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CONTROLLED PRODUCTION AND DEGRADATION OF SELECTED BIOMATERIALS

Doctoral thesis statement for obtaining the academic title of "Doctor", abbreviated to "Ph.D."

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CONTENT

SUMMARY

Proposed work is aimed at the study on production and degradation of polymeric materials using microorganisms. In the production way, the main attention is given to polyesters of bacterial origin – polyhydroxyalkanoates. These materials are accumulated by a wide variety of bacterial strains which use polyhydroxyalkanoates as a storage of carbon, energy and reducing power. Thanks to their mechanical properties, which are similar to those of traditional synthetic plastics such as polyethelene or polypropylene, and thanks to their biodegradability, polyhydroxyalkanoates are considered to be environmental-friendly alternative to traditional plastics of petrochemical origin. Thus, polyhydroxyalkanoates have many potential applications in industry, agriculture as well as in medicine.

Important part of this work is focused on production of polyhydroxyalkanoates from waste substrates coming from food industry. Among tested substrates were waste cheese whey and waste plant edible oils of different origin. Utilization of cheap waste substrates for polyhydroxyalkanoates production could facilitate economically feasible process of large scale production of polyhydroxyalkanoates. According to the results, waste oils are very promising substrates for biotechnological production of polyhydroxyalkanoates.

Next part of the thesis deals with involvement of polyhydroxyalkanoates into stress response of bacteria. It was observed, that exposition of bacterial culture to controlled dose of ethanol or hydrogen peroxide resulted in significantly enhanced yields (about 30 %). After stress factors application, particular metabolic pathways involved in stress response were activated in order to endure stress conditions. Subsequently, ratio $NAD(P)H/NAD(P)^+$ increased and, thus, Krebs cycle was partially inhibited whereas polyhydroxyalkanoates synthetic pathway was activated. Moreover, application of stress factors increased molecular weights of polymers. Therefore, strategy based on application of controlled dose of stress factors not only enhanced polymer yields, but, moreover, improved properties of materials.

The last part of thesis is focused on biodegradation experiments. The investigation of biodegradation of polyurethane elastomeric films modified by various biopolymers in presence of mixed thermophillic culture as a model of natural bacterial consortium is discussed. The presence of materials in cultivation medium resulted in delayed but intensive growth of bacterial culture. The unusually long lag-phase was caused by release of un-reacted polyether polyol and tin catalyst from materials. The main part of material degradation was caused by abiotic degradation of elastomeric films, nevertheless, also bacterial culture slightly contributed to material decomposition. The measure of biotic degradation strongly depended on the type of used modification agent. The highest tendency to undergo biotic degradation was observed for elastomeric film modified by acetylated cellulose.

1. CURRENT KNOWLEDGE OF THE STUDIED PROBLEMATIC

1.1. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are polyesters of natural origin accumulated in the form of intracellular granules by a wide variety of bacterial strains. The first example of PHA to be discovered was poly(3-hydroxybutyrate) (PHB). In 1926, Lemoigne isolated and characterized PHB from bacterial strain *Bacillus megaterium*. Since that, PHA accumulation ability has been reported for many microorganisms including Gram-negative and Gram-positive species (i.e. autotrophic, heterotrophic and phototrophic microrganisms, aerobes and anaerobes) as well as for some archae strains. Microorganisms usually synthesize and accumulate PHA as carbon, energy and reducing power storage material under conditions of the nutrients limitation (such as nitrogen, phosphorus or iron limitation) and in presence of excess of carbon source. Under the conditions of carbon source starvation, PHA are degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy sources [1].

Homopolymer PHB, the best characterized member of wide family of polyhydroxyalkanoates, is not only present in microorganisms as storage material but is also very ubiquitous in nature in different roles. A fascinating development in recent years has been the discovery of the very wide distribution of PHB as a low molecular weight oligomer (120-200 monomers units) in microorganisms, plants and animal, including humans. In many cases this form of PHB is found as a PHB–calcium polyphosphate complex in membranes that seems to function as an ion channel through cell membranes. In *Escherichia coli*, the complex has been found to occur in large amounts in the membrane of cells made competent for genetic transformations [1].

1.1.1. Biosynthesis of polyhydroxyalkanoates

The biochemistry of SCL-PHA biosynthesis is well studied [1, 2]. Bacterial strain *Cupriavidus necator* H16 (formerly known as *Hydrogenomonas eutropha, Alcaligenes eutrophus, Ralstonia eutropha and Wautersia eutropha)* is often in use as a model organism for SCL-PHA metabolism. In *C. necator*, PHB is synthesized in a three-step reaction starting with acetyl-CoA when cultivated on carbohydrates, pyruvate, or acetate. Two acetyl-CoA molecules are coupled to form acetoacetyl-CoA in a condensation reaction catalyzed by β-ketothiolase. The product is subsequently stereoselectively reduced to *R*-3-hydroxybutyryl-CoA in a reaction catalyzed by NADPH-dependent acetoacetyl-

CoA reductase. Finally, PHB is synthesized by polymerization of *R*-3-hydroxybutyryl-CoA molecules by the SCL-PHA synthase [3].

A few exceptions to this general pathway are known, such as *Rhodospirillum rubrum*, where *S*-3-hydroxybutyryl-CoA is generated and converted to *R*-3-hydroxybutyryl-CoA by two stereospecific enoyl-CoA hydratases [1].

A copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate can be synthesized by *C. necator* and many other microorganisms from either a mixed substrate of glucose and propionic acid or a direct precursor of 3-hydroxyvalerate (e.g., valeric acid). If propionate is fed, essentially the same biochemical pathway as for PHB synthesis is used, but propionyl-CoA and acetyl-CoA are condensed by the β-ketothiolase to give 3-ketovaleryl-CoA, which leads to the incorporation of 3-hydroxyvalerate monomers into the polymer. Alkanoic acids of odd number carbon chain length can also serve as carbon source. In this case, the 3-hydroxyvalerate in the polymer arises directly from the β-oxidation of these fatty acids [1, 4].

1.1.2. Involvement of polyhydroxyalkanoates in stress response of bacteria

Although it is generally assumed that the ability of PHA synthesis improves bacterial stress resistance, the reasons and mechanisms of PHA involvement in stress response have not been fully understood yet. Ayub et al. studied cold resistance of Antarctic bacterium *Pseudomonas* sp. 14-3 [5]. Its stress tolerance was analyzed in PHA accumulating and non-accumulating conditions and significantly higher levels of stress resistance were observed when PHA were produced. In the recent study, the same authors tested PHA synthase negative mutants of *Psedomonas* sp. 14-3 in order to investigate the mechanism of involvement of PHA in stress resistance [6]. Mutant strain was unable of growing at 10° C and was more susceptible to freezing than its parental strain. PHA were necessary for the development of the oxidative stress response induced by cold treatment. The NADH/NAD^+ ratio and NADPH content decreased strongly in the mutant while only minor changes were observed in parental strain. Authors proposed that PHA metabolism modulated the availability of reducing equivalents, contributing to alleviate the oxidative stress produced by low temperature.

The connections between PHA and stress endurance were demonstrated in many other cases. Mutant strain of *Aeromonas hydrophila* 4AK4 unable of PHA production and wild type were tested under stress conditions. It was found that ability of PHA synthesis improved resistance against environmental stress factors such as heat and cold treatment, hydrogen peroxide, UV irradiation, ethanol and high osmotic pressure [7]. In other studies, wild type of

Azospirillum brasilense Sp7 was compared with PHA synthase minus and PHA depolymerise minus mutant strains that were unable of synthesis and degradation of PHA, respectively. The ability of the wild type to endure starvation conditions, ultraviolet irradiation, heat, and osmotic shock and to grow in the presence of hydrogen peroxide was always higher than that of the mutants [8]. Breedveld et al. reported that *Rhizobium leguminosarum* TA-1 and *Rhizobium meliloti* SU-47 cell cultures responded to osmotic stress exposition by augmenting the cellular trehalose content of the cells. Its synthesis paralleled the breakdown of the reserve materials glycogen and PHB [9].

According to studies mentioned above, it could be proposed that exposition of culture to stress conditions lead to the mobilization of PHA and resulting energy and carbon material is subsequently used for stress survival. Nevertheless, the explanation is not so simple, because also increases in PHA accumulation were observed in bacterial cultures exposed to stress conditions. In non-endophyte strain *Azospirillum brasilense* Sp7 heavy metals induced an enhanced accumulation of PHB. In contrast, the response of the endophytic strain *Azospirillum brasilense* Sp245 to heavy metal uptake was found to be much less pronounced [10]. Natarajan et al. reported that NaCl stress resulted in the accumulation of PHB in *Rhizobium* DDSS-69 cultures grown under unbalanced growth conditions [11].

