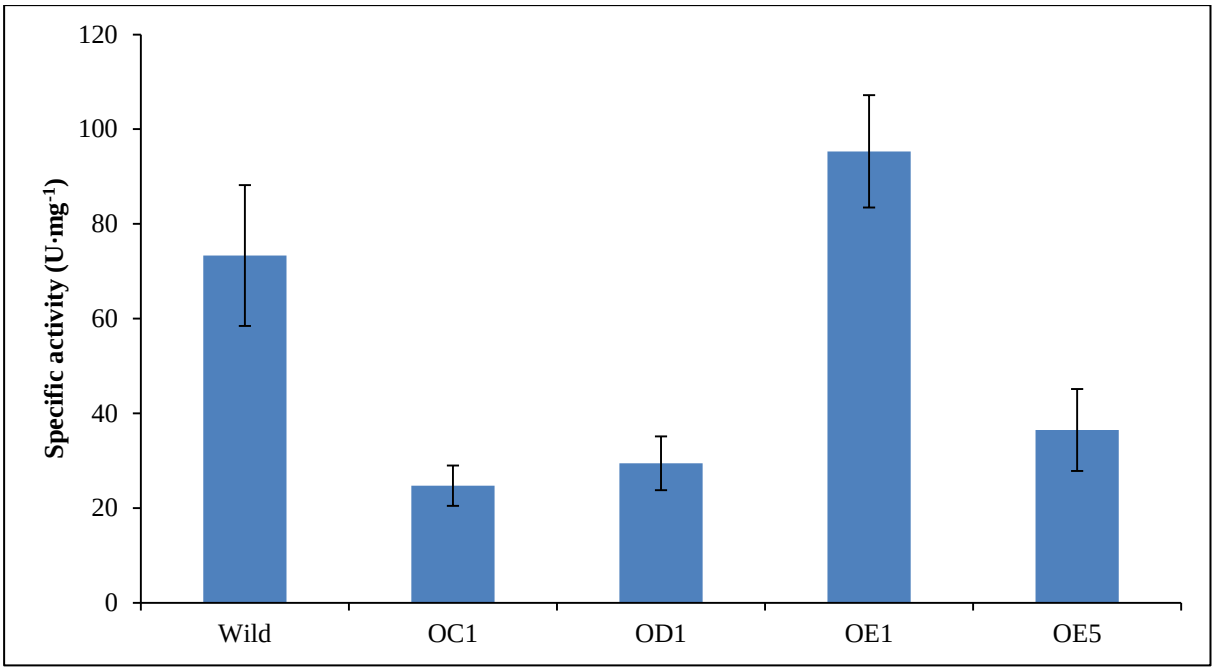


Figure 29: *Specific activity of extracellular secretion of mutant strains at the 72nd hour of cultivation in mineral medium with propanol*

Specific activity of extracellular secretion of mutant strains OC1 OD1 and OE5 at the 72nd hour of cultivation was lower than specific activity of extracellular secretion of wild strain. Specific activity of extracellular secretion of mutant strain OE1 at the 72nd hour of cultivation was higher than specific activity of extracellular secretion of wild strain.

From the comparison of specific activity of extracellular secretion of mutant strains at the 72nd hour of cultivation with PHA content in cells of these mutants at the 72nd hour of cultivation it can be seen that total specific activity of extracellular secretion is not dependent on PHA content.

4.2.2 SDS-PAGE electrophoresis

Intracellular extract contains many proteins of different molecular weights. SDS-PAGE electrophoresis of intracellular extract of mutant strains was performed to separate these proteins according to their sizes and to see differences in protein composition between mutant strains. Electrophoresis was performed as describe previously (3.5.1.3). SERVA Recombinant SDS PAGE Protein Marker was used as a protein standard. Gel was stained with Coomassie blue and analyzed with Scion image (3.5.1.4).

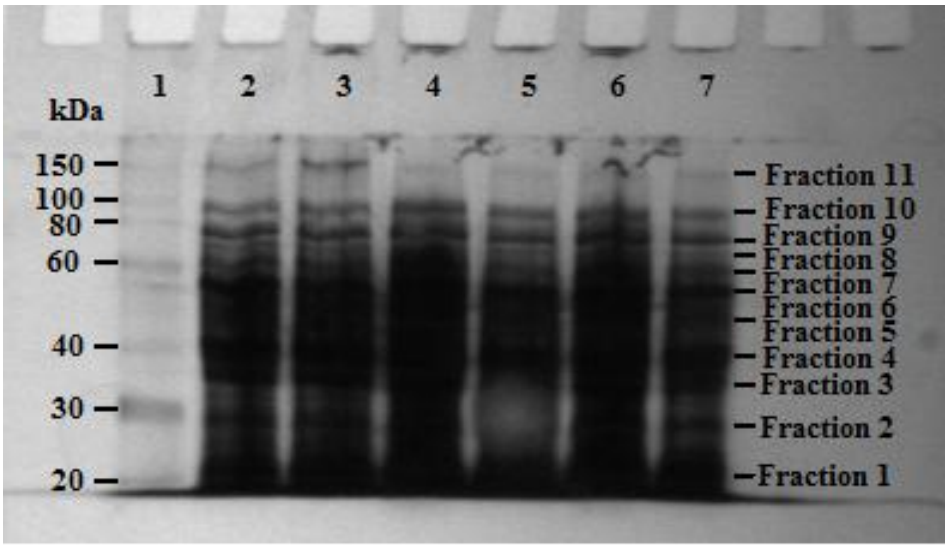


Figure 30: *SDS-PAGE electrophoresis of intracellular extract of mutant strains*

Line	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Sample	Standard	Wild	OE1	Wild	OD1	OE1	OE5

Table 18: *Main protein fractions of intracellular extract of mutant strains*

Bend	**Molar mass (Da·10^3)**
Protein fraction 1	19,42
Protein fraction 2	26,86
Protein fraction 3	34,00
Protein fraction 4	40,58
Protein fraction 5	49,88
Protein fraction 6	60,42
Protein fraction 7	68,99
Protein fraction 8	76,48
Protein fraction 9	83,56
Protein fraction 10	96,82
Protein fraction 11	124,39

Intracellular extract of mutant strains and wild strain contains many proteins that were separated by SDS-PAGE electrophoresis. Surprisingly protein composition of mutant strains and wild strain didn't differ much from each other. 11 protein fractions of different molar masses were identified in intracellular extract of mutant strains and wild strain. Some protein fractions were very distinctive and some were almost indistinctive.

Protein fractions with molar masses 19,42 kDa; 34,00 kDa; 40,58 kDa; 60,42 kDa; 83,56 kDa and 96,82 kDa were distinctive in both wild strain and mutant strains. Protein fractions with molar mass 26,86 kDa; 49,88 kDa 68,99 kDa; 76,48 kDa and 124,39 kDa were less distinctive in both wild strain and mutant strains.

Differences in protein composition of mutant strains and wild strain were not significant. It is likely that differences between mutant strains could not be detected by SDS-PAGE electrophoresis. For future study of protein composition of mutant strains and wild strain 2D electrophoresis and MALDI-TOF analysis of selected bends would be needed.

4.2.3 Activity of intracellular enzymes

Mutant strains OC1, OD1, OE1 and OE5 were overproducing PHA. Activity of intracellular enzymes of these mutant strains was measured in order to better understand these mutants and their capability to overproduce PHB.

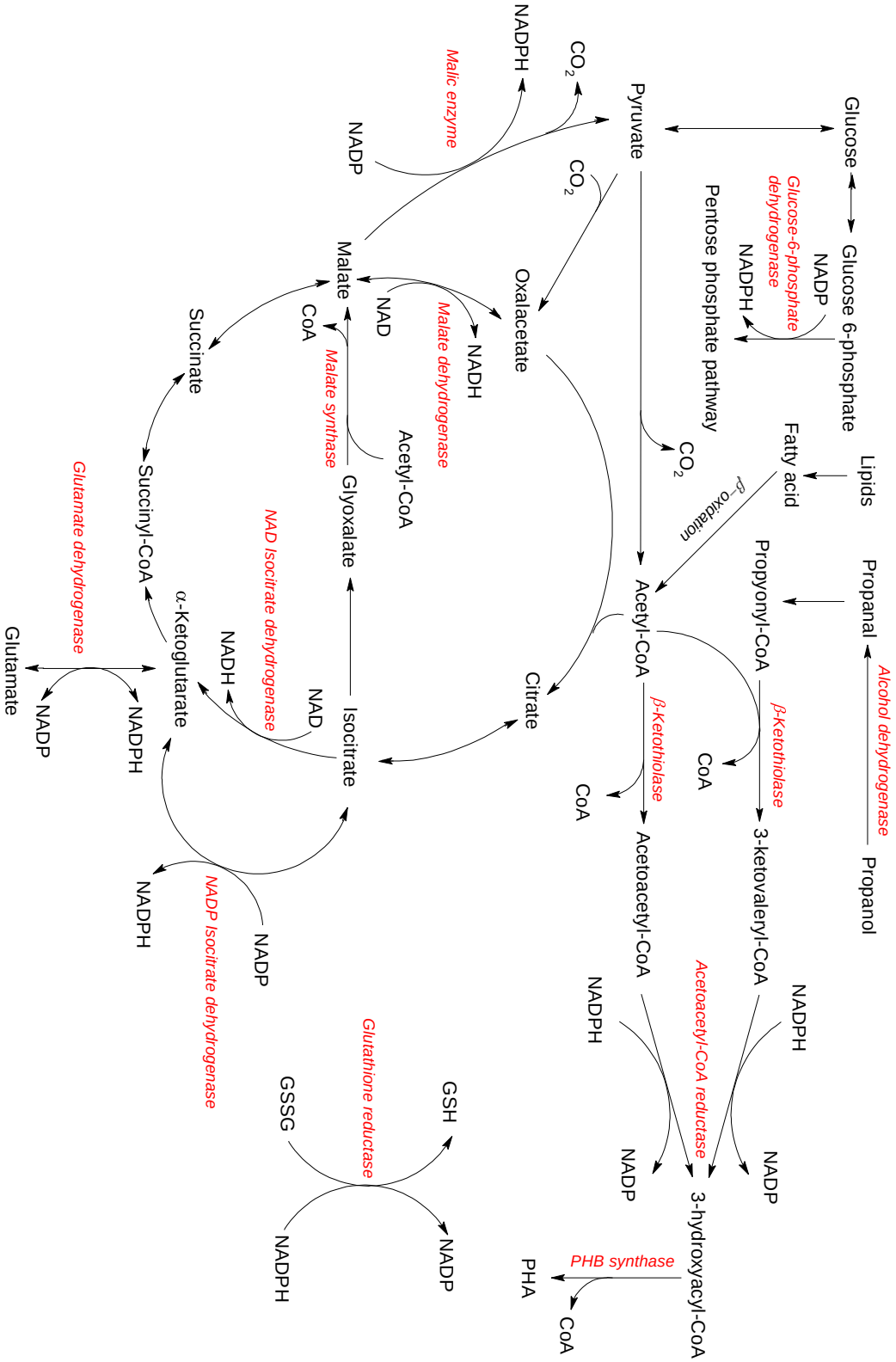


Figure 31: *Overview of intracellular enzymes whose activity was measured and their metabolic roles.*

4.2.3.1 Alcohol dehydrogenase EC 1.1.1.1

Alcohol dehydrogenase (ADH) is an enzyme catalyzing oxidation of alcohol using NAD or NADP as electron acceptor. The reaction is reversible and substrates can be a variety of primary or secondary alcohols [32]. Activity of ADH of *Cupriavidus necator* H16 at the 48th hour of cultivation in mineral medium with propan-1-ol and ethanol as substrates and NAD and NADP as cofactors was measured. In the second experiment activity the substrate specificity of ADH of *Cupriavidus necator* towards various alcohols was tested as well. Finally, activity of ADH of mutant strains at the 48th hour of cultivation in mineral medium with propan-1-ol and butan-1,4-diol as substrates was measured. These alcohols were tested, because their utilization might result in incorporation of other monomers into structure of PHA – 3HV and 4-hydroxybutyrate which would significantly improve mechanical properties of produced materials. Activity of ADH was measured as described previously (3.3.3.3).

Table 19: *Relative activity of intracellular ADH propan-1-ol and ethanol as substrates and NAD and NADP as cofactors*

Substrate	NAD Relative activity (%)	NADP Relative activity (%)
Propan-1-ol	100 ± 13,65	8,98 ± 3,03
Ethanol	22,89 ± 4,75	4,75 ± 4,34

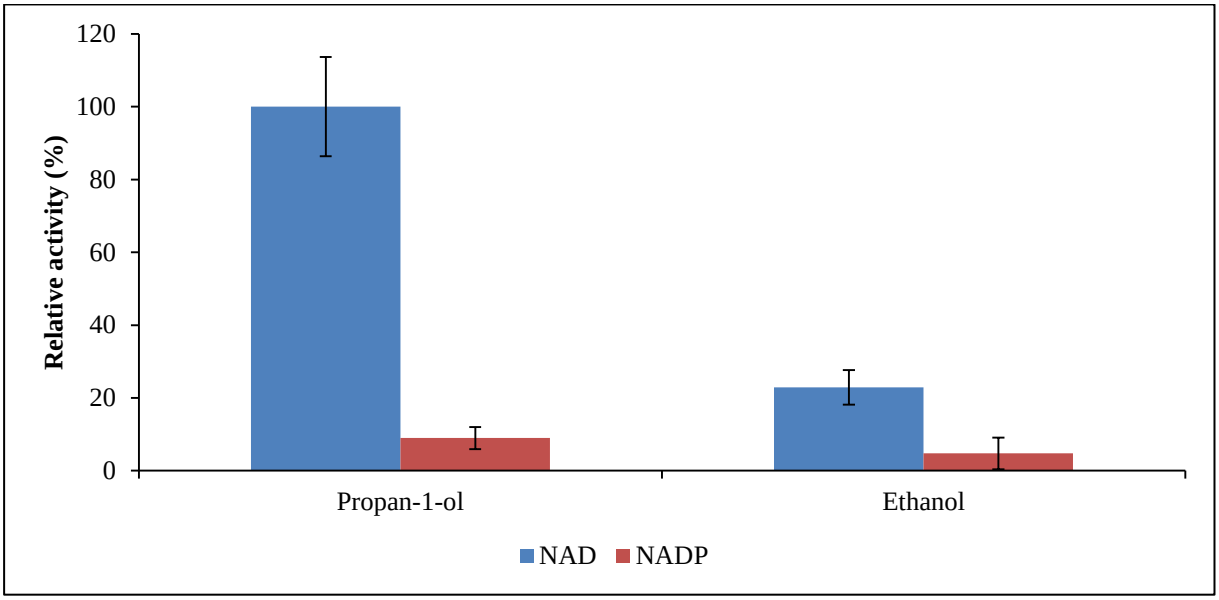


Figure 32: *Relative activity of ADH at the 48th hour of cultivation in mineral medium with propan-1-ol and ethanol as substrates and NAD and NADP as cofactors*

Activity of alcohol dehydrogenase of *Cupriavidus necator* H16 with NAD as a cofactor was much higher as compare to activity of ADH with NADP. ADH catalyzed oxidation of both alcohols used in this experiment (propan-1-ol, ethanol). ADH of *Cupriavidus necator* uses NAD as a cofactor in oxidation of alcohols. There was some small activity of ADH with NADP as a cofactor. It is very likely that some other enzyme caused this activity (intracellular extraction contains wide variety of proteins). Surprisingly, activity of ADH with NAD as a cofactor and with propan-1-ol as a substrate was higher than activity of ADH with ethanol as a substrate. Because NAD is preferred coenzyme of studied ADH, NAD was used as a cofactor in all the other experiments with ADH.

