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CONTROLLED PRODUCTION AND DEGRADATION OF SELECTED BIOMATERIALS

REGULOVANÁ PRODUKCE A BIODEGRADACE VYBRANÝCH TYPŮ BIOMATERIÁLŮ

DOKTORSKÁ PRÁCE
DOCTORAL THESIS

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SOUHRN

Předložená disertační práce se zabývá studiem produkce a degradace polymerních materiálů s využitím mikroorganismů. V oblasti produkce je hlavní pozornost zaměřena na studium tvorby polyesterů bakteriálního původu – polyhydroxyalkanoátů. Tyto materiály jsou akumulovány celou řadou bakterií jako zásobní zdroj uhlíku, energie a redukční síly. Díky svým mechanickým vlastnostem, kterými silně připomínají tradiční syntetické polymery jako jsou polyetylén nebo polypropylén, a také díky své snadné odbouratelnosti v přírodním prostředí, jsou polyhydroxyalkanoáty považovány za ekologickou alternativu k tradičním plastům vyráběným z ropy. Polyhydroxyalkanoáty mají řadu potenciálních aplikací v průmyslu, zemědělství, ale také v medicíně.

Významná část předložené práce je zaměřena na produkci polyhydroxyalkanoátů z odpadních substrátů pocházejících především z potravinářských výrob. Testována byla odpadní syrovátka nebo odpadní oleje z různých zdrojů. Právě využití levných odpadních substrátů je strategií, která by mohla přispět ke snížení ceny polyhydroxyalkanoátů a tím usnadnit jejich širší využití. Podle výsledků dosažených v této práci jsou právě odpadní olejové substráty velmi perspektivní cestou k ekonomicky rentabilní biotechnologické produkci polyhydroxyalkanoátů.

Další část předložené práce se zabývá studiem vztahu mezi metabolickou rolí polyhydroxyalkanoátů a stresové odezvou bakterií. V této práci bylo zjištěno, že expozice bakteriální kultury řízené dávce etanolu nebo peroxidu vodíku významně navýší dosažené výtěžky a to přibližně o 30 %. Po aplikaci výše zmíněných stresových faktorů došlo k aktivaci metabolických drah vedoucích k odbourání stresového faktoru z média. Výsledkem bylo navýšení poměru NAD(P)H/NAD(P)$^+$, což vedlo k částečné inhibici Krebsova cyklu a naopak aktivaci biosyntetické dráhy polyhydroxyalkanoátů. Dále došlo k významnému navýšení molekulové hmotnosti vyprodukovaných materiálů. Podle těchto výsledků se regulovaná aplikace vhodně zvolených stresových podmínek zdá být zajímavou strategií, která vede nejen k navýšení celkových výtěžků, ale také k významnému zlepšení vlastností polymeru.

Poslední část disertační práce byla zaměřena na biodegradační experimenty. Podrobněji byl studován proces biodegradace polyuretanových materiálů. Polyuretanové eleastomery byly modifikovány rozličnými biopolymery za účelem navýšení jejich biodegradability. Tyto materiály byly posléze vystaveny působení směsné termofilní kultury jako modelového systému, který simuluje přirozené konsorcium bakterií. Přítomnost testovaných materiálů v kultivačním médiu vedla k neobvyklým růstovým charakteristikám bakteriální kultury. V průběhu prvních několika dní byl růst kultury silně inhibován, nicméně po překonání této neobvykle dlouhé lag-fáze došlo k intenzivnímu nárůstu. Během experimentů měl hlavní podíl na hmotnostním úbytku testovaných materiálů jejich samovolný rozpad, nicméně byl pozorován i vliv bakteriální kultury, kdy míra biotické degradace závisela na použitém modifikačním činidle. Nejvyšší míra biotické degradace byla pozorována u polyuretanového materiálu modifikovaného acetylovanou celulózou. Neobvykle dlouhá lag-fáze byla způsobena uvolněním nezreagovaného katalyzátoru (dibutylcín laurát) a polyolu do kultivačního média. Bakteriální kultura se však po čase dokázala na přítomnost toxických látek v médiu adaptovat nebo je dokázala eliminovat.

Disertační práce předložená ve formě komentovaného souboru již uveřejněných publikací a rukopisů v recenzním řízení je v souladu s nastupujícím zájmem o biodegradabilní materiály, jejichž širší uplatnění v každodenním životě moderní společnosti by mělo výrazný pozitivní dopad na životní prostředí.

SUMMARY

Proposed dissertation thesis is aimed at the study of 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 thesis is focused on production of polyhydroxyalknotes 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 presented in this thesis, 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 pathaways 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.

Doctoral thesis, which is in form of commented published papers and abstracts and manuscripts currently under review process, is in agreement with recent interest in biodegradable materials. Wider application of these materials in life of modern society would be beneficial for environment.

Klíčová slova:
Polyhydroxyalkanoáty, stressová odpověď bakterií, *Cupriavidus necator* H16, *Bacillus megaterium*, biodegradace, polyurethany

Key words:
Polyhydroxyalkanoates, stress response of bacteria, *Cupriavidus necator* H16, *Bacillus megaterium*, biodegradation, polyurethanes

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PROHLÁŠENÍ

Prohlašuji, že jsem disertační práci vypracoval samostatně a že všechny použité literární zdroje jsem správně a úplně citoval. Disertační práce je z hlediska obsahu majetkem Fakulty chemické VUT v Brně a může být využita ke komerčním účelům jen se souhlasem vedoucího disertační práce a děkana FCH VUT.