Interesting contribution was given by Wang et al. [12] who tested stress durability of recombinant strains of *E. coli*. The first of tested strains harbored only PHA synthetic genes whereas the second recombinant strain harbored PHA synthetic genes and intracellular PHA depolymerase as well. It was observed that both recombinant strains were more resistant against heat shock, UV radiation, acid and osmotic pressure than wild type of *E. coli* unable of PHA synthesis. However, recombinant strain which was able to synthesize as well as degradate PHA was slightly more resistant than strain unable of PHA degradation. Thus, only the presence of PHA in cytoplasm enhanced stress resistance to stress factors and ability of PHA degradation even improved this effect. Nevertheless, mechanisms have not been understood yet.

Actually, more is known about PHA involvement in stress resistance of *Pseudomonas*. Ruiz et al. studied stress resistance in carbon starved cells of *Pseudomonas oleovorans* [13]. It was observed that PHA degradation is accompanied by an increase of ATP and guanosin tetraphosphate (ppGpp) levels in cells. ppGpp serves in cells as nutritional alarmone, which significantly enhances expression of *rpoS* gene and, therefore, plays important role in stress resistance regulation. In following study the same authors observed, that carbon starvation resulted in PHA degradation, which was accompanied by increased expression of *rpoS* gene and enhanced tolerance to H_2O_2 and heat shock [14]. Thus, PHA seem to be involved in stress response under conditions of carbon starvation through RpoS, which consequently enhanced stress tolerance of culture. However, RpoS and PHA seem to be even closely connected. In

comparison of *rpoS* negative mutant and wild type of *Psudomonas putida*, mutant strain showed a higher PHA degradation rate. These results suggested, that RpoS might control the genes involved in PHA metabolism [15]. Similar results were obtained by Schuster et al. who looked for genes under control of RpoS in *Pseudomonas aeruginosa*. One of such genes was gene *phaC2* encoding for PHA synthase [16].

1.2. Biodegradation of polymeric materials

Microorganisms are involved in the deterioration and degradation of both synthetic and natural polymers. Up to now, little is known about the biodegradation of synthetic polymeric materials. It is due to the recent development and manufacture of this class of materials and the relatively slow rate of degradation in natural environments. Since chemically synthesized polymeric materials have become an important part of our human society and have more diversified applications than traditional metals, issues related to polymer deterioration and protection will receive increasingly attention in the time to come. Polymeric materials are very unique in chemical composition, physical forms, mechanical properties and applications. High versatility of the carbon to carbon and carbon to non-carbon (C–C, C–R and C–H) bonds and substituent groups, the possible configurations, stereochemistry and orientation provide basis for variations of chemical structures and stereochemistry. Very small variations in the chemical structures may result in large differences in term of biodegradability [17].

Polymers are potential substrates for heterotrophic microorganisms including bacteria and fungi. At least two categories of enzymes are actively involved in biological degradation of polymers: extracellular and intracellular depolymerases. During degradation, exoenzymes from microorganisms break down complex polymers yielding short chains or smaller molecules, e.g., oligomers, dimers, and monomers, that are small enough to pass the semi-permeable outer bacterial membranes, and then to be utilized as carbon and energy sources. The process is called depolymerization. When the end products of the process are inorganic species, e.g., CO_2, H_2O or CH_4 the degradation is called mineralization [18]. A commonly recognized rule is that the closer the similarity of a polymeric structure to a natural molecule is, the easier it is to be degraded and mineralized. Polymers such as cellulose, chitin, pullulan, or PHA are all biologically synthesized and can be completely and rapidly biodegraded by heterotrophic microorganisms in a wide range of natural environment. It is important to point out that mineralization of polymeric substrate can rarely reach 100 % and the reason is that small portion of the polymer will be incorporated into microbial biomass, humus and other natural products [17, 18].

1.2.1. Biodegradation of polyurethanes

After years of PUR production, manufacturer's found them susceptible to degradation. Originally, most studies on the microbial degradation of PUR had been performed with the aim to prevent PUR from microbial hazards, and not much research had been focused on the enzymes or biochemical mechanisms involved in PUR degradation by microorganisms. Nevertheless, with increasing focus on environmental impact of PUR production, biochemical aspects has became more important. PUR biodegradation ability was observed in fungal, bacterial and yeasts strains as well [19].

PUR can be divided into two families according to the type of used polyol: polyester polyol based PUR and polyether polyol based PUR. Generally, it was observed that polyester PUR is relatively easily biodegradedable and, on the contrary, polyether PUR is relatively resistant to enzymatic attack. Labrow et al. treated both polyester and polyether PUR with human neutrophil elastase and porcine pancreatic elastase [20]. The rate of polyester PUR degradation by porcine pancreatic elastase was 10 times higher than its activity against the polyether PUR. Furthermore, human neutrophill elastase had no significant activity against the polyether PUR. Similarly, Jansen et al. reported that some kinds of polyether based PUR were degraded by *Staphylococcus epidermis* KH11, but the degradation progressed very slowly [21]. The integrity of polyether PUR foam was investigated by Urgun-Demirtas et al. using short-term accelerated laboratory experiments including bioavailability assays, soil burial experiments, and accelerated bioreactors to determine the fate of PUR foam in the soil where anaerobic processes are dominant [22]. The experimental results have shown that the polyether PUR foam is likely not biodegradable under anaerobic conditions. The relative resistance of polyether PUR to microbial degradation is considered to be due to its degradation mechanism, which involves exo-type depolymerization, whereas that of polyester PUR degradation involves endo-type depolymerization [23].

2. THE AIMS

The first goal of proposed work was to study biotechnological production of selected biomaterials, particularly polyhydroxyalkanaotes, employing selected bacterial strains. Further, the second part of the work deals with process of biodegradation of polymeric materials, especially polyurethanes.

In order to reach goals of the thesis, they can be divided into the following partial aims:

- Review on current knowledge of problematic – biodegradation and biotechnological production of polymeric materials.

- Study on stability and process of biodegradation of selected types of biomaterials and composites, particularly modified polyurethanes. Comparison of biodegradability of polyurethane elastomeric films and polyurethane foams.

- Utilization of various waste substrates for production of selected biomaterials, especially polyhydroxyalkanoates.

- Study on involvement of polyhydroxyalkanoates into stress response of bacteria. Evaluation of potential strategy based on application of controlled stress conditions to improve biotechnological production of bioplastics.

3. MATERIALS AND METHODS

3.1. Bacterial strains

Both *Bacillus megaterium* CCM 2037 and *Cupriavidus necator* H16 (CCM 3726) were purchased from Czech Collection of Microorganisms. Mixed aerobic thermophillic culture was obtained for sludge of waste water treatment in Bystřice pod Hostýnem.

3.2. Cultivations in Erlenmeyer flasks

Cultivations were carried out in flasks under permanent shaking (usually 150-200 rpm), temperature was set at 30°C (*C. necator* and *B. megaterium*) and 60°C (thermophillic mixed culture). For inoculation, 24 hour inoculums were used. In experiments focused on PHA production under stress conditions, stress factors were applied at particular times of cultivations to get desired concentration of stress factor in medium. Further details are provided in particular manuscripts and papers attached to the thesis (Chapter 9).

3.3. PHA production in laboratory fermentor

Fermentor vessel (2 l, BioFlo Celligen 115, New Brunswick) containing 1.2 l of MS medium with waste rapeseed oil (20 g l^{-1}) was inoculated with 60 ml of 24 h culture. The culture temperature was set at 30° C, pH was maintained at 7 by 0.5 M $NaOH/H_2SO_4$. The dissolved oxygen (DO) concentration was monitored with DO electrode and was maintained at the value of 50 % to air saturation by varying the agitation speed and/or air flow rate automatically.

3.4. Analytical methods

3.4.1. Biomass determination

Concentration of biomass was estimated by measuring the absorbance of culture broth at 630 nm on Helios Alpha (Unicam, UK). Calculation was done using the calibration curve (cell dry weight vs. absorbance) prepared by dilution of sample of known cell dry weight content. For cell dry weight determination, the cells obtained after centrifugation (8 000 rpm, 10 min.) were dried (105°C) until constant weight was obtained.

3.4.2. Analysis of polyhydroxyalkanoates

PHA content was determined by gas chromatography with mass spectrometry or flame ionization detection. PHA were transformed to methyl-esters of particular hydroxyacids in presence of methanol and sulfuric acids. Resulting methyl- esters were analyzed using gas chromatography with mass spectrometry or flame ionization detector.

Molecular weight data were obtained by gel-permeation chromatography after extraction of PHA using chloroform and precipitation of PHA using mixture of methanol and water (7:3).