Table 20: *Relative activity of ADH at the 48th hour of cultivation in mineral medium with different alcohols as substrates.*

Substrate	Relative activity (%)
Methanol	22,57 ± 4,42
Ethanol	37,74 ± 3,65
Propan-1-ol	100 ± 5,70
Butan-1-ol	17,51 ± 4,70
Pentan-1-ol	45,53 ± 7,57
Isoamylalcohol	8,17 ± 4,64
Butan-1,4-diol	4,28 ± 3,12

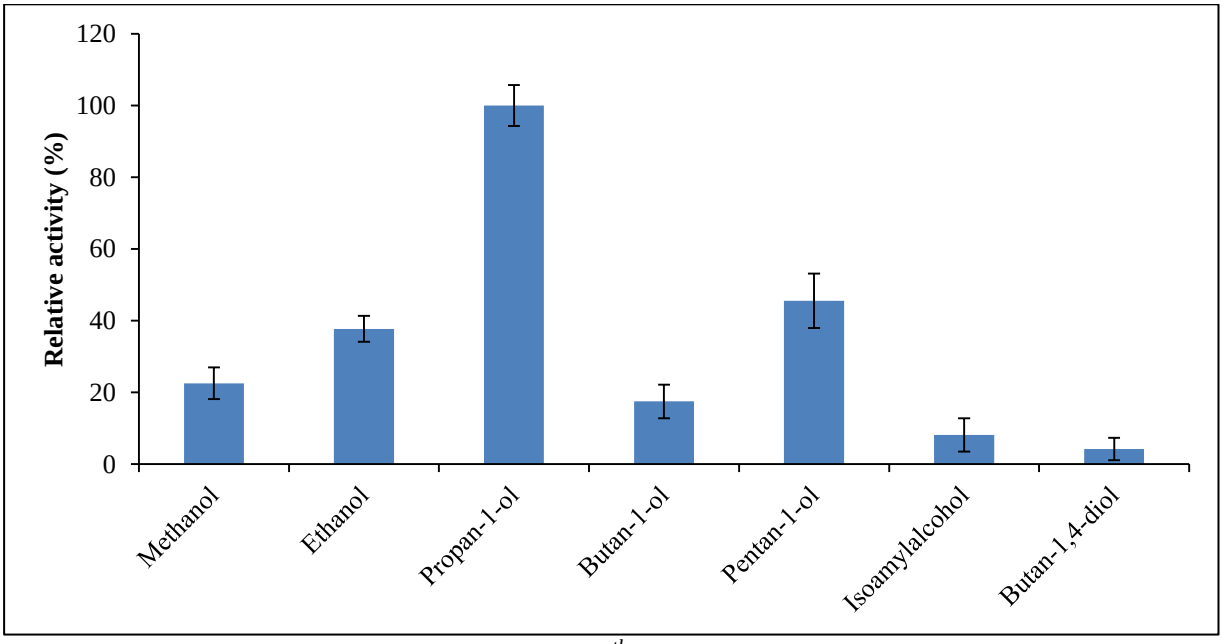


Figure 33: *Relative activity of ADH at the 48th hour of cultivation in mineral medium with different alcohols as substrates.*

Alcohol dehydrogenase of *Cupriavidus necator* H16 could catalyze oxidation of all used alcohols. Activity of ADH was the highest when propan-1-ol was used as a substrate for enzyme. Activity of ADH with petan-1-ol as a substrate was approximately 50 % of activity of ADH with propan-1-ol as a carbon source. Activity of ADH with ethanol was approximately 35% of activity of ADH with propan-1-ol. Activity of ADH with methanol and butan-1-ol was approximately 20% of activity of ADH with propan-1-ol. Activity of ADH with other substrates was really small compared to activity of ADH with propan-1-ol.

ADH of *Cupriavidus necator* H16 easily oxidizes propan-1-ol and pentan-1-ol. Oxidation of these alcohols leads to formation of short-chain fatty acids (propionic acid, valeric acid). These acids are involved in biosynthesis of copolymer P(3HB-*co*-3HV). High activity of ADH with propan-1-ol and pentan-1-ol can be useful for copolymer biosynthesis. To our knowledge, this the first report on substrate specificity of *Cupriavidus necator* ADH.

Table 21: *Specific activity of ADH of mutant strains at the 48th hour of cultivation in mineral medium with propa-1-ol and butan-1,4-diol as substrates*

Mutant	Propan-1-ol (U·mg^{-1})	Butan-1,4-diol (U·mg^{-1})
Wild	10,27 ± 1,46	5,11 ± 0,59
OC1	14,98 ± 1,19	5,29 ± 0,51
OD1	16,77 ± 2,08	6,27 ± 1,09
OE1	8,29 ± 0,94	6,76 ± 0,31
OE5	4,45 ± 0,38	4,97 ± 1,98

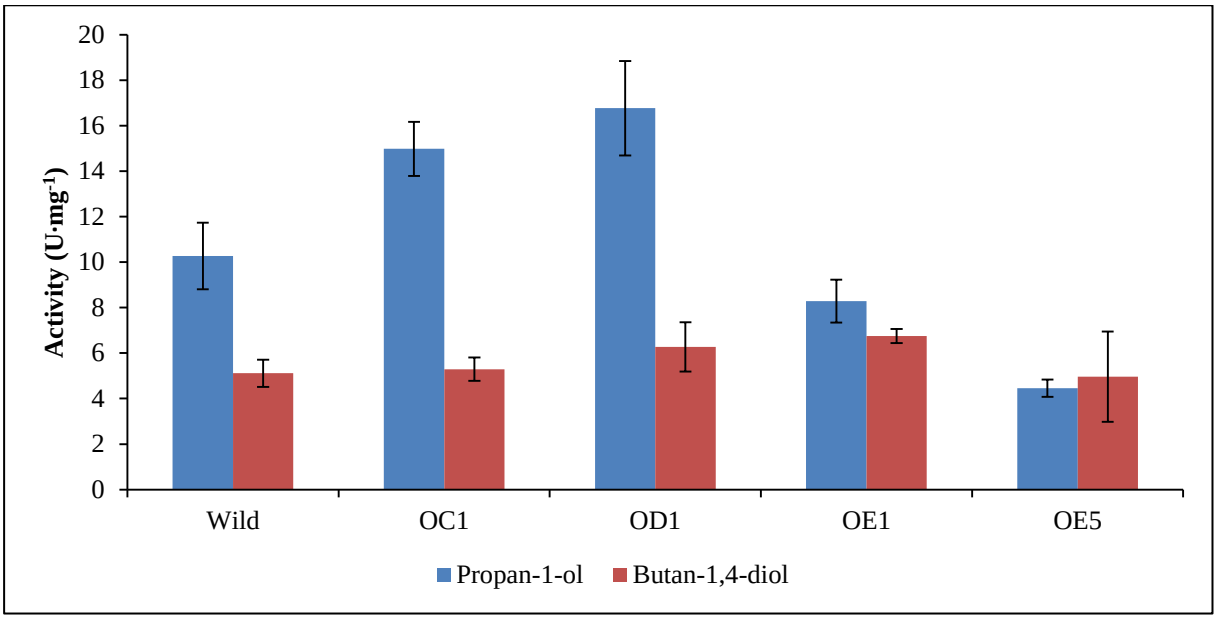


Figure 34: *Specific activity of ADH of mutant strains with propa-1-ol and butan-1,4-diol as substrates.*

The highest specific activity of ADH with propan-1-ol as a substrate at the 48th hour of cultivation had mutant strain OD1. Specific activity of ADH with propan-1-ol as a substrate of mutant strain OC1 at the 48th hour of cultivation was also higher than specific activity of wild strain. Specific activity of ADH with propanol as a substrate of mutants OE1 and OE5 at the 48th hour of cultivation was lower than specific activity of ADH with propan-1-ol as a substrate of wild strain.

Specific activity of ADH with butan-1,4-diol as a substrate at the 48th hour of cultivation of all mutant strains was comparable to specific activity of ADH with butan-1,4-diol as substrate of wild strain.

Specific activity of ADH with propan-1-ol as a substrate of mutant strains at the 48th hour of cultivation was compared with copolymer composition of these mutant strains at the 72nd hour of cultivation. It can be seen that mutant strains with higher specific activity of ADH with propanol as a substrate had statistically higher amount of 3HV in copolymer composition than mutant strains with lower specific activity of ADH with propan-1-ol as a substrate. Composition of copolymer P(3HB-*co*-3HV) is influenced by activity of ADH with propan-1-ol as a substrate. In general it may be stated that the higher ADH activity within cells, the higher 3HV content in polymer This suggest that ADH and level of its expression may be potential target for genetic engineering of *Cupriavidus necator* H16 in order to improve mechanical properties of produced materials.

4.2.3.2 NAD dependent isocitrate dehydrogenase EC 1.1.1.41

NAD dependent isocitrate dehydrogenase is enzyme of TCA cycle. This enzyme catalyzes oxidation of isocitrate to α-ketoglutarate using NAD as cofactor [41]. Activity of NAD isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.4).

Table 22: *Specific activity of NAD isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	2,61 ± 0,56
OC1	3,91 ± 0,75
OD1	4,04 ± 1,12
OE1	3,4 ± 0,99
OE5	1,34 ± 0,41

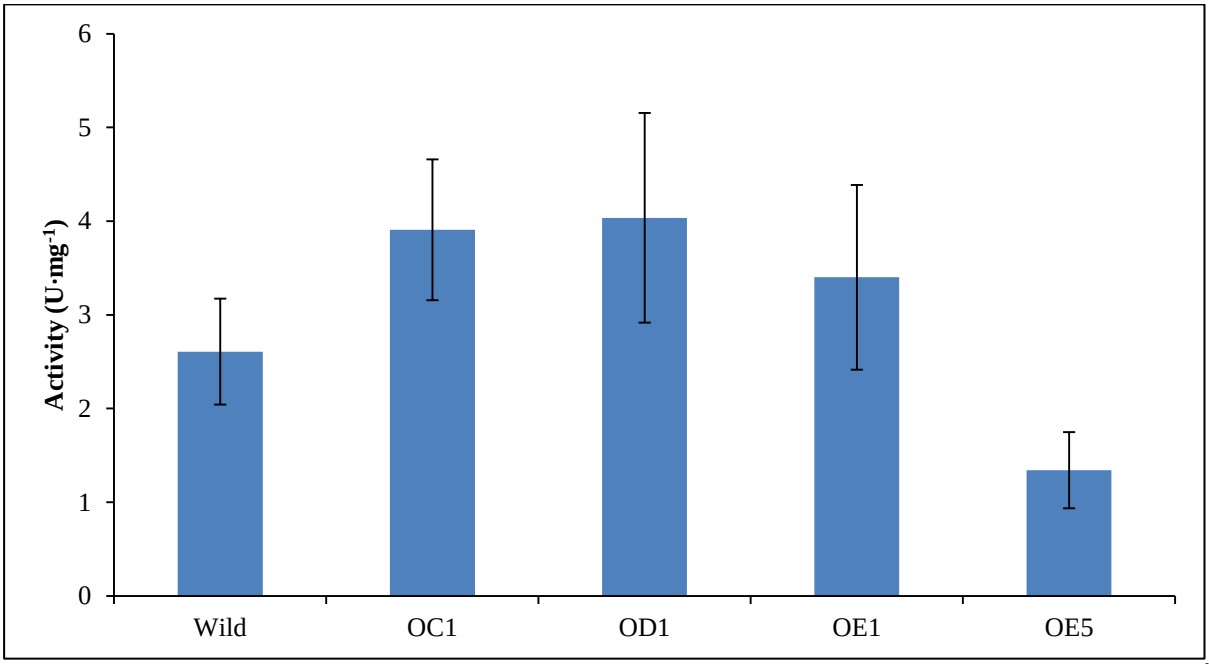


Figure 35: *Specific activity of NAD isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of NAD dependent isocitrate dehydrogenase of mutant strains OC1, OD1 and OE1 at the 48th hour of cultivation was higher than activity of NAD dependent isocitrate dehydrogenase of wild strain at the 48th hour of cultivation. Specific activity of NAD dependent isocitrate dehydrogenase of mutant strain OE5 at the 48th hour of cultivation was lower than activity of NAD isocitrate dehydrogenase of wild strain at the 48th hour of cultivation.

TCA cycle is competing (with PHB biosynthesis) pathway for acetyl-CoA metabolism. TCA cycle inhibition causes acetyl-CoA to be metabolized by β-Ketothiolase to acetoacetyl-CoA. This pathway leads to PHA formation (see figure 31). Lower activity of NAD dependent isocitrate dehydrogenase of OE5 could cause partial inhibition of TCA cycle and activation of anabolic pathway leading to PHB.

4.2.3.3 NADP dependent isocitrate dehydrogenase EC 1.1.1.42

NADP dependent isocitrate dehydrogenase is enzyme that allows cells to generate NADPH. NADP dependent isocitrate dehydrogenase catalyzes oxidation of isocitrate to α-ketoglutarate and reduction of NADP to NADPH in process [42]. Specific activity of NADP dependent isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.5).

Table 23: *Specific activity of NADP isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	29,45 ± 1,04
OC1	586,19 ± 11,07
OD1	169,09 ± 9,04
OE1	111,72 ± 6,80
OE5	151,29 ± 4,51

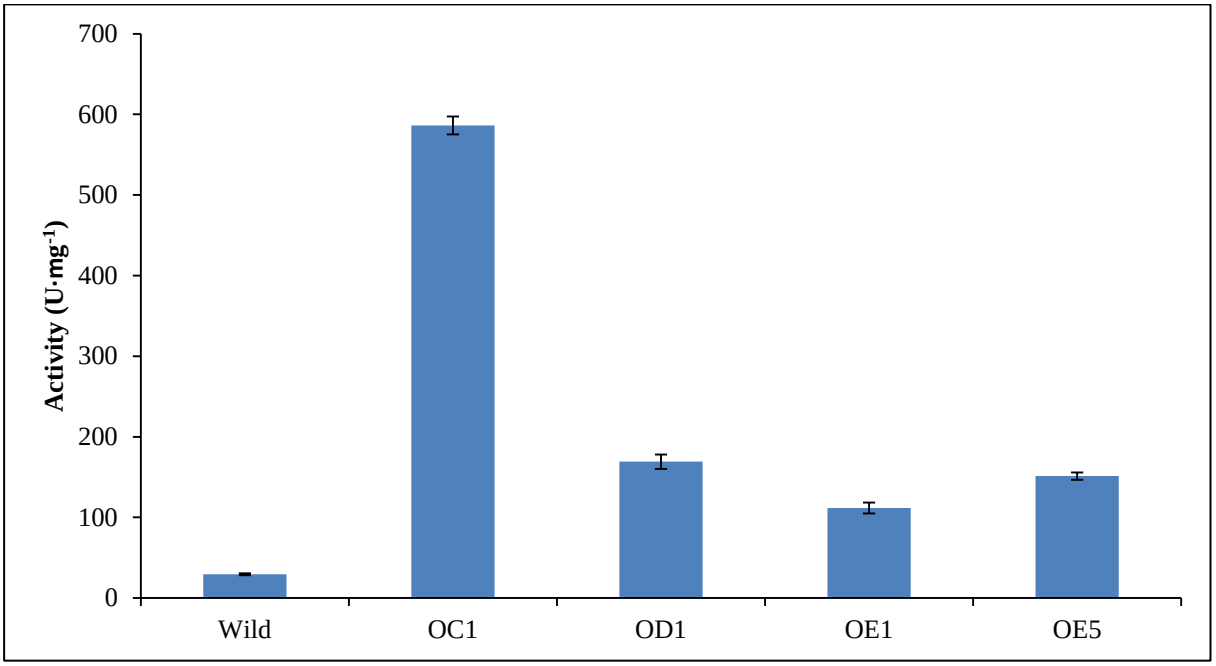


Figure 36: *Specific activity of NADP isocitrate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of NADP isocitrate dehydrogenase of all mutant strains at the 48th hour of cultivation was higher that specific activity of NADP isocitrate dehydrogenase of wild strain at the 48th hour of cultivation. Specific activity of NADP isocitrate dehydrogenase of mutant strain OC1 was distinctively higher than specific activity of NADP isocitrate dehydrogenase of wild strain.

High specific activity of NADP isocitrate dehydrogenase causes formation of high levels of NADPH in cells. High level of NADPH in cell causes inhibition of catabolic pathways in metabolism (TCA cycle) and activation of anabolic pathways (PHA synthesis). High activity of NADP isocitrate dehydrogenase of mutant strains could generate high levels of NADPH that could induce PHA synthesis.

4.2.3.4 Malate dehydrogenase EC 1.1.1.37

Malate dehydrogenase is enzyme of TCA cycle which catalyzes oxidation of malate to oxaloacetate with NAD serving as an electron acceptor [43]. Activity of malate dehydrogenase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.6).

Table 24: *Specific activity of malate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium*

Mutant	Specific activity (U·mg^{-1})
Wild	3,64 ± 0,27
OC1	3,28 ± 1,54
OD1	2,22 ± 0,55
OE1	3,24 ± 0,55
OE5	0,63 ± 0,39

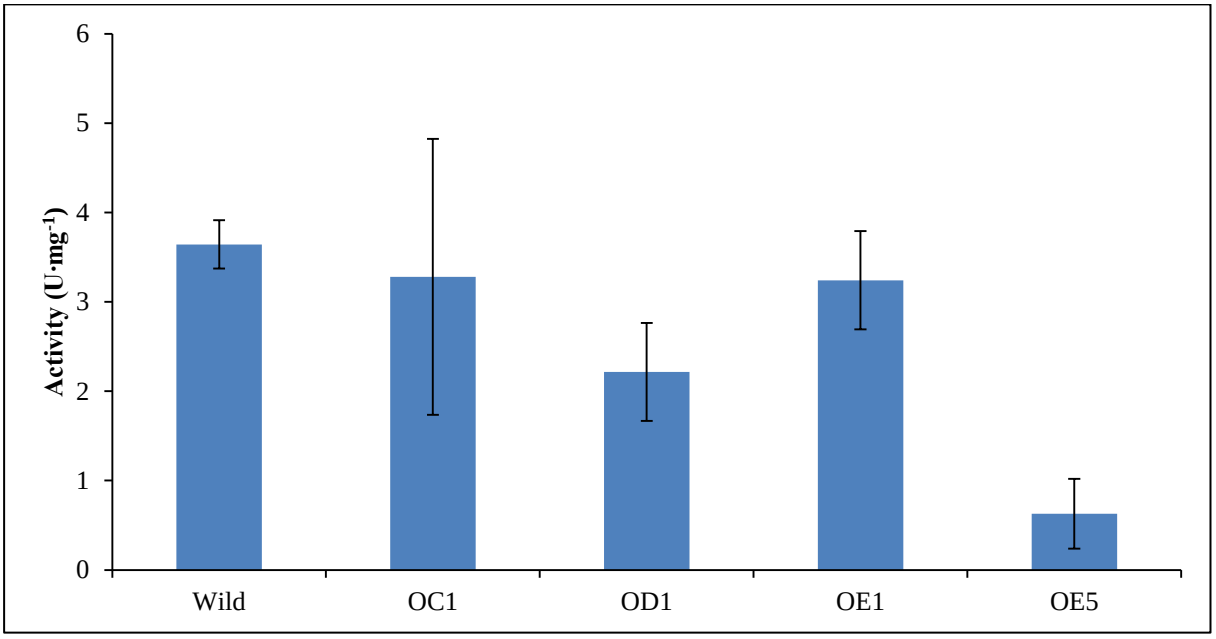


Figure 37: *Specific activity of malate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of malate dehydrogenase of mutant strains OC1 and OE1 at the 48th hour of cultivation was comparable to specific activity of malate dehydrogenase of wild strain at the 48th hour of cultivation. Specific activity of malate dehydrogenase of mutant strains OD1 and OE5 at the 48th hour of cultivation was lower than specific activity of malate dehydrogenase of wild strain at the 48th hour of cultivation.

High activity of malate dehydrogenase causes repression of anabolic pathways in metabolism (PHA synthesis). Low activity of malate dehydrogenase causes repression of TCA cycle. Low activity of malate dehydrogenase of mutant OE5 could cause repression of TCA cycle and activation of PHA synthesis. It seems that mutant OE5 has partially reduced activity of TCA cycle, because similarly to malate dehydrogenase it also revealed lower activity of NAD dependent isocitrate dehydrogenase. Because TCA cycle competes for

acetyl-CoA with PHB biosynthetic pathway, this might be a reason why mutant OE5 possesses better PHB production capabilities than wild strain.

4.2.3.5 Malic enzyme EC 1.1.1.40

Malic enzyme is an enzyme that allows cells to generate NADPH. It catalyzes oxidation of malate to pyruvate. NADPH is released in process [42]. Malic enzyme activity of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.7).

Table 25: *Specific activity of malic enzyme of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	353,46 ± 8,48
OC1	465,42 ± 10,27
OD1	643,79 ± 3,96
OE1	363,71 ± 6,82
OE5	489,69 ± 30,05

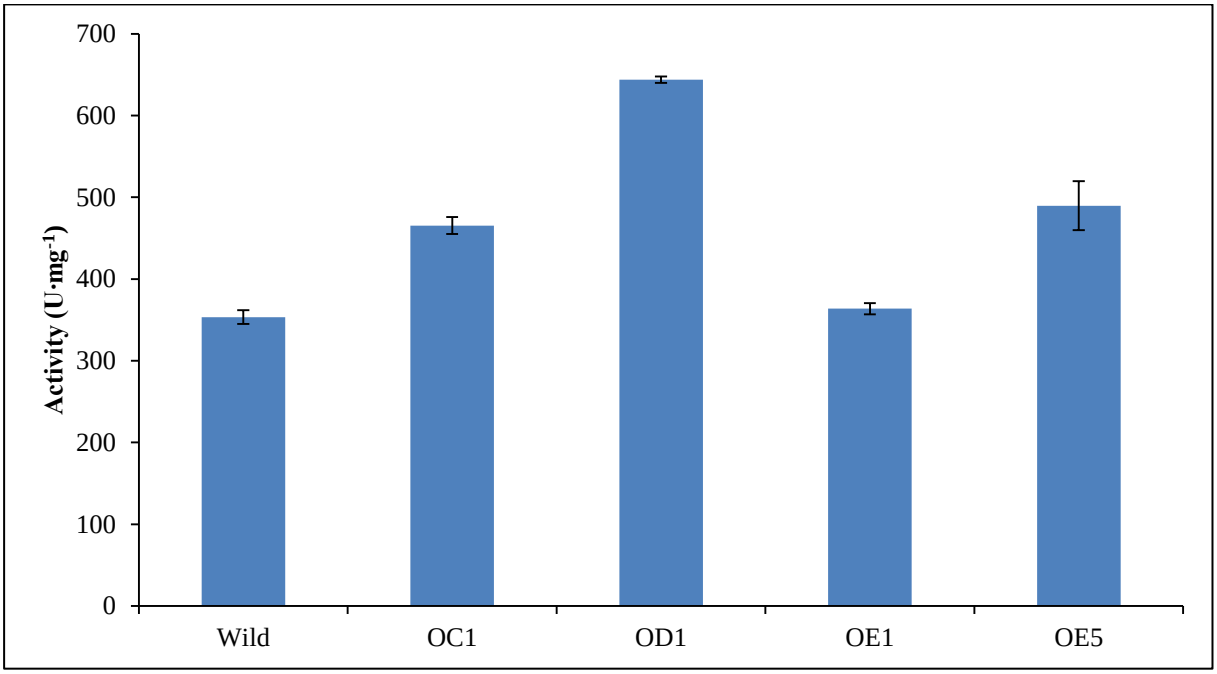


Figure 38: *Specific activity of malic enzyme of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of malic enzyme of mutant strains OC1, OD1 and OE5 at the 48th hour of cultivation was higher than specific activity of wild strain at the 48th hour of cultivation. Specific activity of malic enzyme of mutant strain OE1 at the 48th hour of cultivation was comparable to specific activity of malic enzyme of wild strain at the 48th hour of cultivation.

High specific activity of malic enzyme causes formation of high levels of NADPH in cells. High level of NADPH in cell causes repression of catabolic pathways in metabolism (TCA cycle) and activation of anabolic pathways (PHA synthesis). Therefore, high activity of malic enzyme of mutant strains OC1, OD1 and OE5 could support PHA synthesis.

4.2.3.6 Glucose 6-phosphate dehydrogenase EC 1.1.1.49

Glucose 6-phosphate dehydrogenase (G6PD) is an enzyme of pentose pathway that generates pentoses and NADPH [42]. Activity of G6PD of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.8).

Table 26: *Specific activity of G6PD of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	4,88 ± 0,27
OC1	9,15 ± 0,83
OD1	17,72 ± 1,35
OE1	22,65 ± 0,15
OE5	14,11 ± 0,59

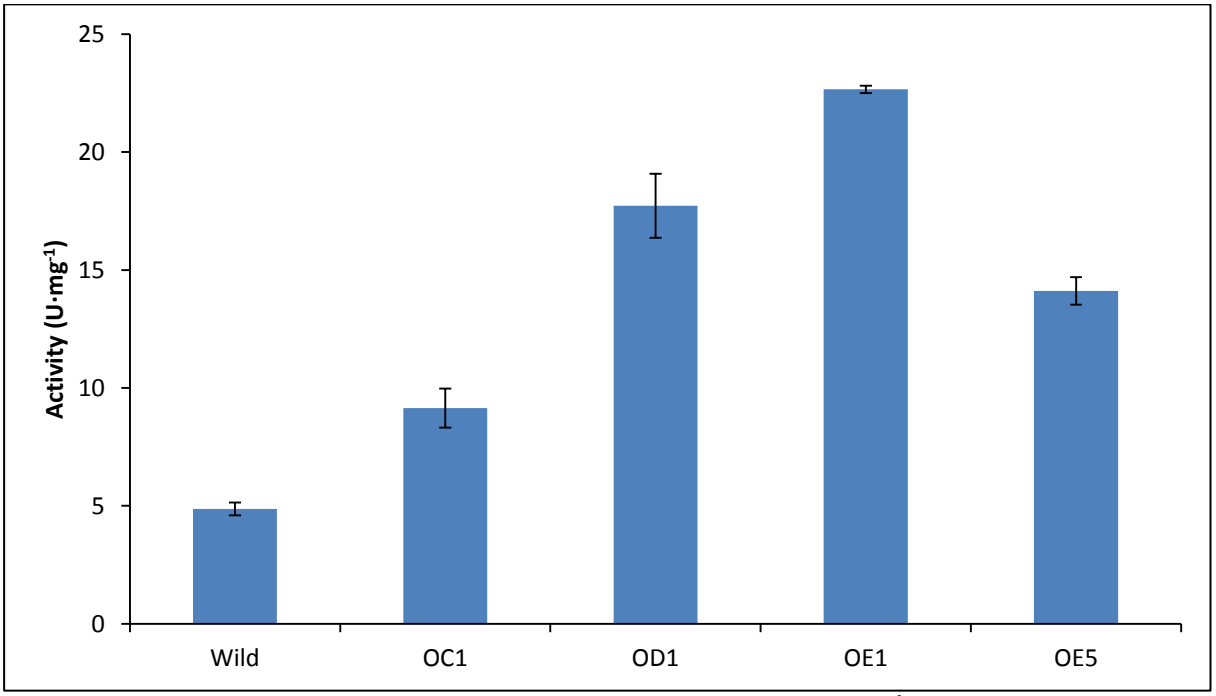


Figure 39: *Specific activity of G6PD of mutant strains at the 48th hour of cultivation in mineral medium.*

Specific activity of G6PD of all mutant strains at the 48th hour of cultivation was higher than specific activity of wild strain at the 48th hour of cultivation. Mutant strain OE1 had highest G6PD activity.

High specific activity of G6PD causes formation of high levels of NADPH in cells. High level of NADPH in cell causes repression of catabolic pathways in metabolism (TCA cycle) and activation of anabolic pathways (PHA synthesis). High activity of G6PD of all mutant strains could cause activation of PHA synthesis.

4.2.3.7 Glutamate dehydrogenase EC 1.4.1.4

Glutamate dehydrogenase is enzyme catalyzing oxidation of glutamate to α-ketoglutarate using NADP as electron acceptor [44]. Activity of glutamate dehydrogenase of mutant strains was measured as described previously (3.3.3.9).

Table 27: *Specific activity of glutamate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium*

Mutant	Specific activity (U·mg^{-1})
Wild	6,54 ± 1,10
OC1	14,44 ± 1,39
OD1	18,66 ± 1,21
OE1	9,91 ± 1,09
OE5	15,59 ± 1,37

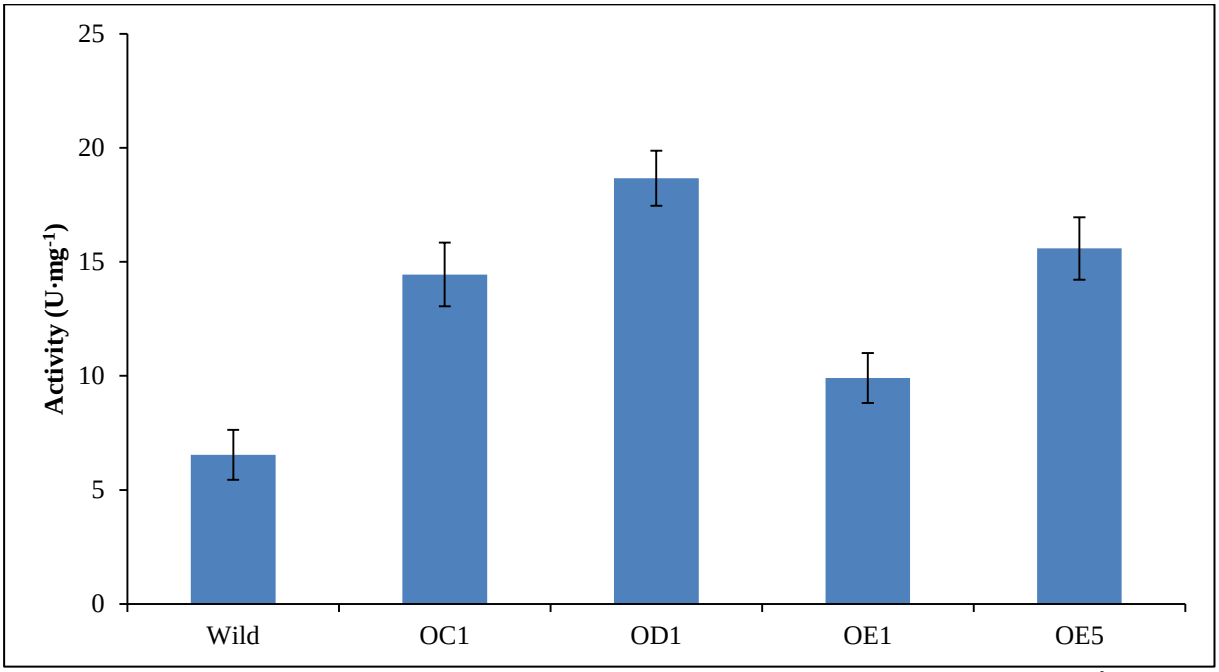


Figure 40: *Specific activity of glutamate dehydrogenase of mutant strains at the 48th hour of cultivation in mineral medium.*

Specific activity of glutamate dehydrogenase of all mutant strains at the 48th hour of cultivation was higher than specific activity of glutamate dehydrogenase of wild strain.