3.4.3. Activities of enzymes of PHA biosynthetic pathway analysis

The activities of β-ketothiolase (β-KT) and acetoacetyl-CoA reductase (AACR) were analyzed based on measurement of absorbance of acetoacetyl-CoA at $A_{303\ nm}$ and NADPH at $A_{340\ nm}$ in crude cell extract. The activity of PHB synthase was determined using measurement of the absorbance of released CoA at $A_{412\ nm}$. The activity of glucose-6-phosphate dehydrogenase and $NAD(P)^+$ dependent alcohol dehydrogenase was analysed by measuring the released NAD(P)H at $A_{340\ nm}$. For protein concentration Bradford method was used.

3.4.4. Analysis of residual nutrients in cultivation medium

Residual sugars were estimated by Somogyi-Nelson method. The content of residual ammonia nitrogen was determined using Nessler reagent by measuring the absorbance of diluted sample at 436 nm. Concentration of residual oil in cultivation media was analyzed gravimetrically, propanol concentration was measured as amount of NADH (A_{340nm}) formed after 5 min treatment of sample with 30 units of alcohol dehydrogenase.

3.5. Biodegradation experiments

A series of batch cultures was set up to screen PUR biodegradability. In a batch assays, plugs of PUR were placed in Erlenmeyer flask containing medium for cultivation of mixed thermophillic culture. Flasks were kept under 60°C and constantly shaken (120 rpm). For each of PUR, six identical samples were used. Three of them were inoculated by bacterial culture and three flasks were used as controls. Bacterial growth was monitored during the test. At the end of cultivation (about 300 hours) PUR weight losses were measured gravimetrically and changes of material surfaces were analyzed microscopically.

4. RESULTS AND DISCUSSION

Proposed PhD. thesis is in the form of commented articles, manuscripts and abstracts. The articles and abstracts have already been published; manuscripts have been submitted or accepted for publication. All materials are attached to thesis. In this chapter, detail links to particular articles or manuscripts are always provided.

4.1. Screening for strategies improving PHA production process

Despite many satisfactory properties of polyhydroxyalkanoates, there are still several problems preventing them from entering everyday life of consumers. Of course, the main reasons are economical, because the final price of polyhydroxyalkanoates is still too high to allow their wider application in many fields of industry. Therefore, a lot of effort has been made in research on PHA in last two decades and a lot of concern is aimed at improvement of processes of PHA production and, thus, reduction of their cost. A number of studies have been focused on looking for new candidates for industrial production of PHA, utilization of cheap carbon sources including those of waste origin and also many works have aimed at optimization of medium composition and cultivation conditions to reach maximal PHA yields. Moreover, promising strategies involve genetic engineering of microorganisms and plants to introduce or improve particular biosynthetic pathways [24].

In order to contribute to development of economically feasible process, we also tried testing and comparing few strategies with potential to decrease PHA price and improve the process of PHA production. Our first experiments were carried out with *Bacillus megaterium* CCM 2037 (culture was purchased from Czech Collection of Microorganisms). At first, we tested the influence of various limitations (nitrogen, phosphorus or combination of both) applied at the beginning of stationary phase. Secondly, the new strategy of exposition of bacterial culture to exogenous stress in order to enhance PHB production was screened. Finally, various media including waste cheese whey or starch-based medium were introduced to test whether *B. megaterium* could be used for biotechnological production of PHA using cheaper carbon substrate. The results obtained in this work were published ***(Obruca et al., Chemicke Listy 102 (2008) p. 1255-1256)***.

Nitrogen and phosphorous limitation is widely used for enhancement of PHA yields, on the contrary, application of exogenous stress factors in order to support PHA accumulation would be a new approach in PHA production. Controlled stress conditions are known to enhance microbial production of several industrially interesting metabolites such as carotenoids [25] proline [26] or ectoines [27], but to our knowledge only Natarajan et al. used controlled

osmotic pressure to increase PHB yields in *Rhizobium* DDSS-69 [11]. Influences of other stress factors such as ethanol, heavy metals, hydrogen peroxide etc. on microbial production of PHA have not been studied yet. Hence, we applied selected stress factors (H_2O_2, ethanol, NaCl, $NiCl_2$, citrate and Na_2SO_3) at the beginning of cultivation and analyzed PHB contents in cells after 35 hours of cultivation. Because particularly ethanol and also Na_2SO_3 supported PHB biosynthesis as compared to control cultivation, we assumed that application of stress factors may have been potential strategy improving process of PHA production. Nevertheless, time of stress factor application as well as its concentration had to be optimized. Thus, the first important part of our further work was focused on PHA production under controlled stress conditions as a tool allowing improvement of PHA production process.

Bacillus megaterium is bacterial strain able to utilize wide range of carbon substrates and for this reason we performed cultivation of this bacterial strain on selected carbon sources. The highest PHB yields were obtained for BM medium (Bacillus Medium according to Czech Collection of Microorganisms) supplemented with glucose, however, *B. megaterium* was also able to utilize cheaper starch and, moreover, waste cheese whey. Only limited number of bacterial strains is able to turn lactose, the main organic constituent of cheese whey, directly into PHA. Thereby, next part of our work was focused on biotechnological conversion of waste cheese whey into the high value materials employing *B. megaterium* monoculture.

4.2. The influence of exogenous stress on PHA production

In our further experiments we focused on the study on influence of exogenous stress conditions on PHA production. As mentioned above, exposition of culture to particular stress factor enhanced PHB accumulation in cells. Nevertheless, the PHA contents in *Bacillus megaterium* cells as well as total PHB yields were rather low, therefore, we decided to employ other bacterial strain – *Cupriavidus necator* H16 (CCM 3726). *C. necator* H16 is considered as a model microorganism for SCL-PHA production. So that, for us it was the strain of choice to study the connection between stress response to particular stress and PHA metabolism. In addition, biomass and PHA productions in *C. necator* are much higher as compared to *B. megaterium*.

Another published paper ***(Obruca et al., Folia Microbiol 55 (2010) p. 17-23)***, describes the effect of different times of applications and concentration levels of selected stress factors on PHB production in *C. necator* H16. Among tested stress factors (ethanol, hydrogen peroxide, NaCl, $NiCl_2$ and $CoCl_2$) predominantly both ethanol and hydrogen peroxide enhanced PHB accumulation in cells. The stress factor concentration and the time of stress application were observed to be crucial in terms of gained PHB yields.

It was interesting to observe that exposition of bacterial culture to mild stress resulted in unaffected biomass formation and increased PHB biosynthesis, but higher stress dose resulted in inhibited bacterial growth and lowered PHB yields. For example, application of higher osmotic stress (5 $g.l^{-1}$of NaCl) significantly reduced PHB content in cells as compared to control culture (55.3 % stressed culture and 65.2 % control). This may have been caused by inhibition of PHB biosynthetic pathway in stressed culture and simultaneous activation of other metabolic processes related to osmotic stress response. Moreover, also partial degradation of PHB might have occurred in order to produce energy to endure osmotic stress, as was reported by Breedveld et al. [9].

Therefore, optimizations of stress factors concentrations were performed to achieve maximal PHB yields. Finally, the total yields obtained in this work were compared with those published in literature. It could be stated, that our yields belonged among the highest reported for batch cultivation of *C. necator* H16 (see **Tab. 1**). Furthermore, controlled application of stress factors not only enhanced total PHB yields, but also increased PHB content in cells. This effect would reduce costs of PHB recovery, because PHB content in cells strongly affects the efficiency and the cost of down-stream processing [29]. Hence, controlled application of either ethanol or hydrogen peroxide seems to be very effective and simple strategy to improve PHB yields and reduce its production cost.

Tab.1. *Comparison of PHB yields and PHB contents in cells obtained in our work and those published in literature for Cupriavidus necator in batch culture.*

Substrate (concentration)	Cultivation*	Biomass [g l^{-1}]	PHA [g l^{-1}]	PHA %	Reference
Fructose (40 g l^{-1})	E	13.40	6.75	50.37	Khanna & Srivastava 2005
Fructose (40 g l^{-1})	F	20.73	9.35	45.10	Khanna & Srivastava 2005
Glucose (40 g l^{-1})	E	20.50	12.70	61.95	Wang & Yu 2007
L-Thr (15 g l^{-1}), Butyric acid (5 g l^{-1})	E	13.00	5.73	44.30	Kimura et al. 2008
Glucose (20 g l^{-1})	E	8.78	2.80	31.90	Zhang et al. 2004
Glucose (20 g l^{-1})	E	3.22	2.50	77.80	Zhang et al. 2004
Fructose (20 g l^{-1}) ethanol stress	E	14.16	11.23	79.30	This study
Fructose (20 g l^{-1}) peroxide stress	E	14.30	10.90	77.41	This study

* E – erlenmmeyer flasks, F - fermentor

The enhancement of PHA production under stress conditions is interesting phenomenon with potential practical outcome, so that, we decided to look into the metabolic aspects of exposition of bacterial culture to stress conditions. Our

findings are summarized in the next paper ***(Obruca et al., World J. Microbiol. Biotechnol. 26 (2010) p. 1261-1267)***. In this work we describe the consequences of stress response of *C. necator* to ethanol and hydrogen peroxide from PHB production point of view. Application of optimal concentrations of either ethanol or hydrogen peroxide at the beginning of the stationary phase of growth had resulted in stress response of bacterial strain which subsequently increased activity of PHB biosynthetic pathway.

Exposition of bacterial culture to oxidative stress results in activation of several metabolic pathways involved in oxidative stress response. Under oxidative stress conditions, some molecules are constitutively present in cell and help to maintain intracellular reducing environment or to scavenge chemically reactive oxygen species. For instance, reduced glutathione present in cells at high concentrations maintains a strong reducing intracellular environment. Its reduced form is regenerated by glutathione reductase using NADPH as a source of reducing power. Therefore, under oxidative stress cells need to maintain high ratio NAD(P)H/NAD(P)$^+$. It was the reason why exposition of *Escherichia coli* [29] and *Saccharomyces cerevisiae* [30] to hydrogen peroxide increased activity of glucose-6-phosphate dehydrogenase, the first enzyme of pentose phosphate pathway generating NADPH for various metabolic processes. We observed, that also exposition of *Cupriavidus necator* to stress induced by hydrogen peroxide resulted in increased activity of glucose-6-phosphate dehydrogenase. Thus, it is likely that resulting high intracellular ratio NADPH/NADP$^+$ partially inhibited the Krebs cycle and supported both flux of acetyl-CoA into PHB biosynthetic pathway and activity of NADPH dependent acetoacetyl-Co reductase. This was probably the reason why exposition of *C. necator* to oxidative stress enhanced PHB biosynthesis in bacterial cells. From this point of view, enhancement of PHB accumulation in cells could be considered as a side effect of stress response. Nevertheless, according to Ayub et al., PHA are directly involved in oxidative stress response because PHA serve as a modulator of the pool of reducing equivalents NADPH and NADH in cells, which helps to alleviate oxidative stress [6]. Generally, stress response of bacteria is complex metabolic process involving number of pathways and regulation points. Also PHA seems to belong among molecules contributing to survival under harmful conditions caused by reactive oxygen species, but the exact mechanisms have not been clearly explained so far.

According to our results, ethanol pronounced PHB production in *C. necator* even more intensively than hydrogen peroxide. In order to understand this effect, we looked into connection of PHB biosynthesis and stress response to ethanol as well. Addition of ethanol into cultivation media activated alcohol dehydrogenase. Then final product of ethanol metabolization is acetyl-CoA while reduced coenzymes NAD(P)H are formed and free CoA is built into acetyl-CoA. Similarly to hydrogen peroxide, the consequence of such a stress response is partial inhibition of Krebs cycle and enhanced flux of acetyl-CoA

into PHB biosynthetic pathway. Moreover, enhanced level of reduced coenzymes supported activity of acetoacetyl-CoA reductase and decreased free CoA concentration activated β-ketothiolase.

Interconnection between stress response (ethanol and hydrogen peroxide) and PHB biosynthetic pathway of *C. necator* is displayed in **Fig. 1**.

Application of both stress factors enhanced PHB yields and activities of β-ketothiolase and acetoacetyl-CoA reductase, but did not affect activity of the last enzyme of the PHB biosynthetic pathway – PHB synthase. These results indicate that the key enzymes regulating PHB content in cells are β-ketothiolase and/or acetoacetyl-CoA reductase rather than PHB synthase. This conclusion is in consistence with results of Oeding and Schlegen [31] as well as Senior and Dawes [32]. In both works they suggested β-ketothiolose rather than PHB synthase being the crucial enzyme regulating PHB synthesis in cells. On the contrary, Doi et al. proposed acetoacetyl-CoA reductase to be the main enzyme regulating PHB content in cell [33]. It is also possible that both enzymes are involved equally in regulation of PHB synthetic pathway. However, the fact that the application of ethanol or hydrogen peroxide enhances activities of both of them is beneficial in terms of production parameters.

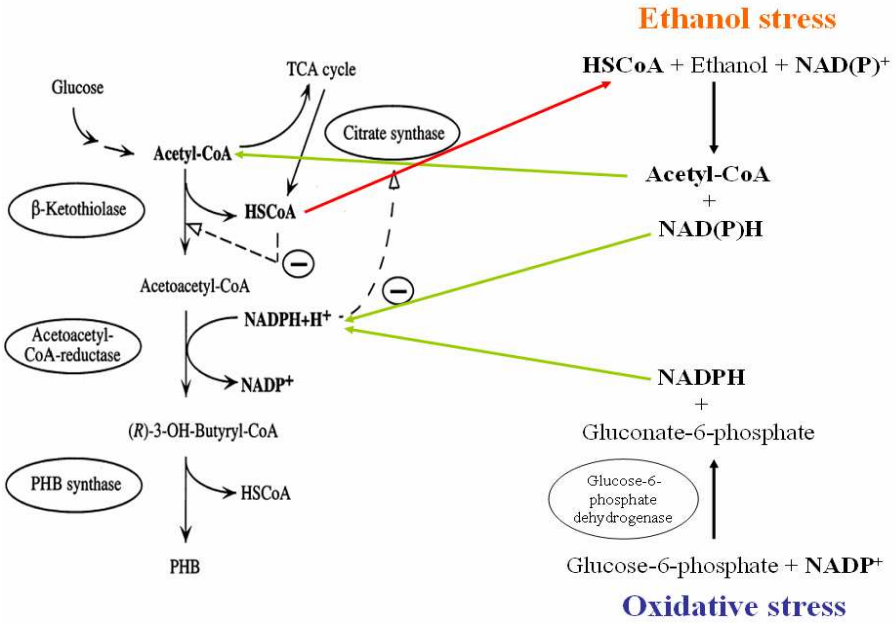

***Fig.1**. Schema of interaction between stress response (ethanol and oxidative pressure) and PHB biosynthesis.*

Although the exposition of culture to stress did not influence activity of PHB synthase, which is considered to be enzyme responsible for length of polyester chain, molecular weights of materials produced under stress conditions were observed to be significantly higher as compared to control. Our results are similar to those of Kichise et al. who reported that molecular weight of PHB is independent of PHB synthase activity [34]. Oppositely, *in vitro* experiments of Gerngross and Martin demonstrated that molecular weight of PHB decreased

with increase in the initial activity of PHB synthase [35]. Kawaguchi and Doi proposed the presence of chain-transfer agent generated in *Cupriavidus necator* cells which reacts with propagating polyester chain to regulate the chain length of PHB. In vivo, this regulating factor is probably more important than activity of PHB synthase [36]. Our results could indicate that besides PHB synthase also β-ketothiolase and acetoacetyl-CoA reductase may contribute to regulation of molecular weight of PHB. Potential explanation could be that increased intracellular level of 3-hydroxybutyryl-CoA, the consequence of increased activities of β-ketothiolase and acetoacetyl-CoA reductase, might be another factor stimulating PHB synthase to form longer polyester chains.

Generally, the fact that controlled application of stress conditions leads to enhanced PHB yields could bring practical biotechnological outcomes. Simple addition of cheap substance enhanced PHB yields about 30 %. Moreover, molecular weight of PHB produced under stress conditions was significantly higher. Thereby, application of ethanol and hydrogen peroxide did not only enhance PHB yields, but also improved mechanical properties of produced material. Furthermore, in the manuscript attached as ***(Obruca et al. Biotechnol. Letters, Accepted for Publication)*** we reported that the application of propanol had similar effect on PHA biosynthesis in terms of increased PHA yields, but, in addition, also other monomer unit - 3-hydroxyvalerate - was built into polyester chain. This again significantly improves mechanical properties of PHA. For instance, incorporation of 10 % of 3-hydroxyvalerate increases elongation to break from 3 % (homopolymer PHB) to 20 % (copolymer P(HB-co-HV)) [1].

To our knowledge, the use of propanol as a precursor of 3-hydroxyvalerate in P(HB-co-HV) has not been reported so far. We assumed that the way of propanol metabolization in *C. neactor* is the same as that of ethanol. Hence, propanol is oxidized and, finally, transformed to propionyl-CoA. During oxidation, reduced coenzymes are formed, which again supports PHA synthesis in the same way as in ethanol. Further, propionyl-CoA is coupled with acetyl-CoA in reaction catalyzed by β-ketothiolase. β-ketothiolase encoded by gene *phbA* is specific only for acetyl-CoA, therefore, this step must be catalyzed by enzyme encoded by gene *bktB* which is placed out of *phaCAB* operon of *C. necator* [37]. Following NADPH dependent stereospecific reduction of 3-ketovareryl-CoA could be catalyzed by acetoacetyl-CoA, nevertheless, the rate of 3-ketovaleryl-CoA reduction is only 16 % of that of acetoacetyl-CoA [38]. The final step of copolymer synthesis is catalyzed by PHB synthase (also called PHA synthase) which is, thanks to its low substrate specificity, able to built 3-hydroxyvalerate units into PHA structure. Mechanism that we propose for the metabolization of propanol and its subsequent incorporation (*via* propionate and 3-hydroxyvaleryl-CoA) into PHA structure is shown in **Fig. 2**.