It is generally considered, that NADPH-dependent isocitrate dehydrogenase, malic enzyme, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase are the main enzymes involved in NADPH formation when bacterial cell is exposed to oxidative pressure. All the mutants exhibited significantly higher activity of all these enzymes. Resulting reduced coenzymes NADPH supply necessary reductive power to quell the oxidative potential of reactive oxygen species [42]. Thus it seems that the mutagenesis induced "fake" oxidative stress response in mutants which consequently resulted in overproduction of PHA. The fact, that introduction of oxidative pressure supported accumulation of PHA in cells has already been proved [47].

4.2.3.8 Glutathione reductase EC 1.8.1.7

Glutathione reductase catalyzes reduction of oxidized glutathione to reduced glutathione with NADPH serving as electron donor. Reduced glutathione serves as an important antioxidant in cell [45], therefore, we tested intracellular activity of this enzyme as well. Activity of glutathione reductase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.10).

Table 28: *Specific activity of glutathione reductase of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	14,11 ± 0,20
OC1	19,50 ± 1,63
OD1	15,34 ± 0,66
OE1	10,57 ± 2,37
OE5	13,34 ± 0,53

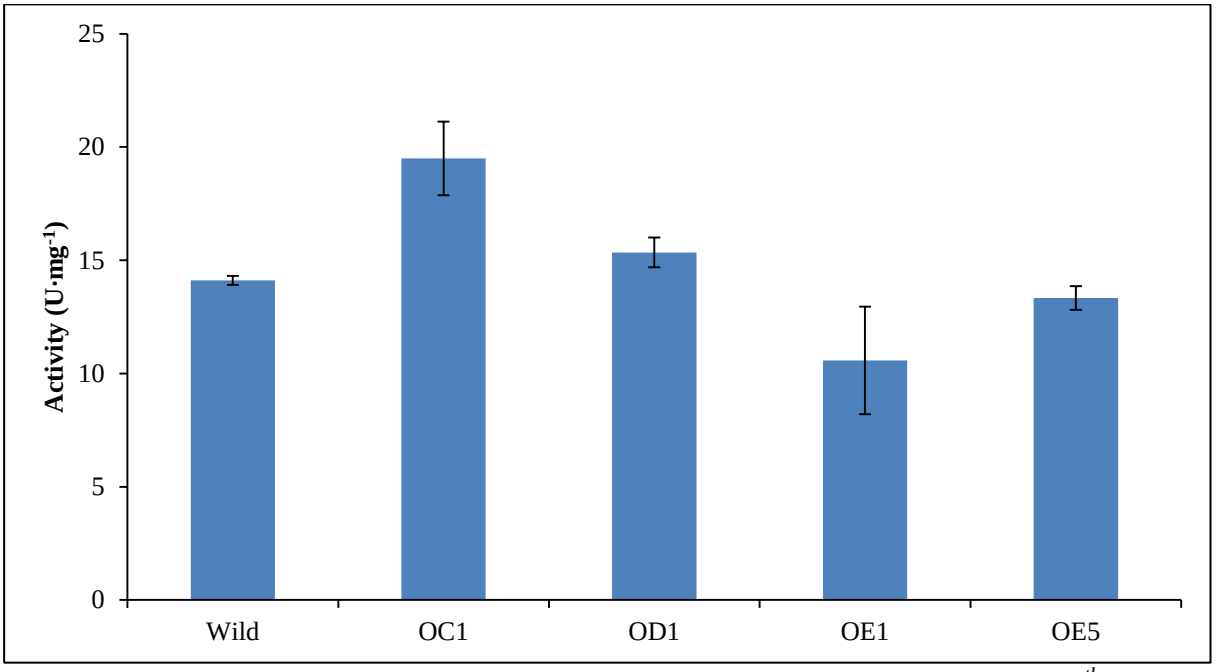


Figure 41: *Specific activity of glutathione reductase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of glutathione reductase of mutant strains OD1 and OE5 at the 48th hour of cultivation was comparable to specific activity of glutathione reductase of wild strain at the 48th hour of cultivation. Specific activity of glutathione reductase of mutant strain OE1 at the 48th hour of cultivation was lower than specific activity of glutathione reductase of wild strain at the 48th hour of cultivation. Specific activity of glutathione reductase of mutant strain OC1 at the 48th hour of cultivation was higher than specific activity of glutathione reductase of wild strain at the 48th hour of cultivation.

High activity of glutathione reductase causes formation of high levels of reduced glutathione (GSH) in cells which might support oxidative stress survival. The activities of glutathione reductase among mutants and wild strain were very similar; therefore, activity of

glutathione reductase is probably independent of activities of other enzymes involved in oxidative stress response and NADPH formation. To confirm our theory, that mutants are partially adapted to face oxidative stress we conducted experiment focused on their survival in presence of hydrogen peroxide (see further 4.2.4).

4.2.3.9 Malate synthase EC 2.3.3.9

Malate synthase is enzyme of glyoxalate cycle. Enzyme catalyzes aldol condensation of glyoxylate with acetyl-CoA during which malate is formed [46]. Specific activity of malate synthase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.11).

Table 29: *Specific activity of malate synthase of mutant strains at the 48th hour of cultivation in mineral medium*

Mutant	Specific activity (U·mg^{-1})
Wild	24,11 ± 1,19
OC1	29,79 ± 1,67
OD1	33,63 ± 2,12
OE1	14,89 ± 0,39
OE5	26,89 ± 1,67

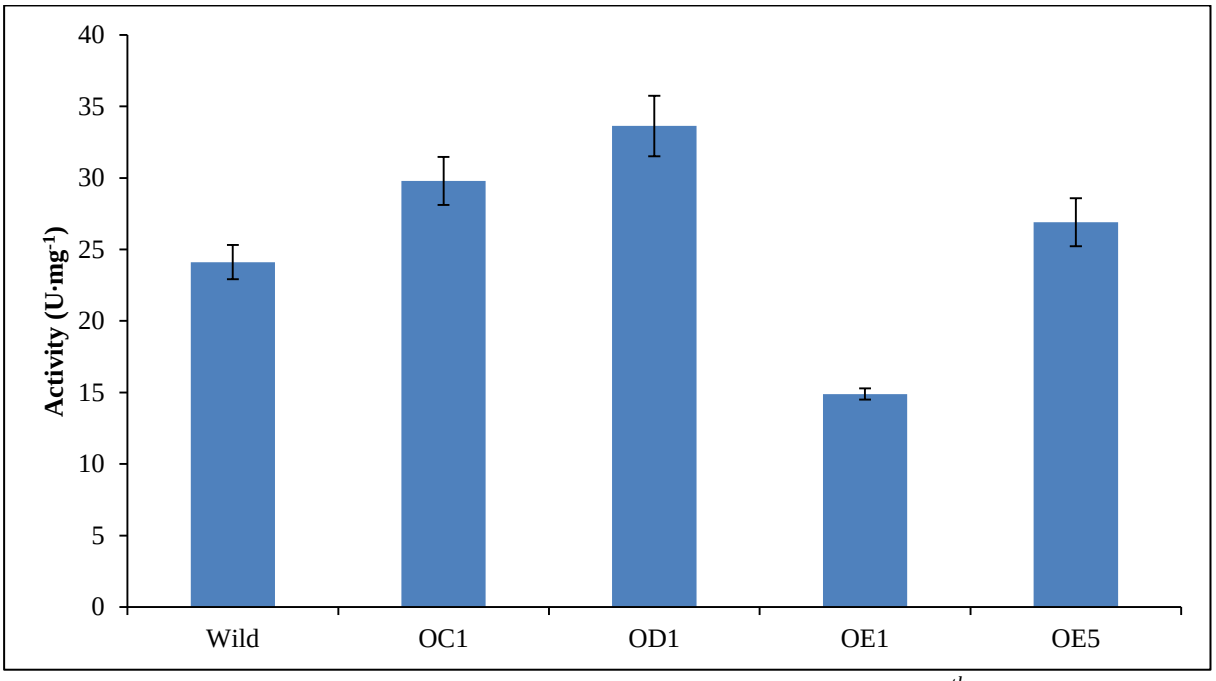


Figure 42: *Specific activity of malate synthase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of malate synthase of mutant strains OC1, OD1 and OE5 at the 48th hour of cultivation was higher than specific activity of malate synthase of wild strain at the 48th hour of cultivation. Specific activity of malate synthase of mutant strain OE1 at the 48th hour of cultivation was lower than specific activity of malate synthase of wild strain at the 48th hour of cultivation.

Enzymes of glyoxalate cycle are repressed during cultivation on glucose and induced during cultivation on oil. According to Wang et al., for sufficient PHA production by *Cupriavidus necator*, glyoxalate cycle should be active [46]. This prediction was confirmed in our mutants, because all of them revealed relatively high activity of malate synthase.

4.2.3.10 β-Ketothiolase EC 2.3.1.9

β-Ketothiolase is the first enzyme involved in PHA synthesis. This enzyme catalyzes biosynthesis of two acetyl-CoA into acetoacetyl-CoA [33]. Activity of β-ketothiolase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.12)

Table 30: *Specific activity of β-ketothiolase of mutant strains at the 48th hour of cultivation in mineral medium.*

Mutant	Specific activity (U·mg^{-1})
Wild	5,55 ± 0,54
OC1	10,28 ± 0,8
OD1	5,49 ± 0,89
OE1	11,02 ± 0,68
OE5	4,93 ± 0,58

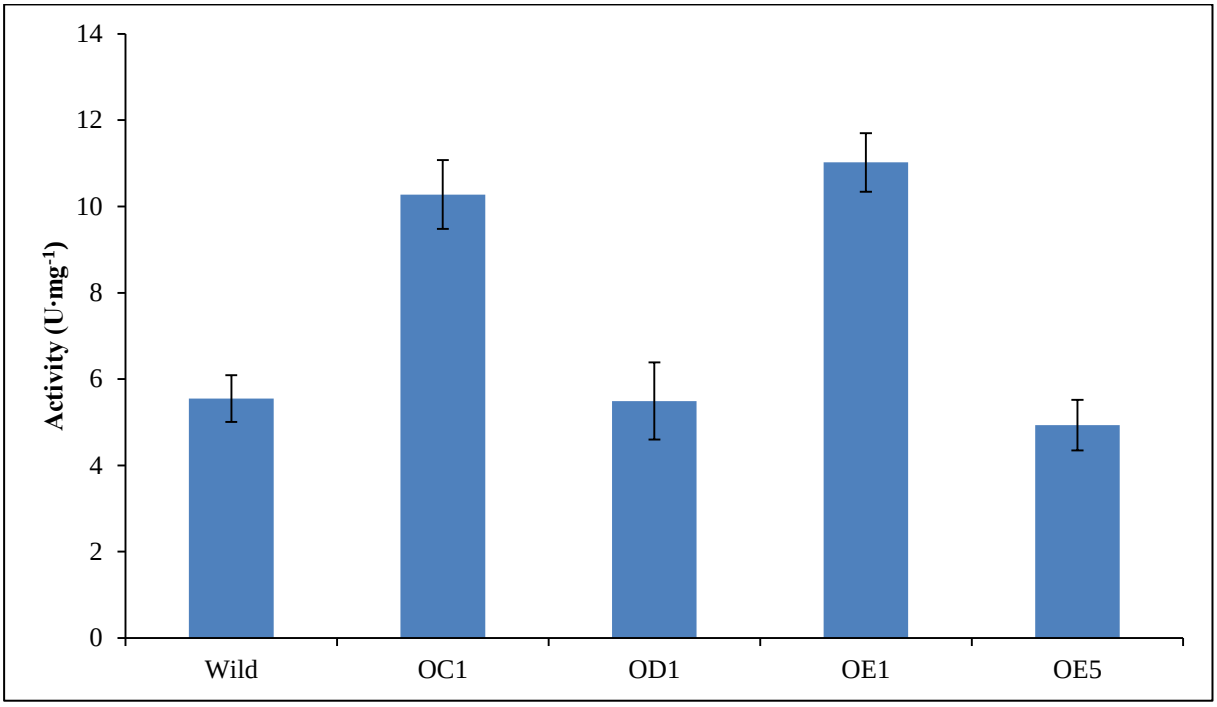


Figure 43: *Specific activity of β-ketothiolase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of β-ketothiolase of mutant strains OD1 and OE5 at the 48th hour of cultivation was comparable to specific activity of β-ketothiolase of wild strain at the 48th hour of cultivation. Specific activity of β-ketothiolase of mutant strains OC1 and OE5 at the 48th hour of cultivation was higher than specific activity of β-ketothiolase of wild strain at the 48th hour of cultivation.

β-ketothiolase is enzyme directly involved in PHA synthesis. Higher activity of this enzyme causes higher amount of PHA to be formed in cells. High activity of β-ketothiolase of mutant strains OC1 and OE1 could increase amount of PHA accumulated in cells of these mutant strains. β-ketothiolase is inhibited by free CoA [8], which is formed mainly in TCA cycle. Therefore, it is likely that partial inhibition of TCA cycle by reduced coenzymes NADPH, formed by action of NADP-dependent isocitrate dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase or glutamate dehydrogenase occurred

4.2.3.11 Acetoacetyl-CoA reductase EC 1.1.1.36

Acetoacetyl-CoA reductase is the second enzyme involved in PHA synthesis. This enzyme catalyzes reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA [8,33]. Activity of acetoacetyl-CoA reductase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.12).

Table 31: *Specific activity of acetoacetyl-CoA reductase of mutant strains at the 48th hour of cultivation in mineral medium*

Mutant	Specific activity (U·mg^{-1})
Wild	916,06 ± 17,37
OC1	2095,5 ± 80,62
OD1	1194,13 ± 28,12
OE1	1040,84 ± 33,68
OE5	700,52 ± 52,69

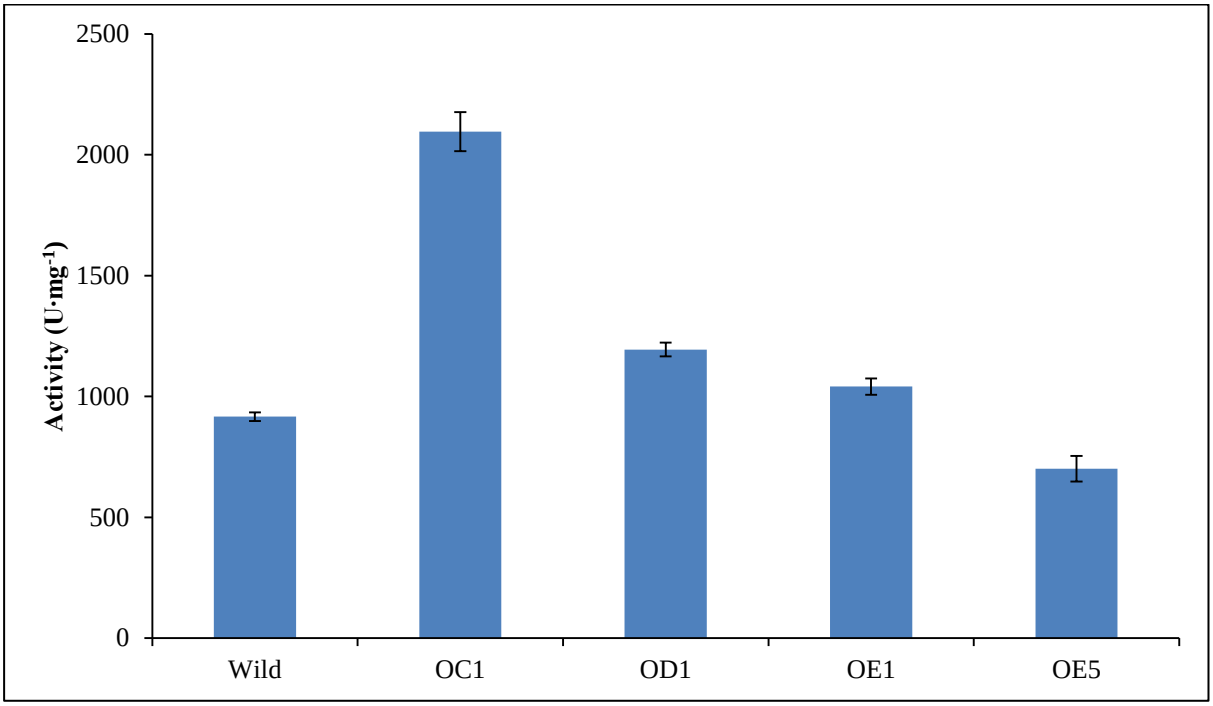


Figure 44: *Specific activity of acetoacetyl-CoA reductase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of acetoacetyl-CoA reductase of mutant strains OC1, OD1 and OE1 at the 48th hour of cultivation was higher than specific activity of acetoacetyl-CoA of wild strain at the 48th hour of cultivation. Specific activity of acetoacetyl-CoA reductase of mutant strain OE5 at the 48th hour of cultivation was lower than specific activity of acetoacetyl-CoA of wild strain at the 48th hour of cultivation.