To conclude this chapter, exposition of culture to controlled stress conditions caused by alcohols (ethanol or propanol) and hydrogen peroxide could be used as a novel strategy for improvement of PHA production process

as well as mechanical properties of produced materials. It can be assumed, that the mechanism of stress response against these stress factors is more or less similar in most of bacteria. Thus, this strategy could be effective also in other PHA producing strains than in *C. necator* and might be used also for improvement of PHA production from waste cheep substrates. We believe that synergic effect of application of cheap stress factor enhancing PHA yields and utilization of cheap waste substrates could reduce final cost of polyesters and make them more susceptible for various application and consumers. Therefore, in our next work we focused on PHA production using waste substrates.

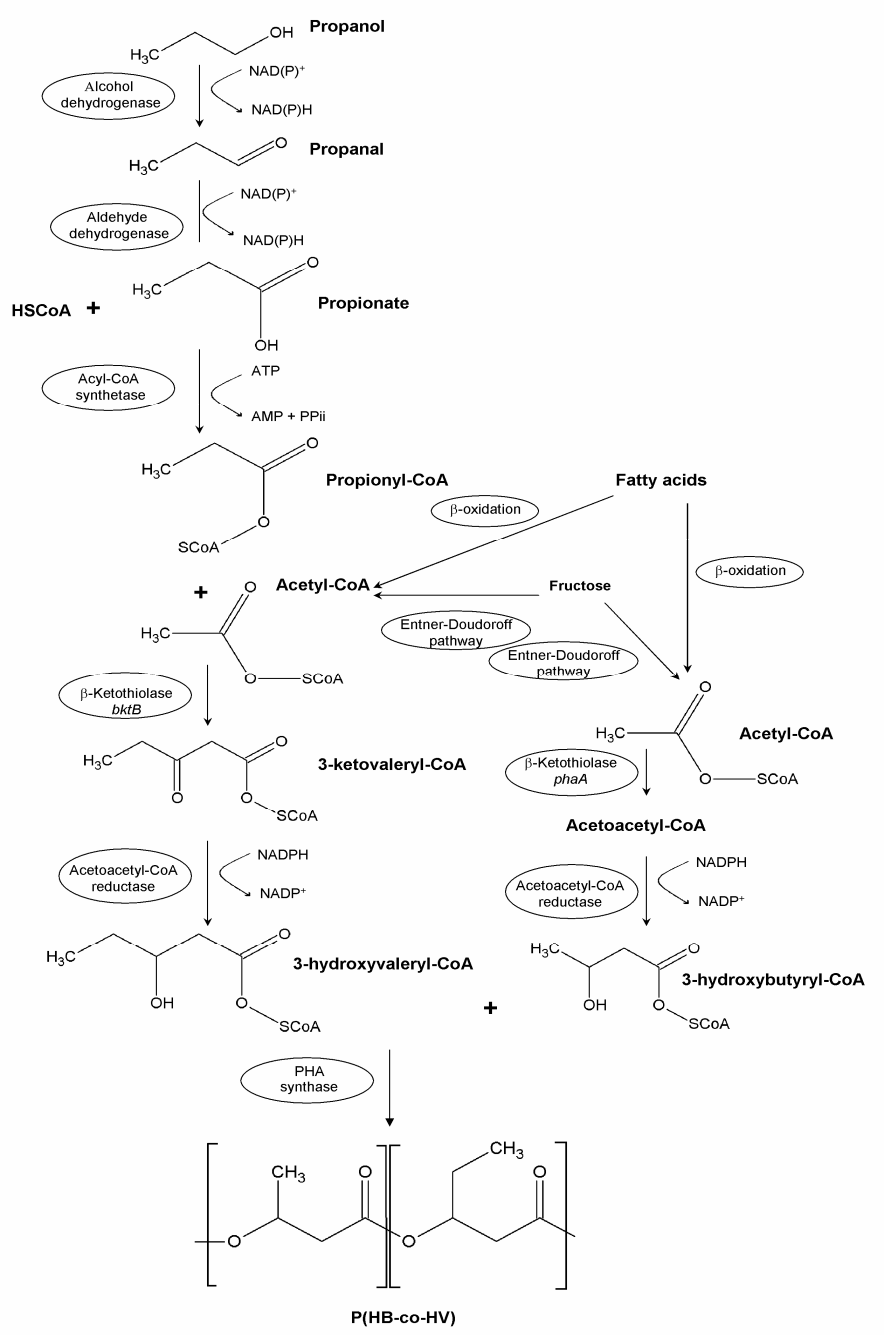

***Fig. 2**. Proposed mechanism of metabolization of propanol by C. necator H16 and its incorporation into P(HB-co-HV) copolymer structure.*

4.3. Production of PHA from cheap/waste substrates

Bacillus megaterium is able to convert lactose from cheese whey directly into PHB. Thanks to high amount of cheap cheese whey produced daily in cheese manufactories, this carbon source represents attractive substrate for economically feasible production of PHA in large amounts. However, apart from transgenic *E. coli*, only few PHA producing strains are capable of turning lactose directly into PHA. *B. megaterium* is one of them. Therefore, we decided to optimize the whey medium to enhance PHB and biomass yields.

Optimization of cheese whey medium was performed using Placket-Burman experimental design. This statistical tool allows optimizing multivariable process in which numerous potentially influencing factors are involved. Placket-Burman experimental design is often used to screen and evaluate factors that influence studied process and it helps to identify the crucial ones. In our process of PHB production from cheese whey, dilution of whey was identified to be the most important factor. Therefore, we made optimization in order to find the best dilution of whey expressed as lactose concentration ($g.l^{-1}$). Undiluted cheese whey contained 40 $g.l^{-1}$ of lactose, for our purpose it was optimal to dilute it to 20 $g.l^{-1}$. Also supplementation of medium with some inorganic salts pronounced biomass as well as PHB formation. Finally, medium optimization enhanced PHB yields about 50 times **(see Fig. 3)**.

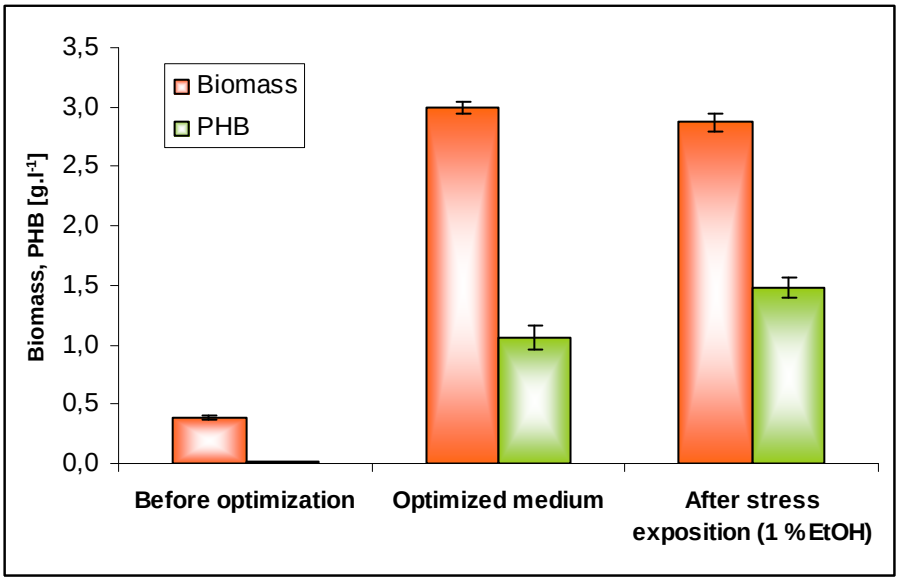

Fig. 3 *Biomass and PHB yields obtained on cheese whey medium.*

After that, we tried adding ethanol and hydrogen peroxides into cultivation media in order to even increase PHB yields. Both stress factors were applied at the beginning of stationary phase at several concentration levels. The most efficient, in term of enhancement of PHB yields, was ethanol which increased yields about 40 %. This fact supports our presumption that the stress

response against alcohols, especially ethanol, is more or less similar among bacteria. Hence, ethanol can be used as universal factor stimulating PHB biosynthetic pathway.

Furthermore, our results indicate the potential of *B. megaterium* for industrial production of PHB from cheep waste whey. However, further experiments must be aimed at cultivation in laboratory and semi-industrial fermentors, because for industrial production of PHB it is necessary to reach high cell density (more than 100 $g.l^{-1}$ of cell dry weight). To obtain such a high cell concentration, it is esential to control closely many factors such as agitation speed and aeration, pH, temperature etc., which is possible only in fermentor. To overcome substrate inhibition, it is necessary to work in fed-batch mode. As a feeding solution, concentrated whey can be used, but there might be a problem with raising osmotic pressure in medium (cheese whey itself contains a lot of salts) especially at late period of cultivation. Finally, introduction of stress factors into well aerated fermentor could lead to different response of culture than was observed in Erlenmeyer flaks. Thus, further experiments are needed to face all potential problems.

Results regarding PHB production on cheese whey employing *B. megaterium* were presented ***(Obruca et. al., New Biotech. 25 (2009) p. S257)*** and also manuscript ***(Obruca et. al., Annals Microbiol., submitted)*** has been recently submitted for publication.

Due to a lot of problems related to PHB production from waste cheese whey, we decided to test other attractive feedstock for PHA production – edible oils. In contrast to the other carbon sources, the theoretical yield coefficients of PHA production from plant oils are as high as over 1.0 g-PHA per g-plant oil, since they compose much higher number of carbon atoms per weight [39].

For PHA production from plant edible oils we employed bacterial strain *C. necator* H16 ***(Obruca et al. Biotechnol. Letters, Accepted for Publication)***. First of all, we decided to test various edible oils which were available at shops and also waste oils from different sources (household, university canteen, restaurant and fried chips producing manufactory in Strážnice). According to our results, PHA yields are dependent on the type of oil. Relatively high PHA yields were obtained on rapeseed oil, which is typical edible oil for our region. However, even better yields were obtained when bacterial culture was cultivated on waste oils. Our results are showed in **Tab. 2**.

Table 2. *Growth and PHB production of C. necator H16 on various oils as carbon sources*

Oil	Biomass [g.l^{-1}]	PHB [g.l^{-1}]	PHB [% w/w]
Olive	8.14 ± 0.15	3.27 ± 0.25	40.20
Corn	10.85 ± 0.61	4.36 ± 0.38	40.18
Soybean	7.47 ± 0.43	2.08 ± 0.11	27.79
Sunflower	9.37 ± 0.15	4.63 ± 0.44	49.37
Rapeseed	9.54 ± 0.40	4.77 ± 0.40	50.02
Waste - Household (sunflower)	10.28 ± 0.57	5.82 ± 0.39	56.60
Waste - University canteen (rapeseed)	12.77 ± 0.49	7.69 ± 0.37	60.22
Waste - Restaurant (sunflower)	11.43 ± 0.52	6.73 ± 0.08	58.87
Waste – Chips manufactory (rapeseed)	12.22 ± 0.63	6.76 ± 0.50	55.28

Waste edible oils exhausted from the food industry and food service industry are recovered legally as industrial wastes and can be converted to an animal-based feed, fatty acids, soaps or biodiesel etc. However, a lot of waste oils (especially those from households) are not recovered and are disposed of by incinerators as inflammable waste after being absorbed in papers or coagulation with a certain coagulants which cause serious environmental problems, such as waste management or global warming. In addition, a part of waste oils flows up into the sewage, resulting in water pollution [40]. On the other side, waste edible oils represent promising source for production of polyhydroxyalkanoates. The idea is to convert problematic waste, which is really low in price, into high value environmental friendly product. Despite all the advantages of such a process, there are only few reports on PHA production from waste oils. Taniguchi et al. reported PHA production from waste sesame oil employing *C. necator*, achieved yields were about 4.6 g.l^{-1} [40]. Chan et al. studied MCL-PHA production from various plant oils including waste oil using *Pseudomonas aeruginosa*, but the PHA content in cell was only about 5 % of dry cell weight [41]. Finally, Mas et al. cultivated *Pseudomonas aeruginosa* on waste frying oil and reached MCL-PHA yields 3 g.l^{-1} [42]. PHA yields we obtained on waste oils in batch culture in flasks were almost twice as high as those reported in literature. The highest PHA yields were obtained on waste rapeseed oil coming from university canteen, therefore, this oil was used as carbon substrate in all following experiments.

On the basis of our results we tried evaluating the most promising carbon substrate from the economical point of view. The yield coefficients were calculated from results of experiments carried out in batch mode. It should be taken into account that the value obtained in fed-batch mode would be significantly higher and our economic analysis is, therefore, only approximate. The prices of pure substrates were taken from web pages of International Trade and Business Knowledge. Final calculation was based on the presumption that the costs of carbon substrates represent 40 % of the final cost of PHA produced

by bacterial fermentation. The commercial price of PHA is 2-4 $.kg^{-1}, for this economical consideration we took mean of this values 3 $.kg^{-1}. The price of waste oil was taken from web pages of company Trafin Oil a.s., which buys out waste edible oils for price about 2,- CZK per 1 liter of oil. So, in **Tab. 3** there are shown the calculated theoretical prices of PHA and these prices are compared to the prices of synthetic polymers with properties similar to PHA – polyethylene and polypropylene.

Tab 3. *Basic economical consideration of PHA production based on results obtained in our work (Cupriavidus necator H16, batch culture, Erlenmayer flask, 30°C, constant shaking 150-200 rpm, substrate concentration 20 g.l^{-1}).*

Substrate	PHA yield [g.l^{-1}]	Yield coefficient[a] [g.g^{-1}]	Price of substrate[b] [EUR.kg^{-1}]	Calculated price of PHA[c] [EUR.kg^{-1}]
Fructose	11.20	0.56	1.58	3.81
Olive oil	3.27	0.16	2.25	14.73
Corn oil	4.36	0.22	1.03	5.71
Soybean oil	2.08	0.10	1.12	11.77
Sunflower oil	4.63	0.23	0.87	4.76
Rapeseed oil	4.77	0.24	0.60	3.51
Waste oil - Household (s)	5.82	0.29	0.10	1.33
Waste oil - University canteen (r)	7.69	0.38	0.10	1.24
Waste oil - Restaurant (s)	6.73	0.34	0.10	1.28
Waste oil - Chips manufactory (r)	6.76	0.34	0.10	1.28
Synthetic polymer				**Price [EUR/kg]**
Polypropylene				1.47
Polyethylene				1.15

[a] – coefficients were calculated as PHA produced (g) per substrate added (g)

[b] – prices of substrate were taken from web pages of Intenational Trade and Business Knowledge (www.alibaba.com), only price of waste oils were taken from web pages of company Trafin Oil a.s. which does business with waste edible oils of plant origin. 1.00 $ was taken as 21.00 CZK, 1 EURO was taken as 25.75 CZK

[c] - final prices of PHA were calculated based on presumption that the cost of carbon source represent 40 % of the final cost, price of PHA was taken as 3 $ per kg.

(s) – sunflower waste oil, **(r)** – rapeseed waste oil

If pure carbon substrates such as fructose or oils were used, the theoretical prices of produced materials are significantly higher than that of synthetic polymers. Oppositely, if waste edible oils are used as the substrate, final cost of PHA is comparable with price of polypropylene. Of course, other factors such as the costs of up-stream and down-stream processes contribute to the final cost of

PHA and, hence, the cost of produced material would be probably higher. However, we believe that the price of PHA produced from waste edible oils could be, at least, competitive to the price of polypropylene or polyethylene. It is likely that the limiting factor could be the amount of available waste oil. Its delivery transportation from far distances could be logistically problematic and also expensive. On the other side, the idea of conversion of problematic waste into high value product is worth of such an effort.

In our further experiments we tested exposition of bacterial culture cultivated on the waste rapeseed oil to controlled stress conditions caused by methanol, ethanol and propanol. Surprisingly, application of all stress factors at the 24^{th} hour of cultivation not only enhanced PHA yields, but, moreover, also significantly supported the growth of bacterial culture. The more non-polar alcohol, the more pronounced growth was observed (biomass yields were: control 11.17, methanol 12.09, ethanol 13.03, propanol 14.68 $g.l^{-1}$). The explanation of such an effect could be that alcohols supported solubilization of triacylglerols, which made them more susceptible to action of extracellular lipases. This is likely to have increased concentrations of products of lipases action – glycerol and fatty acids in medium which could support bacterial growth.

All tested alcohols also enhanced the PHA synthesis in cells. Application of both ethanol and propanol caused accumulation of really high contents of PHA in cells (about 79 % of cell dry weight). In addition, application of propanol resulted in incorporation of 3-hydroxyvalerate into PHA structure, which significantly improves mechanical properties of produced material. As far as we know, we are the first who have reported that propanol could be used as a precursor of 3-hydroxyvalerate. In comparison with other commonly used precursors of 3-hydroxyvalerate, such as propionate or valerate, propanol has also other advantages – it is much cheaper and it enhances PHA yields and supports the growth of bacterial culture on oils as carbon substrates.