Acetoacetyl-CoA reductase is NADPH-dependent enzyme; therefore its activity is increased by NADPH generated by NADP-dependent isocitrate dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase or glutamate dehydrogenase. Higher activity of these enzymes was observed in almost all the mutants.

4.2.3.12 PHB synthase EC 2.3.1.B2

PHB synthase is the last enzyme involved in PHA synthesis. Enzyme catalyzes synthesis of PHB [33]. Activity of PHB synthase of mutant strains at the 48th hour of cultivation was measured as described previously (3.3.3.14).

Table 32: *Specific activity of PHB synthase of mutant strains at the 48th hour of cultivation in mineral medium*

Mutant	Specific activity (U·mg^{-1})
Wild	3,50 ± 0,57
OC1	18,82 ± 0,51
OD1	19,79 ± 1,34
OE1	15,19 ± 1,28
OE5	13,27 ± 0,62

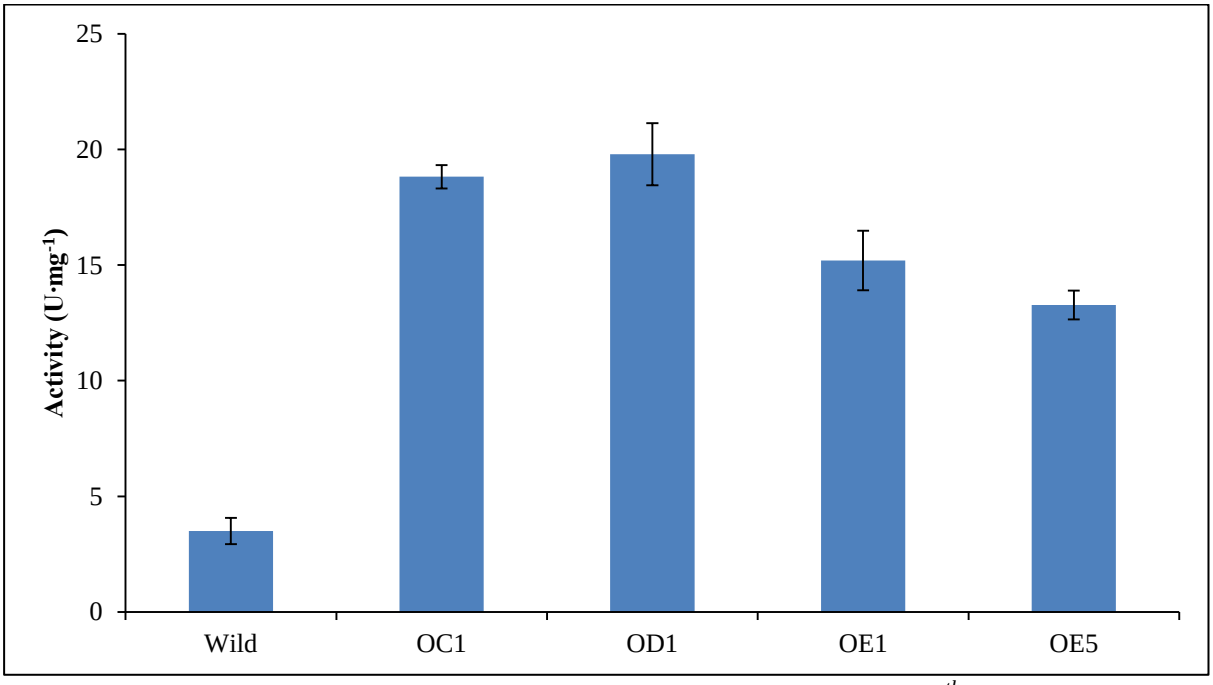


Figure 45: *Specific activity of PHB synthase of mutant strains at the 48th hour of cultivation in mineral medium*

Specific activity of PHB synthase of all mutant strains at the 48th hour of cultivation was higher than specific activity of PHB synthase of wild strain at the 48th hour of cultivation.

PHB synthase is enzyme directly involved in PHA synthesis. Higher activity of this enzyme causes higher amount of PHA to be formed in cells. High activity of PHB synthase of all mutant strains could increase amount of PHA accumulated in cells of these mutant strains.

4.2.4 Resistance of mutant strains against oxidative stress

Because all the mutants prepared by random mutagenesis revealed oxidative stress-like set of metabolism we decided to test their survival in highly challenging conditions of 50 mmol.l^{-1} of hydrogen peroxide. Reducing environment in cell enhances anabolic pathways and PHA formation, and; moreover, it also protects microorganism from reactive oxygen species (ROS). Resistance of mutant strains against oxidative stress was measured as described previously. (3.3.4).

Table 33: *Survivability of mutant strains in 50 mmol·l^{-1} hydrogen peroxide (oxidative stress)*

Mutant	Survivability after 5 minutes (%)	Survivability after 30 minutes (%)
Wild	3,95 ± 1,29	2,12 ± 0,24
OC1	1,98 ± 0,42	1,14 ± 0,07
OD1	20,48 ± 2,91	12,1 ± 1,53
OE1	70,58 ± 6,90	15,4 ± 2,73
OE5	30,18 ± 2,33	14,41 ± 0,24

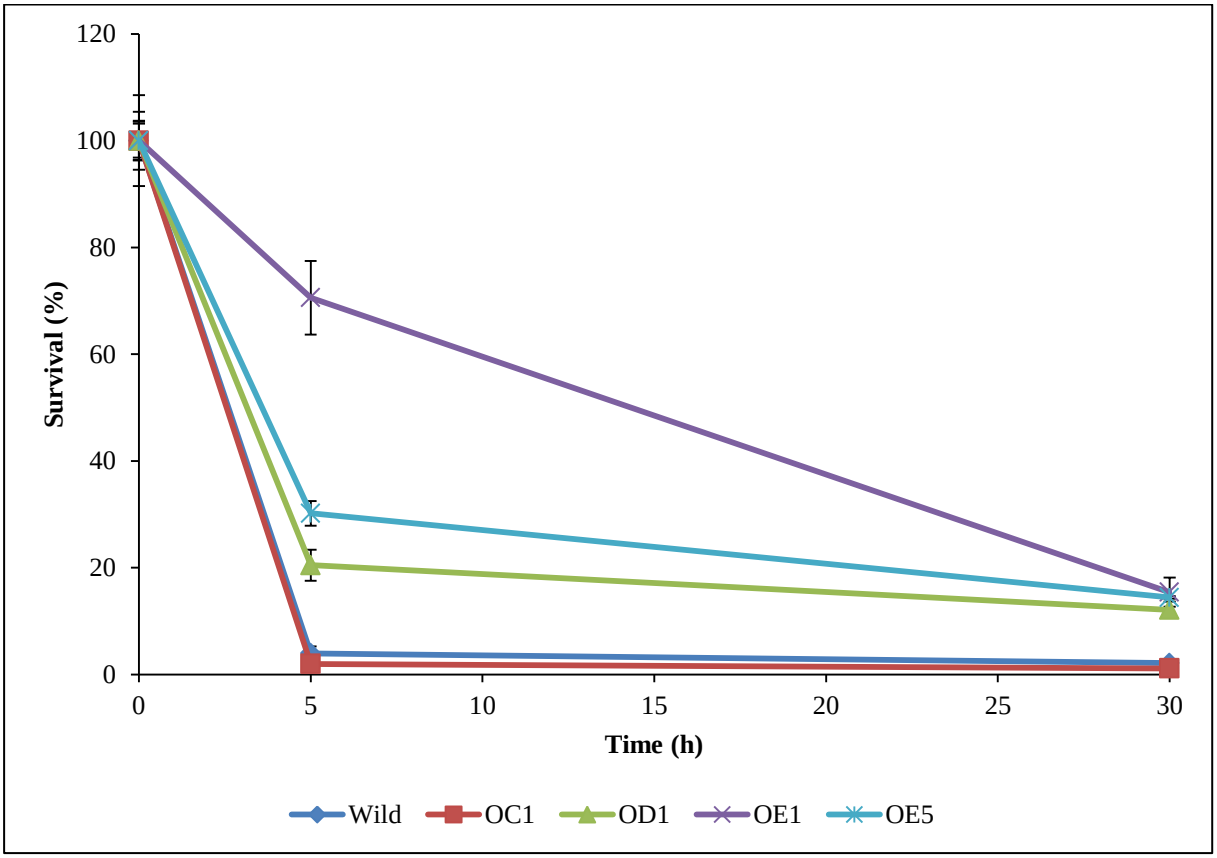


Figure 46: *Survivability of mutant strains in 50 mmol·l^{-1} hydrogen peroxide (oxidative stress)*

Survivability of mutant strains OD1, OE1 and OE5 during oxidative stress was significantly higher than survivability of wild strain. Survivability of mutant strain OC1 during oxidative stress was lower than survivability of wild strain. Survivability of mutant strain OE1 during oxidative stress was the highest of all other mutant strains.

Survivability of mutant strains during oxidative stress corresponds with enzymatic activities of NADPH producing enzymes. We could also hypothesize that higher stress durability may be also contribution of higher PHA content of mutant; however, the connections between stress response of microorganism and its intracellular PHB content are still not fully understood.

4.2.5 Polymer analysis by GPC

Gel permeation chromatography (GPC) is a technic often used in analysis of polymers. Polymers can be characterized by a variety of definitions for molecular weight including the number average molecular weight (M_n), the weight average molecular weight (M_w). Polydispersity index (PDI) can be calculated from number average molecular weight and weight average molecular weight [48]. Mechanic properties of polymer are dependent on its dispersity. Number average molecular weight and weight average molecular weight of mutant strains was measured and PDI of mutant strains was calculated as described previously (3.3.5).

Table 34: *Polymer analysis by gel permeation chromatography*

Mutant	M_n (Da·10^5)	M_w (Da·10^5)	PDI (-)
Wild	2,19	6,27	2,87
OC1	2,94	7,24	2,46
OD1	2,41	6,73	2,79
OE1	1,95	5,42	2,78
OE5	2,75	7,35	2,67

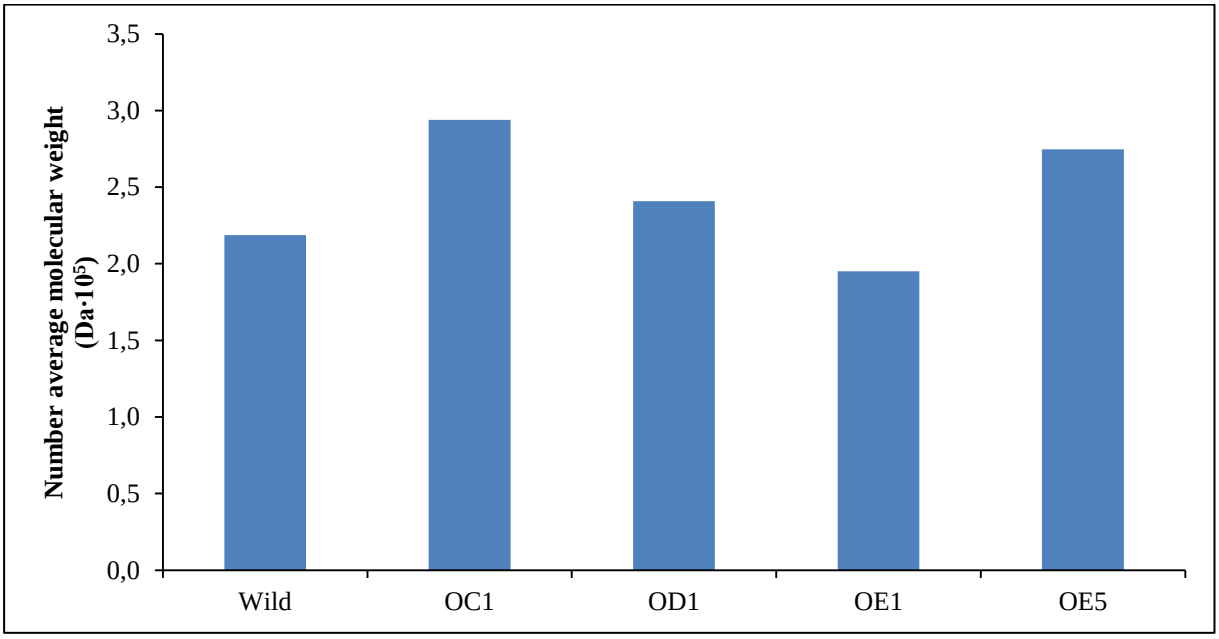


Figure 47: *Number average molecular weight of PHB of mutant strains*

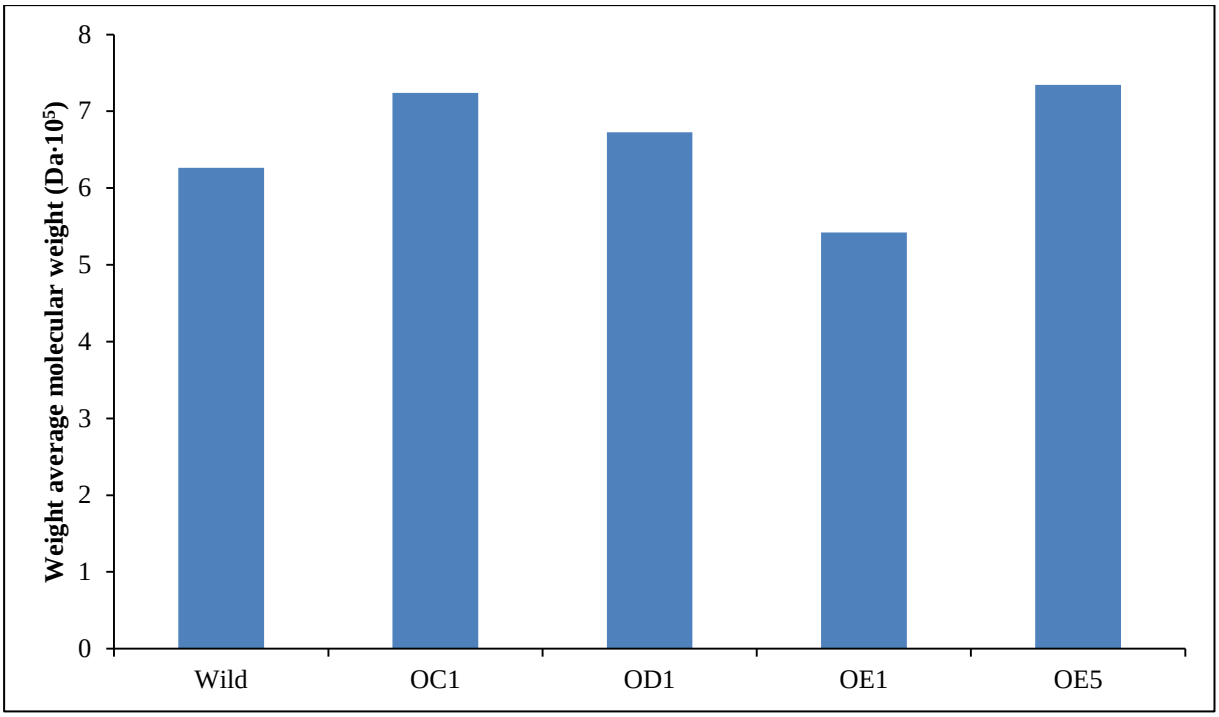


Figure 48: *Weight average molecular weight of PHB of mutant strains*

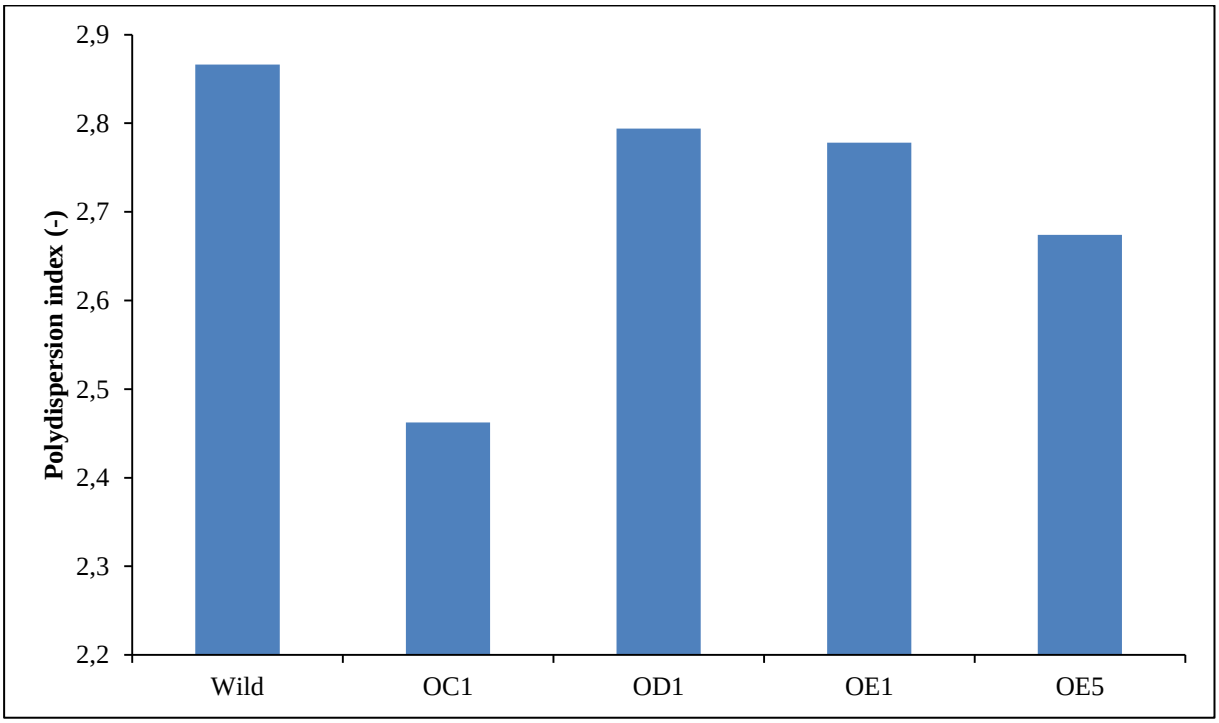


Figure 49: *Polydispersion index of mutant strains*

Number average molecular weight of PHB of mutant strains OC1, OD1 and OE5 was higher than number average molecular weight of PHB of wild strain. Number average molecular weight of PHB of mutant stain OE1 was lower than wild strain.

Weight average molecular weight of PHB of mutant strains OC1, OD1 and OE5 was higher than weight average molecular weight of PHB of wild strain. Weight average molecular weight of PHB of mutant stain OE1 was lower than wild strain.

It was reported that weight of PHB decreased with increase in the activity of PHB synthase [49]. However, in our case the molecular weight of polymer was independent of activity of PHB synthase of these mutants. On the other side, there are reports on positive influence of oxidative pressure on molecular weight of PHB produced by *Cupriavidus necator* [50]. Because mutants prepared in this work exhibited oxidative stress like metabolic response, this might be an explanation of increase of Mw and Mn of materials produced by most of mutants.

4.3 Extracellular secretion characterization

Several experiments were performed in order to characterize and purify extracellular secretion of *Cupriavidus necator* H16. Ionic strength optimum and pH optimum of extracellular secretion were measured to characterize it. Acetone precipitation, ammonium sulfate precipitation, dialysis and ultrafiltration were used for purification of extracellular secretion. Finally SDS-PAGE electrophoresis was performed to determine protein composition of extracellular secretion.

4.3.1 Extracellular secretion characterization

4.3.1.1 Extracellular secretion pH optimum

Activity of extracellular secretion of *Cupriavidus necator* H16 was measured with several buffers as described previously (3.4.1.1).

Table 35: *Extracellular secretion pH optimum.*

pH	Activity (%)
3,0	7,42 ± 2,22
4,0	6,52 ± 1,96
4,8	9,59 ± 0,31
5,8	18,8 0± 1,13
6,6	27,49 ± 1,48
7,0	37,21 ± 2,36
7,4	76,60 ± 9,89
8,0	79,28 ± 5,69
8,3	54,6 ± 1,78
9,0	10,1 ± 2,66
9,4	16,88 ± 2,49
9,8	68,54 ± 3,49
10,6	100 ± 17,47
11,0	60,23 ± 8,70
11,5	2,05 ± 0,96
12,0	6,14 ± 5,04
13,0	11,13 ± 6,93

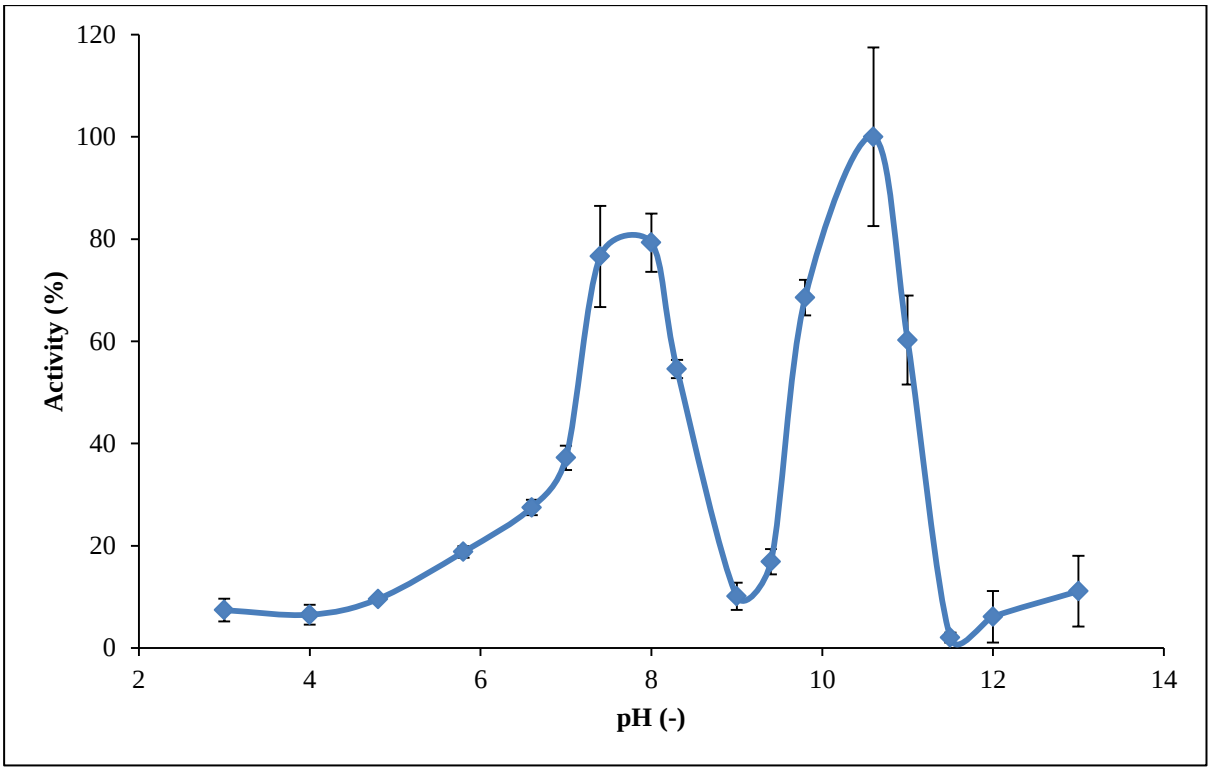


Figure 50: *Extracellular secretion pH optimum.*

It can be seen that there are two pH optimums of extracellular secretion. First pH optimum of extracellular secretion was measured at pH 8,0. Second pH optimum of extracellular secretion was measured at pH 10,6.

4.3.1.2 Extracellular secretion ionic strength optimum

Activity of extracellular secretion of *Cupriavidus necator* H16 was measured with several buffers with different concentration of NaCl as described previously (3.4.1.2).

Table 36: *Extracellular secretion ionic strength optimum*

NaCl concentration (mol·l^{-1})	Activity (%)
0,0	100 ± 12,76
0,5	74,46 ± 11,93
1,0	63,85 ± 9,03
1,5	50,43 ± 2,01
2,0	46,10 ± 1,91
2,5	40,04 ± 4,51
3,0	36,15 ± 1,62
3,5	29,65 ± 0,81
4,0	33,77 ± 3,67
4,5	27,49 ± 1,53
5,0	28,14 ± 11,33

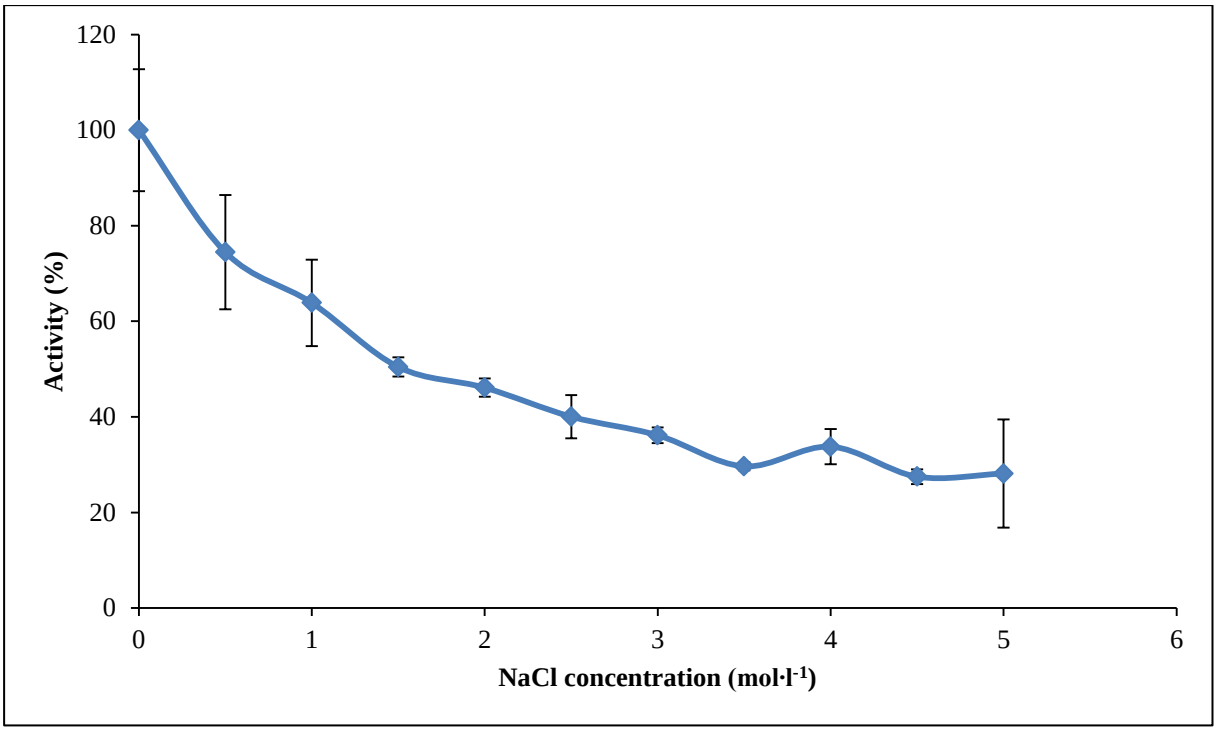


Figure 51: *Extracellular secretion ionic strength optimum*

The highest activity of extracellular secretion was measured with buffer with ionic strength 0 mol·l^{-1}. Activity of extracellular secretion afterwards descended with increase of ionic strength of buffer.

4.3.2 Extracellular secretion purification

Extracellular secretion contains number of protein and other substances. All the substances in secretion are also much diluted. Series of experiment were performed to purify the extracellular secretion and to concentrate it.

4.3.2.1 Acetone precipitation

Acetone precipitation was used to concentrate extracellular secretion. Experiment was performed as described previously (3.4.2.1).

Table 37: *Total secretion concentration, specific activity and recovery of extracellular secretion after acetone precipitation*

Step	Secretion (mg·ml^{-1})	Activity (U·mg^{-1})	Volume (ml)	Recovery (-)
-	0,30 ± 0,01	221,52 ± 10,19	1	1,00
Acetone	0,92 ± 0,00	30,04 ± 0,83	0,05	0,01

Purification of extracellular secretion with acetone precipitation wasn't very successful. Specific activity of extracellular secretion after purification process was lower than before purification process. Recovery was only 1 % of activity. Acetone purification didn't turn out to be the best choice for extracellular secretion purification.

4.3.2.2 Ammonium sulfate precipitation and dialysis

Ammonium sulfate precipitation was used to concentrate proteins, extracellular polymeric substances and other active components of extracellular secretion. Ammonium sulfate was added to extracellular secretion to 60%, 70% and 80% of saturation. Excessive ammonium sulfate in sample was removed thought dialysis. Both experiments were performed as described previously (3.4.2.2 and 3.4.2.3).

Table 38: *Total secretion concentration, specific activity and recovery of extracellular secretion after ammonium sulfate precipitation and dialysis.*

Step	Secretion concentration ($mg \cdot ml^{-1}$)	Activity ($U \cdot mg^{-1}$)	Volume (ml)	Recovery (-)
-	0,30 ± 0,01	221,52 ± 10,19	400	1
60% saturation	0,18 ± 0,00	338,38 ± 13,96	40	0,09
70% saturation	0,22 ± 0,02	301,62 ± 28,31	40	0,10
80% saturation	0,11 ± 0,01	381,62 ± 20,57	40	0,06
Dialysis	0,50 ± 0,02	155,27 ± 19,88	10	0,03

Purification of extracellular secretion with ammonium sulfate precipitation was more successful than purification of extracellular secretion with acetone precipitation. Ammonium sulfate was firstly added to sample to 60%, 70% and 80% saturation. Amount of precipitated secretion in samples first increased and then decreased. This decrease is probably caused by some mistake in sample preparation. Amount of precipitated secretion in sample during precipitation should increase. A lot of activity of extracellular secretion was lost during dialysis. Recovery after dialysis was only 3%.

4.3.2.3 Ultrafiltration

Ultrafiltration was last method used to concentrate extracellular secretion. Experiment was performed as described previously (3.4.2.4).