Because production of P(HB-co-HV) from waste rapeseed oil using propanol as a stress agent seemed to be very prosing strategy, we decided to perform fed-batch cultivation in laboratory fermentor. Propanol was applied at the 18^{th} hour of cultivation and after that its concentration was maintained at 1 %. Nitrogen source ($(NH_4)_2SO_4$) level was maintained at 3 $g.l^{-1}$ until the 30^{th} hour of cultivation when nitrogen feeding was stopped to induce nitrogen limitation. Concentration of carbon source (waste rapeseed oil) was kept at 20 $g.l^{-1}$.

Application of propanol resulted in incorporation of 3-hydroxyvalerate units into PHA chain, thus, we can assume that exposition of bacterial culture to stress condition is effective strategy even if culture is cultivated in fermentor. After 72 hours of cultivation, we gained high cell density (cell dry weight was 138.46 $g.l^{-1}$), moreover, cells contained 76 % of PHA with 8 % of 3-hydroxyvalerate. Total yield of PHA was 105.05 $g.l^{-1}$, volumetric productivity

yield was 1.46 $g.l^{-1}h^{-1}$ and yield coefficient was 0.83 g-PHA per g-oil. Production parameters obtained in our work belong among the highest reported for PHA production from fatty acids. Furthermore, it should be taken into account that we worked with waste oil while most of other authors used pure substrates.

If we use the yield coefficient obtained in fed-batch mode (0,83 g-PHA per g-oil) for calculation of final price of PHA in the same way as in **Tab.3**, the theoretical price of PHA produced in fed-batch mode from waste rapeseed oil is 1.11 $EUR.kg^{-1}$. It is about 30 % less than the price of polypropylene. According to this calculation, waste oils can be considered to be the most promising carbon substrates facilitating economically feasible process of PHA production.

If we compare both types of waste substrates we tested – waste plat oils and waste cheese whey, we can state that waste oils seem to be superior to whey for number of reasons. First of all, the yields obtained on waste oils were much higher than those on cheese whey. This is due to employment of *C. necator* H16, which is highly productive strain that grows very well on oils, but it is unable of utilization of lactose. On the contrary, *B. megaterium* was able to utilize lactose, but biomass yields were rather low, as compared to *C. necator*. Because PHA are intracellular metabolites, high cell density is the first condition of high total yields of PHA. Secondly, important advantage of waste oil is its stability and practically none demands on up-stream processing. The only step required prior cultivation is the removal of solid particles by filtration. Oppositely, cheese whey had to be treated in order to remove excessive proteins, which represent another complication of production process. Furthermore, cheese whey tends to spoil, therefore, it needs being kept frozen or at least chilled. This fact could really complicate and raise price of logistical part of PHA production from cheese whey. On the other side, oils could be stored at room temperature and they are also microbially stable. Finally, as mentioned above, there are many problems concerning using whey as feeding in fed-batch mode because of high osmotic pressure of concentrated whey solution. On the other side, it is not a problem to feed edible oils in fed-batch mode. For all these reasons, waste oils are very promising cheap substrates for PHA production.

4.4. Biodegradation of polyurethane materials

Apart from production of polyhydroxyalkanoates, the second subject of proposed doctoral thesis dealt with biodegradation of selected modified biomaterials, particularly polyurethanes. Because of their resistance against various biotic and abiotic agents and large scale of worldwide production of polyurethane materials of diverse kinds, waste polyurethane materials represent serious environmental issue. In order to overcome such problems, new polyurethane materials were prepared at Department of Material Science, Faculty of Chemistry, Brno University of Technology. Biodegradability of

newly synthesized materials was enhanced by partial replacement of resistant polyether polyol by biopolymer of renewable origin such as carboxymethyl-, hydroxyethyl- or acetylated cellulose, acetylated starch and wheat protein [43]. These materials were prepared in two forms - PUR foams and PUR elastomeric films. Our goal was to investigate the biodegradability of these materials employing selected bacterial cultures. The first part of biodegradation experiments was performed with polyurethane foams and the results were presented as part of the diploma thesis [44] and further published in paper ***(Obruca et al., Chemicke Listy 102 (2008) p. 1219-1220).*** Another manuscript has been recently submitted for publication ***(Obruca et al., J. Environ. Manage., submitted)***. The second part of experiments was focused on research on biodegradability of PUR elastomeric materials. Also this research resulted in two papers, the first one has been already published ***(Obruca et al., Chemicke Listy 102 (2008) p. 1257-1258)*** and the second one was accepted for publication ***(Obruca et al., Environ. Technol., Accepted for Publication)***.

The second form of PUR materials – elastomeric films - was prepared using the same raw materials as for PUR foams, only the foaming agent (water) was omitted. Also in this case PUR materials were modified by partial replacement (10 %) of polyether polyol by biopolymers. For testing of materials biodegradability only mixed thermophillic culture was used**.**

The exposition of PUR elastomers to mixed culture resulted in surprising growth character of bacterial culture. Whereas PUR foams supported the growth of bacterial culture from the beginning of cultivation [44], the presence of PUR elastomeric films in the cultivation media strongly inhibited the growth of bacterial culture for unusually long period. Nevertheless, after this long lag-phase, bacterial growth started and was very intensive, so that the biomass content was higher than in control culture. It can be expected that this long lag-phase was caused by release of some toxic substance(s) from PUR elastomeric films into cultivation media. This substance(s) revealed toxic effect on bacterial culture, because it inhibited its growth. However, after some time culture was able either adapt to harmful condition or eliminate toxic substance and growth of culture was started. The fact that this effect was not observed in PUR foams, which were prepared using the same raw materials, may indicate that the toxic substance(s) responsible for long lag-phase might have been removed during process of foaming.

The release of toxic substances from waste PUR materials, as far as we know, has not been reported yet. Due to huge amounts of PUR materials produced worldwide, this could present another problem related to impact of PUR on environment. Asides its resistance and durability which cause an accumulation of solid waste, PUR materials can be also considered to be potential sources of toxic substances. Thus, more attention should be paid to this group of materials in order to prevent both accumulation of solid waste and potential release of toxic substances.

Finally, we decided to look for the raw material responsible for the long lag-phase and, therefore, we applied individual chemicals into cultivation media and monitored bacterial growth. Surprisingly, none of tested chemicals presented solely in the medium prolonged lag-phase. So, we decided to test combinations of them and we observed that if both catalyst (dibutyltin laurate, DBTL) and polyether polyol (PEP) had been added into cultivation media, the prolonged lag-phase occurred. Hence, our results can be interpreted that prolonged lag-phase was caused by small residues DBTL and PEP, which were not built well into PUR matrix. In water environment they were released into surrounding cultivation medium. After that they interacted which each other or with some components of medium and formed substances prevented bacterial culture from dynamic growth. To our knowledge, the effect of prolonged lag-phase caused by these substances, which are commonly used for synthesis of PUR materials, has not been reported so far.

Because PEP also contributes to toxic effect of PUR elastomers, partial replacement of this component by biopolymer could serve as a strategy decreasing toxic effect of PUR elastomeric films. Replacement of 10 % of PEP did not suppressed lag-phase. Nevertheless, the lag-phase observe in PUR materials modified by biopolymer was significantly shorter than that of non-modified material. For instance, simple replacement of 10 % of PEP by acetylated starch shortened the lag-phase about 3 times. Furthermore, modification of PUR elastomeric by biopolymers supported the growth of bacteria in all cases in comparison with either control or reference non-modified sample. In addition, we observed that modification of elastomers by biopolymers resulted in significantly enhanced mass losses of samples during cultivations both with and without presence of bacterial culture. Although the main part of mass losses was probably caused by mechanical disruption of PUR samples or by solubilization of biopolymer, the fact that mass losses were always higher in presence of bacterial culture indicates that also the bacterial culture contributed to degradation process.

Similarly to PUR foams, also degradation of PUR elastomeric films probably involves two steps. The first step is abiotic degradation, during which PUR elastomeric films exposed to water environment spontaneously degraded and the main part of released degradation products is constituted of modifying agent. Therefore, the mass losses of samples were strongly dependent on the type of modifying agent used and the highest mass losses were observed in PUR materials modified by in water well soluble carboxy- and hydroxyethylcellulose. The second step of process was subsequent utilization of products of abiotic degradation. Apart from this mechanism observed also in PUR foams, direct bacterial utilization of PUR elastomers was observed as well. This is in consistence with Albertosson et al. who reported that the abiotic and biotic degradation of polymeric materials can occur simultaneously or subsequently [45]. Nevertheless, direct utilization of PUR samples modified by

carboxymethyl-, hydroxyethylcellulose and acetylated starch is very slow process as compared to the abiotic degradation. On the contrary, in the case of sample modified by acetylated cellulose, direct utilization of PUR samples represented the main part of degradation process (mass losses - 1.66 % with culture, 0.40 % without culture). Moreover, PUR elastomeric film modified by acetylated cellulose also supported the growth of culture the most intensively of all the tested samples. Because this material does not tend to degrade spontaneously (low mass losses without culture) and it seems to be susceptible to biodegradation, acetylated cellulose could be the modification agent of choice in order to enhance biodegradability of PUR elastomeric films.