Table 39: *Total secretion concentration, specific activity and recovery of extracellular secretion after ultrafiltration.*

Step	secretion concentration ($mg \cdot ml^{-1}$)	Activity ($U \cdot mg^{-1}$)	Volume (ml)	Recovery (-)
-	0,30 ± 0,01	99,74 ± 10,83	400	1,00
Ultrafiltration	0,44 ± 0,01	206,11 ± 3,36	50	0,38

Ultrafiltration was the most successful purification method used. Extracellular secretion was concentrated from 400 ml to 50 ml. Recovery of activity was 38%. Fractions smaller than 10 kDa were discarded during ultrafiltration.

Purification of extracellular secretion with ultrafiltration leads to significant concentration of the active components of secretion with high recovery. This method is best suited for extracellular secretion purification.

4.3.2.4 SDS-PAGE electrophoresis

Extracellular secretion contains many proteins and many other active substances. To characterize its protein composition SDS-PAGE electrophoresis was used for separation of these proteins. Proteins in extracellular secretion were separated according to their size. Experiment was performed as described previously (3.5.1.3). Gel was stained with silver due to low concentration of proteins in extracellular secretion (3.5.1.5).

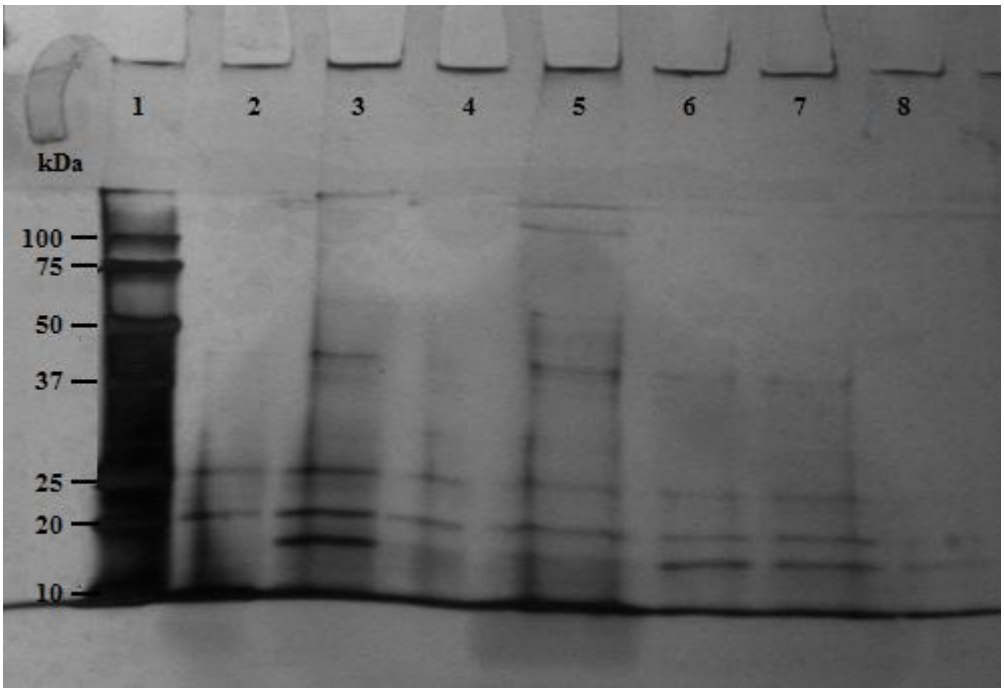


Figure 52: *SDS-PAGE electrophoresis of purified extracellular secretion of Cupriavidus necator H16*

Line	Sample
1	Standard
2	Dialysis
3	Dialysis
4	Ultrafiltration
5	Acetone precipitation
6	$(NH_4)_2SO_4$ precipitation 60%
7	$(NH_4)_2SO_4$ precipitation 70%
8	$(NH_4)_2SO_4$ precipitation 80%

Table 40: *Main protein fractions of extracellular secretion of Cupriavidus necator H16*

Bend	Molar mass ($Da \cdot 10^3$)
Protein fraction 1	15,95
Protein fraction 2	18,80
Protein fraction 3	24,34
Protein fraction 4	47,54
Protein fraction 5	64,52

Extracellular secretion of *Cupriavidus necator* H16 contained 5 protein fractions that were detected. Proteins in extracellular secretion are much diluted; therefore silver staining was used for detection of proteins.

Protein fractions with molar masses 15,95 kDa; 18,80 kDa; 24,34 kDa and 47,54 kDa were visible in sample after dialysis. Extracellular secretion was significantly concentrated during ammonium sulfate precipitation and dialysis. Protein fractions are therefore distinctive.

Protein fractions with molar masses 18,80 kDa and 24,34 kDa were visible in sample after ultrafiltration. Extracellular secretion wasn't concentrated as much with ultrafiltration as with ammonium sulfate precipitation. Proteins with molar mass lower than 10 kDa were discarded during ultrafiltration; therefore protein fraction with molar mass 15,95 kDa was only slightly visible.

Protein fractions with molar masses 15,95 kDa; 18,80 kDa; 24,34 kDa; 47,54 kDa and 64,52 kDa were visible in sample after acetone precipitation. Proteins of extracellular secretion were significantly concentrated during acetone precipitation.

Protein fractions with molar masses 15,95 kDa; 18,80 kDa; 24 kDa and 47,54 kDa were visible in sample after ammonium sulfate precipitation.

5 CONCLUSION

This diploma thesis deals with production of polyhydroxyalkanoates (PHA) by bacterial strain *Cupriavidus necator* H16. Goal of this work was preparation, selection and characterization of mutant strains overproducing PHA.

Theoretical research focused on overview, structure and properties of PHA. Bacteria *Cupriavidus necator* H16 and its ability to produce PHA was studied also together with enzymes involved in PHA synthesis. Last part of theoretical research focused on study of random mutagenesis and its potential for preparation of mutant strains overproducing PHA.

In practical part mutant strains were prepared through physical and chemical mutagenesis. Mutant strains overproducing PHA were selected by cultivation in mineral medium and by cultivation in mineral medium with propanol. Mutant strains OC1, OD1, OE1 and OE5 overproducing PHA were selected for future study. Selected mutants were biochemically characterized. Specific activities of several intracellular enzymes including enzymes involved in PHA biosynthesis were measured. Resistance of mutants against oxidative stress and molecular weight of PHA of mutant strains were measured as well.

Mutants OD1, OE1 and OE5 showed statistically significantly higher PHB production and growth properties as compared to wild strain. The mutant OC1 was selected for its improved ability to incorporate relatively high amount of 3HV into PHA structure.

Mutant strains with higher specific activity of alcohol dehydrogenase with propanol as a substrate had statistically higher amount of 3HV in copolymer composition than mutant strains with lower specific activity of alcohol dehydrogenase with propan-1-ol as a substrate.

All the mutants exhibited significantly higher activity of NADPH-dependent isocitrate dehydrogenase, malic enzyme, glutamate dehydrogenase and glucose-6-phosephate dehydrogenase. These are the main enzymes involved in NADPH formation when cell is exposed to oxidative stress. The mutagenesis induced “fake” oxidative stress response in mutants which consequently resulted in overproduction of PHA.

TCA cycle of mutants was probably partially inhibited by reduced coenzymes NADPH, formed by action of NADPH producing enzymes. TCA cycle inhibition led to activation of PHA synthesis pathway. Activity of β-ketothiolase and acetoacetyl-CoA reductase, enzymes directly involved in PHA synthesis, of some mutant strains was higher as compared to wild strain. All the mutants exhibited significantly higher activity of PHB synthase.

Survivability of mutant strains during oxidative stress corresponded with enzymatic activities of NADPH producing enzymes. Higher stress durability of OE1 and OE5 may be also contribute to higher PHA content of mutant strains.

Molecular weight of PHB of some mutant strains (especially OC1) was higher as compared to wild strain. All the mutant strains exhibited oxidative stress like metabolic response that might contribute to higher weight of PHB of mutants.

Extracellular secretion of mutant strains was purified with several methods. Purification of extracellular secretion with ultrafiltration leads to significant concentration of the active components of secretion with high recovery. This method is best suited for extracellular secretion purification.

It can be concluded that biotechnologically perspective mutagens capable of PHA overproduction can be prepared by application of chemical mutagens. Further molecular characterization of selected mutant strains at the gene level may shed more light on PHA overproduction of these mutants.

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7 LIST OF ABBREVIATIONS AND SYMBOLS

DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
GC-FID	Gas chromatography with flame ionization detector
GPC	Gel Permeation Chromatography
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
PHA	Polyhydroxyalkanoate
PHB	poly(3-hydroxybutyrate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
P4HB	Poly(4-hydroxybutyrate)
SCL PHA	Short-chain-length PHA
MCL PHA	Medium-chain-lenght PHA
PP	Polypropylene
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
EMS	Ethyl methane sulphonate
MMS	Methyl methane sulphonate
DCW	Dry cell weight
CFU	Colony-forming unit
ADH	Alcohol dehydrogenase
G6PD	Glucose-6-phosphate dehydrogenase
PDI	Polydispersity index

8 APENDIX

8.1 Calibration of methods

8.1.1 Dry cell weight determination

Calibration of relationship between optical density and dry cell weight (DCW) was determined by measuring optical density of cell suspension (λ = 630 nm) after suitable dilution. The suspension original dry weight was determined gravimetrically in 3 parallel experiments.

Table 41: *Gravimetrical determination of DCW*

Dry weight (g)	Dry cell weight ($g \cdot l^{-1}$)
0,0445	4,45
0,0474	4,74
0,0465	4,65
mean	4,61±0,12

Table 42: *Absorbance of cell suspension at 630 nm*

Absorbance (630 nm)	Dry cell weight ($g \cdot l^{-1}$)
1,892	0,4610
1,669	0,4149
1,481	0,3688
1,271	0,3227
1,146	0,2766
0,957	0,2305
0,769	0,1844
0,582	0,1383
0,441	0,0922
0,191	0,0461

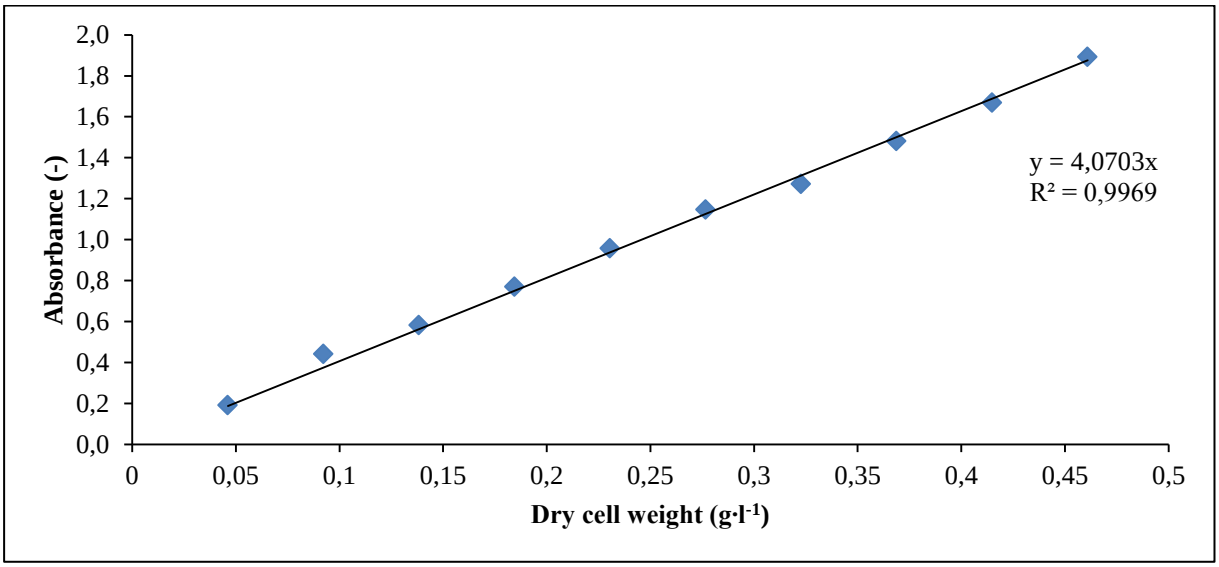

Figure 53: *Relationship between absorbance (630 nm) and dry cell weight*

Standard curve was constructed from relationship between absorbance at 630 nm and dry cell weight (DCW). Curve equation was y = 4,0703x. This equation was used for calculation of DCW of all samples. Regression coefficient was 0,9969.

8.1.2 PHA determination by gas chromatography with FID detection

This method is based on acidic extraction, hydrolysis, and methylation of PHA to 3-hydroxy fatty acid methyl esters. These esters are afterwards separated and analyzed by GC.

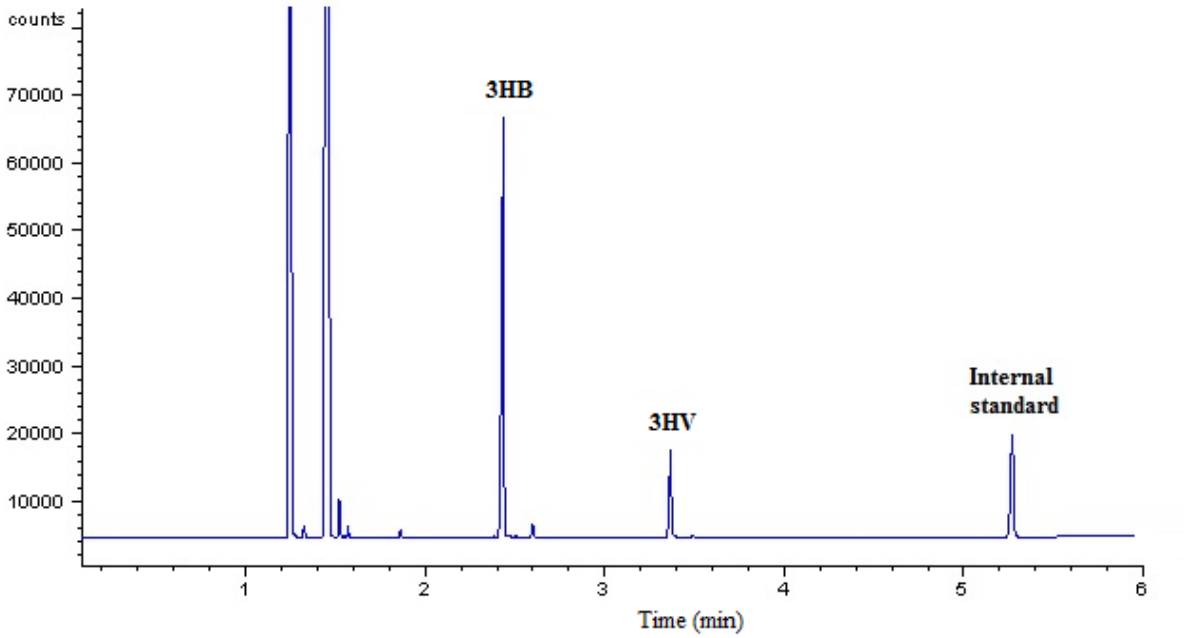

Figure 54: *Example of chromatogram of PHA sample*

3HB and 3HV peaks were integrated for peak area determination. Standard curve was constructed from relationship between peak area and 3HB (3HV) concentration.

Table 43: *Relationship between 3HB (3HV) peak area and 3HB (3HV) concentration*

3HB (mg·ml^{-1})	3HB Area (-)	3HV (mg·ml^{-1})	3HV Area (-)
2,0592	16133,8	0,2808	3076,1
4,1272	31629,3	0,5628	6159,2
6,1952	44894,3	0,8448	8801,3
8,2632	65493,6	1,1268	12837,5
10,296	77344,3	1,4040	15265,1

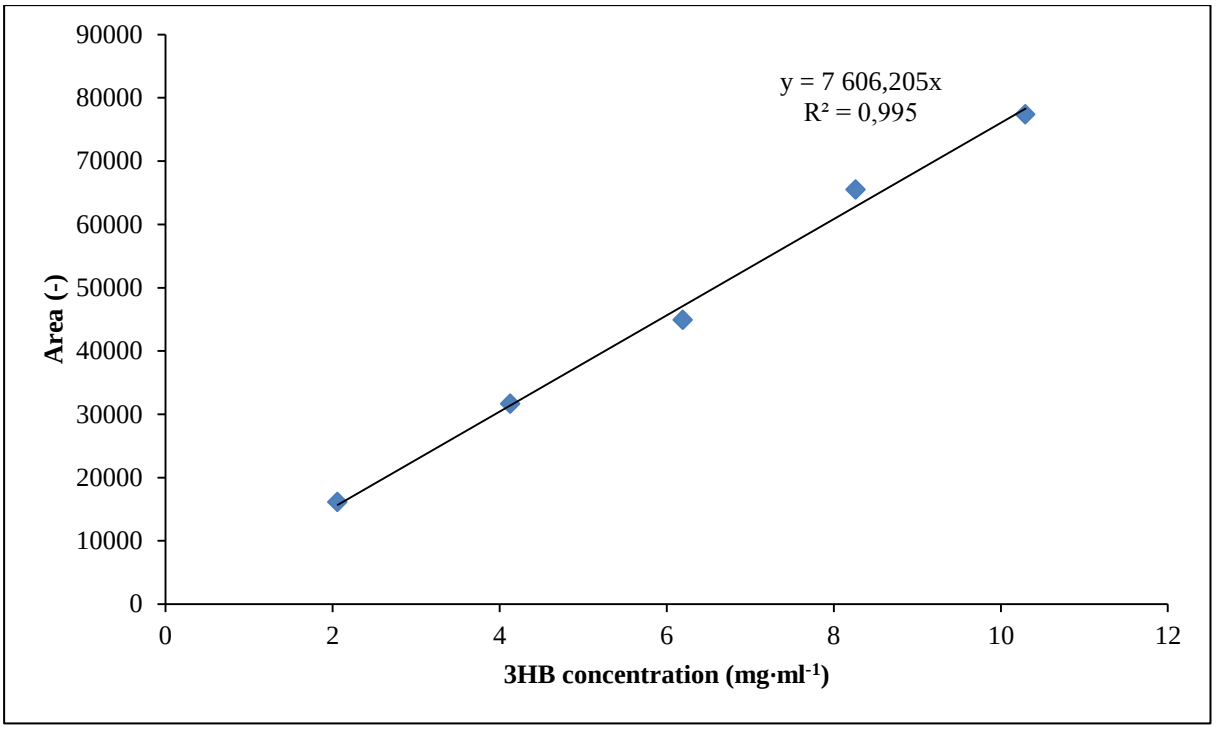

Figure 55: *Relationship between peak area and 3HB concentration*

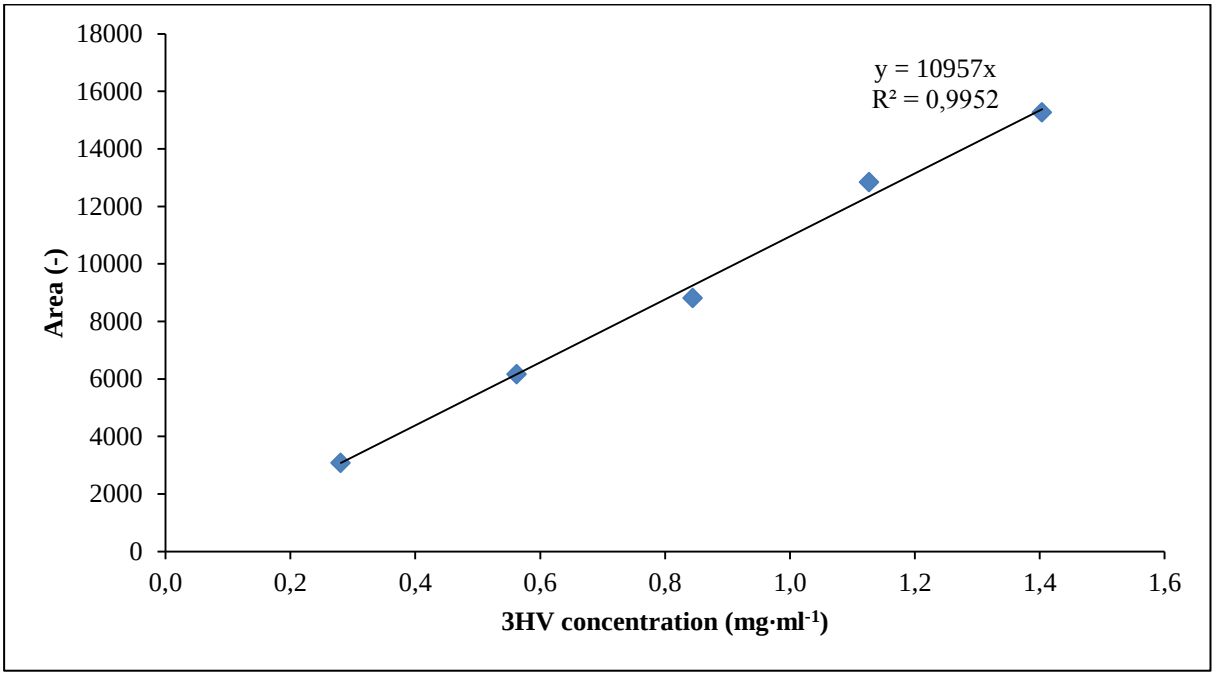

Figure 56: *Relationship between peak area and 3HV concentration*

8.1.3 NADH standard curve determination

Standard curve was constructed from relationship between absorbance at 340 nm and NADH concentration.

Table 44: *Relationship between absorbance and NADH concentration*

Absorbance (340 nm)	NADH concentration (mmol·l^{-1})
1,661	0,50
1,479	0,45
1,405	0,40
1,106	0,35
0,966	0,30
0,801	0,25
0,589	0,20
0,480	0,15
0,314	0,10
0,162	0,05

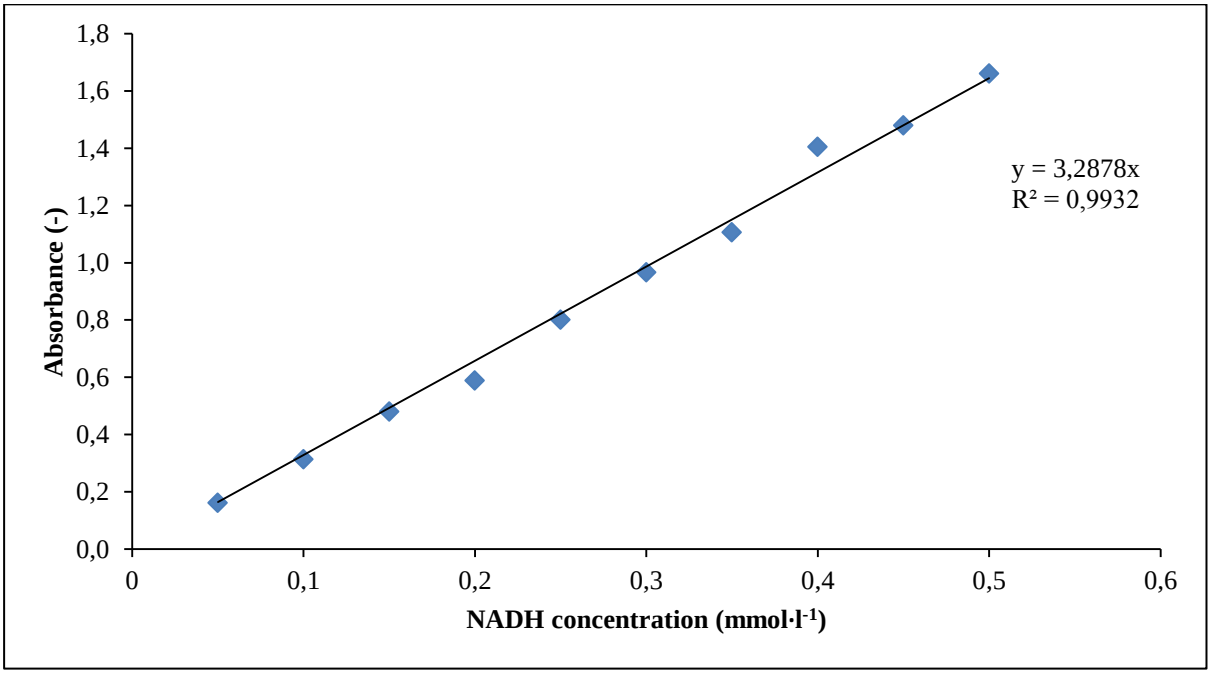

Figure 57: *Relationship between absorbance (340 nm) and NADH concentration*

8.1.4 CoA standard curve determination

Thiol group of CoA reacts quantitatively with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and forms a yellow product. Amount of product can be quantified measuring its absorbance at 405 nm. Standard curve was constructed from relationship between absorbance at 405 nm and CoA concentration.

Table 45: *Relationship between absorbance and CoA concentration*

Absorbance (405 nm)	CoA concentration (mmoll-1)
0,000	0
0,956	0,1
1,882	0,2
2,652	0,3
3,424	0,4

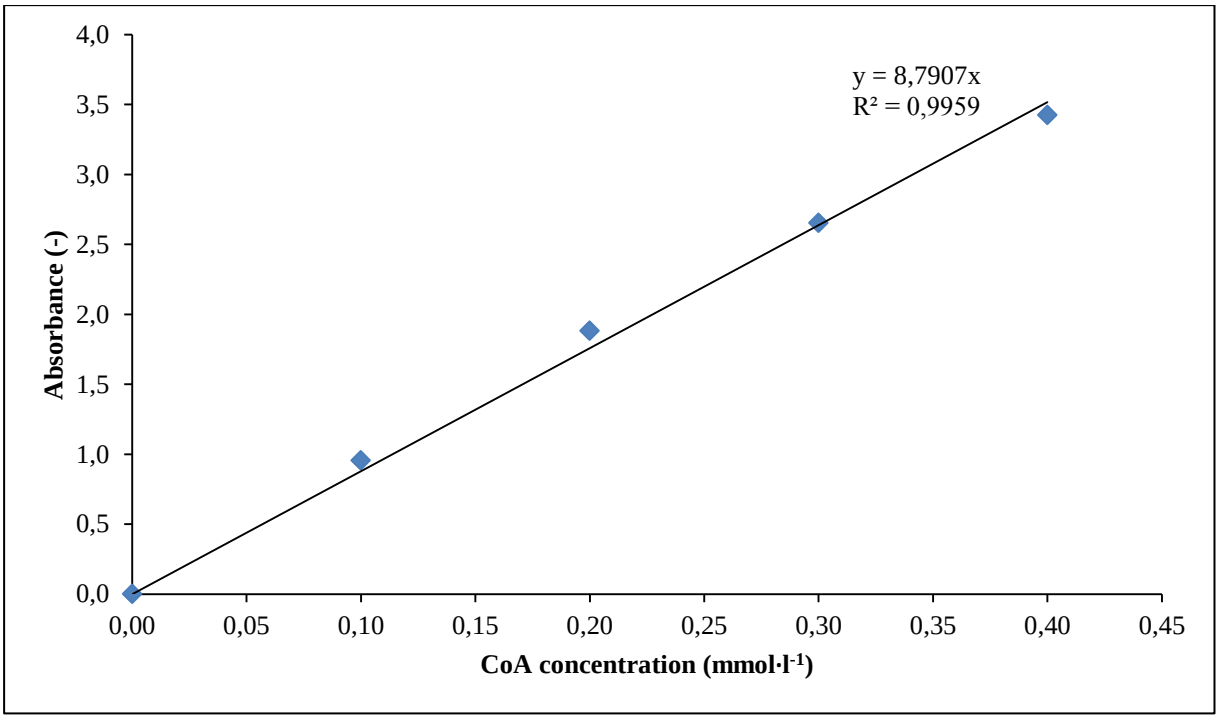

Figure 58: *Relationship between absorbance (405 nm) and CoA concentration*

8.1.5 Total proteins concentration determination by Hartree-Lowry assay

Concentration of total proteins was determined by Hartree Lowry assay. Relationship between absorbance and protein concentration was determined by measuring absorbance of protein samples of various concentrations at 750 nm.

Table 46: *Relationship between absorbance and protein concentration at 750 nm*

Absorbance (750 nm)	Protein concentration (g·l^{-1})
0,088	0,04
0,236	0,08
0,419	0,12
0,524	0,16
0,678	0,20
0,791	0,24
0,897	0,28
1,002	0,32
1,112	0,36
1,210	0,40

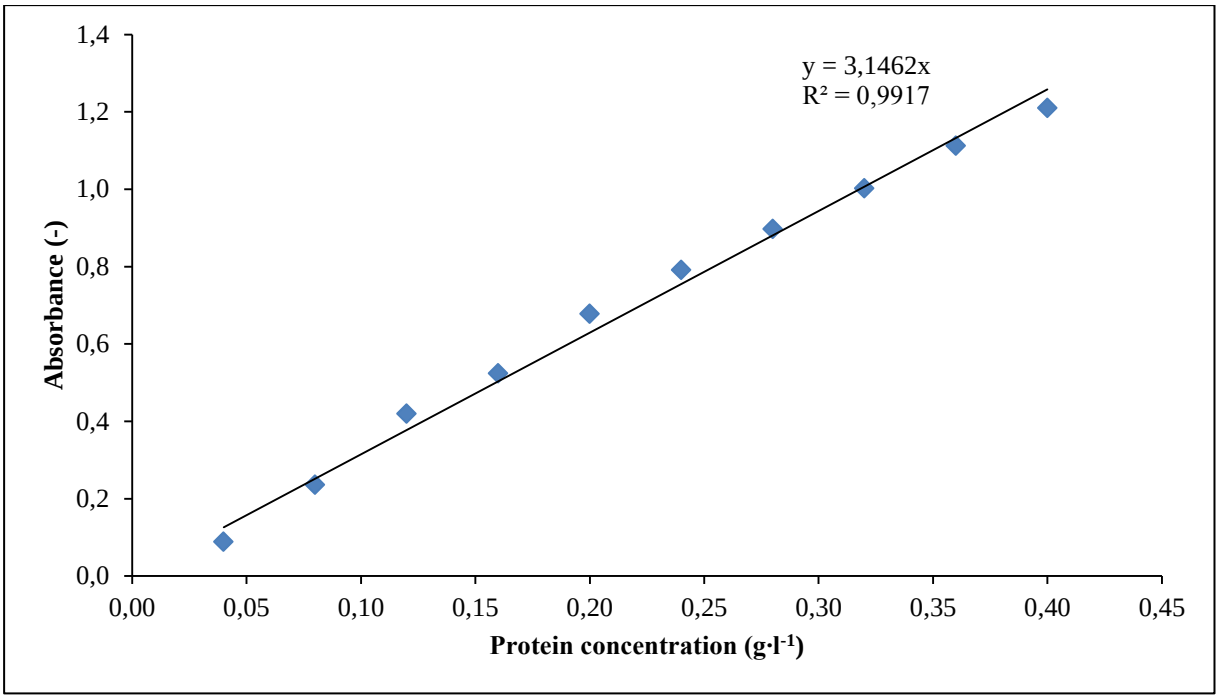

Figure 59: *Relationship between absorbance (750 nm) and protein concentration*

Standard curve was constructed from relationship between absorbance at 750 nm and protein concentration. Curve equation was y = 3,1462x; this equation was used for calculation of protein concentration of all samples. Regression coefficient was 0,9917.