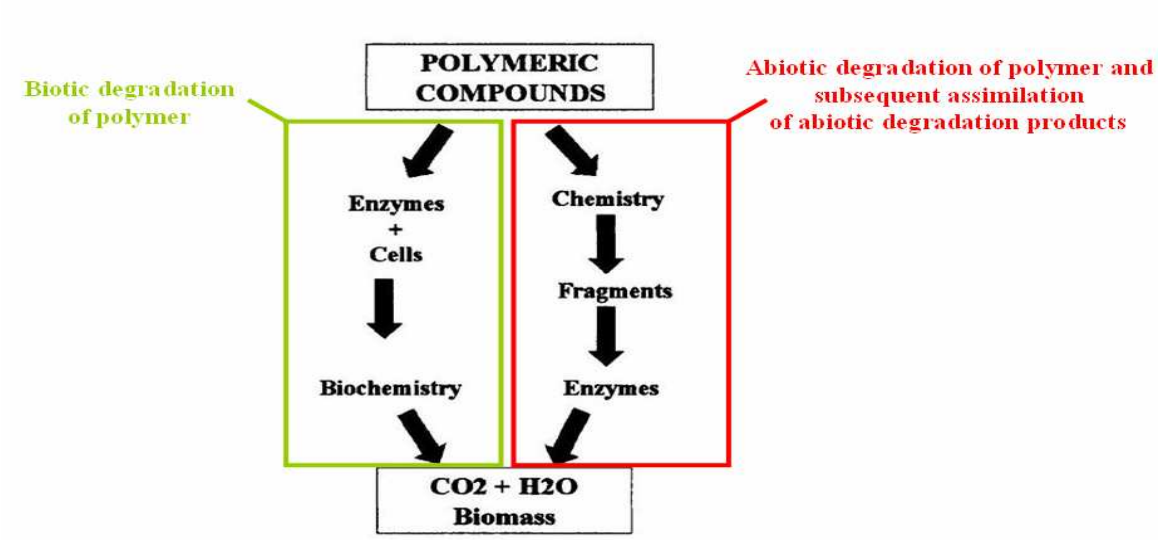

***Fig.19**. Comparison of biotic and abiotic mechanism of degradation of polymeric material [17].*

To sum up the part of the thesis dealing with biodegradation of modified PUR materials, it can be conclude that modification of PUR materials by simple replacement of polyether polyol by biopolymer seems to be promising strategy especially if PUR material is in form of elastomeric film. In PUR elastomers, we observed toxic effect of materials on bacterial culture. This undesirable effect is significantly reduced if polyether polyol is partially replaced by biopolymer. Moreover, in contrast to PUR foams, direct utilization of samples was observed in PUR elastomers, while the degree of bioavailability of material strongly depends on the type of biopolymer used for modification. According to our results, acetylated cellulose is very interesting choice, because this material built into PUR matrix does not tend to degrade spontaneously, but the composite PUR material revealed the highest biodegradability.

5. CONCLUSIONS

5.1. Conclusions 1: Production of polyhydroxyalkanoates

- *Bacillus megaterium* CCM 2037 can be used for PHB production from various carbon substrates including cheese whey or starch based medium. Screening experiments with this strain indicated, that exposition of bacterial culture to controlled stress conditions may have been used for enhancement of PHB yields.

- For our purposes, gas chromatography was the method of choice for analysis of PHA. If Mass Spectrometry detection was used, really sensitive method with the possibility to identify individual monomers was gained. Employment of Flame Ionization Detector resulted in the less labor-intensive protocol for analysis of our samples with optimal sensitivity.

- PHB production in *Cupriavidus necator* can be significantly enhanced if controlled stress conditions caused by ethanol and hydrogen peroxide are induced. In terms of final yields, stress dose and time of stress application should be optimized. The highest PHB yields were observed when stress was applied at the beginning of stationary phase. After optimization of stress dose, PHB yields were improved about 30 % in comparison with control culture.

- Stress response of *C. necator* to hydrogen peroxide involves an increase of activity of pentose phosphate pathway. The result of such a response is increased $NADPH/NADP^+$ ratio which partially inhibits Krebs cycle and supports the flux of acetyl-CoA into PHB biosynthetic pathway. Also activities of □-ketothiolase and acetoacetyl-CoA reductase are enhanced and, thus, PHB accumulation in cells is supported.

- In *C. necator* cells, ethanol is metabolized *via* oxidization while the final product is acetyl-CoA, the key substrate of PHB biosynthesis. During ethanol metabolization, reduced coenzymes are formed as well, which supports PHB synthesis in the same way as in case of hydrogen peroxide.

- Despite the fact that activity of PHB synthase was not influenced under stress conditions, the molecular weights of polyesters produced in the stress environment were significantly higher. This effect was very interesting and it deserves further experiments to understand its

mechanisms. Nevertheless, one of potential explanation could be that also concentration of monomer(s) belongs among factors contributing to control of length of polyester chain.

- Application of propanol as a stress agent also strongly supports PHA accumulation in *C. necator* cells, probably in the same mechanism as in ethanol, but, moreover, 3-hydroxyvalerate units are being built into PHA chain. P(HB-co-HV) copolymer possesses mechanical properties superior to PHB homopolymer.

- Controlled application of stress conditions improves not only PHA yields but also mechanical properties of produced materials - molecular weight and monomer composition. Thereby, our results could be used as a basement for novel strategy in PHA production.

- Production yields of PHB from waste cheese whey employing *B. megaterium* were increased more than 50 times by optimization of medium composition using Placket-Burman methodology. The most important factor, in terms of PHB yields, was whey dilution. Optimal concentration of lactose was found to be 20 $g.l^{-1}$. Subsequent application of stress factors, particularly ethanol, enhanced PHB yields more than 40 %. This indicates that stress response to alcohols is more or less similar among bacteria and application of controlled stress caused by alcohols can be used for improvement of PHB production process employing various bacterial strains.

- Waste edible oils are very promising carbon substrate for PHA production employing *C. necator* as a producing strain. On the basis of the results of our preliminary economical consideration of PHA production process, the price of PHA produced from waste edible oils could be even lower than price of polypropylene.

- Fed-batch cultivation of *C. necator* on waste rapeseed oil in laboratory fermentor under propanol stress provided high biomass and PHB yields - 138 $g.l^{-1}$ and 105 $g.l^{-1}$, respectively. The cell contained 76 % of PHA, 3-hydroxyvalerate content was 8 %. These results are among the best reported for PHA production from fatty substrates.

5.2. Conclusions 2: Biodegradation of polyurethanes

- The mechanism of degradation of PUR materials involves two steps. The first one was abiotic degradation of materials in water environment and

the second step was subsequent utilization of abiotic degradation products by bacterial culture. Unlike in case of PUR foams, in PUR elastomers also direct utilization of PUR materials by mixed culture was observed.

- PUR elastomeric films revealed to be toxic for mixed thermophillic bacterial culture. The cultivation of culture in presence of PUR elastomers was accompanied by unusual long lag-phase. This lag-phase was caused by release of small residues of polyether-polyol and tin catalyst from samples. After few days, bacterial culture was able either to adapt to toxic substances in cultivation medium or to eliminate them. After that bacterial growth started.

- Modification of PUR elastomeric film by partial replacement of polyether polyol by biopolymer significantly reduced toxic effect of PUR samples and increased its biodegradability. The most promising modifying agent seemed to be acetylated cellulose. PUR elastomeric film modified by acetylated cellulose did not tend to undergo abiotic degradation, but, on the other side, the measure of biotic degradation was the highest among all tested samples.

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7. LIST OF ABBREVATIONS

ATP	Adenosine triphosphate
CoA	Coenzyme A
CCM	Czech Collection of Microorganisms
DEG	Diethylene glycol
MCL	Medium Chain Length
NADH	Nicotinamide adenine dinucleotide (reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
P(HB-*co*-HV)	Copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate
ppGpp	Guanosin teraphosphate
PUR	Polyurethane
RpoS	Sigma factor of RNA polymerase
SCL	Short Chain Length
TCA	Tricarboxylic acid cycle, Krebs cycle

8. LIST OF AUTHOR PUBLICATIONS

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11. **Obruca S.**, Marova I., Vojtova L. Biodegradation of polyether-polyol based polyurethane elastomeric films: influence of partial replacement of polyether-polyol by biopolymers of renewable origin. *Environmental Technology, Accepted for Publication.*

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Abstracts - national congresses

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