……..
podpis doktoranda

Poděkování:

Za odborné vedení, všemožnou pomoc a lidský přístup bych chtěl poděkovat mé školitelce doc. RNDr. Ivaně Márové, CSc.
Mé poděkování za pochopení a podporu patří také mé rodině. V neposlední řadě bych rád poděkoval všem přátelům za to, že jsme spolu mohli zažít spoustu neopakovatelného.

CONTENT

1. INTRODUCTION

Synthetic polymers are a group of materials which surrounds us every day of our life. Because of their mechanical properties they are able to possess various forms and serve in many functions, therefore, it is almost impossible to image just one day without them. On the other hand, the dark side of their expansion is their impact on environment. Because of their resistance against action of microorganisms and other environmental factors, half-life of synthetic materials is expected to be tens maybe hundreds of years. Thus, accumulation of solid waste constituting of synthetic polymers has been recently found to be serious environmental issue. It is necessary to find a solution and prevent accumulation of thousands tons of solid waste every year.

There are several possible strategies how to overcome problems with waste of synthetic polymer origin. Among them, biodegradation of synthetic polymers is probably the most promising one. Because synthetic polymers do not tend to undergo microbial degradation, or this process takes long time, it is necessary to enhance biodegradability of polymeric materials.

Microorganisms are able to produce wide variety of industrially interesting metabolites. Some of them reveal mechanical properties similar or superior to synthetic polymers. The most promising representatives are polyhydroxyalkanoates. These polyesters of bacterial origin are comparable with polypropylene or polyethylene in terms of mechanical properties, but, moreover, polyhydroxyalkanoates are biodegradable and biocompatible. Thus, they are considered as an alternative to traditional synthetic polymers in many fields of industry, agriculture and medicine as well.

The second strategy that might reduce accumulation of solid waste is modification of traditional synthetic polymers in order to enhance their biodegradability. It is necessary to develop such a modification strategy which will not influence desirable mechanical properties of material, but, on the other side, which will make material available to enzymatic action.

Proposed doctoral thesis deals with both possible strategies which could contribute to reduction of solid waste accumulation. The first part is focused on improvement of polyhydroxyalkanoates production process employing selected bacterial culture. The second part aims at testing of biodegradability of polyurethane materials which were modified by selected biopolymers. Hopefully, our small contribution will be one of many steps on the way to large scale production and application of environmental – friendly polymeric materials.

This thesis is based on papers and abstracts which were already published and on manuscripts which have been recently submitted for publication. All these documents are supplied in Chapter 9. Furthermore, the results are discussed and commented from point of view of current knowledge in Discussion part (Chapter 4).

2. THEORETICAL PART

2.1 Microorganisms and polymeric materials

Microorganisms are one of the most important factors influencing natural processes in environment. They participate in degradation and transformation of most of organic substances including synthetic polymeric materials. Process of material decomposition by microorganisms is called biodegradation and it could be an innovative solution for the environmental problems caused by accumulation of solid waste of synthetic polymer origin. The main products of such processes are microorganisms themselves – biomass. Important part of biomass is formed by polymeric materials. Probably the most important ones are proteins and nucleic acids, which are responsible for the main part of metabolic processes in cell. Nevertheless, also polymeric storage materials of microorganisms such as polyesters, polysaccharides or polyamides play important role in cells as well as in natural environment. Moreover, thanks to their mechanical properties, which are often similar to those of synthetic polymers and higher biodegradability, these bacterial biopolymers could act as environmental-friendly alternative to polymers of synthetic origin [1, 2].

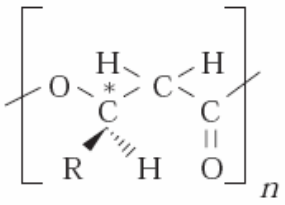
2.2 Polyhydroxyalkanoates – structure and properties

Polyhydroxyalkanoates (PHA) are polyesters of natural origin accumulated in 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].

2.2.1 Chemical and mechanical properties of PHA

From chemical point of view, PHA are polyesters of hydroxyalkanoic acids. PHA can be classified according to number of carbon atoms in monomer unit: short-chain lengths (SCL) PHA consist of 3-5 carbon atoms and medium-chain length (MCL) PHA contain 6-14 carbon atoms per monomer unit. More than 100 monomers have been found in the naturally synthesized polyester polymers. The monomer units in these microbial polyesters are all in the *R*-configuration because of the stereospecificity of the biosynthetic enzymes. Among these are 3-hydroxy- (rarely 4-6 hydroxy-) acids of 3-14 carbon atoms with saturated, unsaturated, straight or branched aliphatic side chains. Furthermore, monomers with various functional groups in the chain, such as halogen, hydroxy-, epoxy-, cyano-, carboxyl- and esterified carboxyl groups, have been discovered in MCL-PHA. It might be expected, because of low substrate specificity of some of PHA synthases, that new constituents will continue to be detected by using different precursor substrates or other bacteria [2].



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

Fig. 1 *General structure of PHA [1].*

A wide range of molecular weights (usually $1.10^4 – 3.10^6$ g.mol^{-1}, polydispersity about 2) are exhibited by PHA from different microorganisms and also from different stages of cultivation. In addition, cultivation conditions strongly influence molecular weight of PHA produced. For instance, it was shown that the pH of the culture medium can greatly affect the molecular weight of the PHA produced. Also the type and concentration of the carbon source supplied can influence the molecular weight of PHA [3].

Mechanical properties of individual PHA strongly depend on monomer unit composition. The PHB homopolymer is a completely stereoregular polyester, with all asymmetric carbon atoms in the *R*-configuration, and it is, therefore, highly crystalline. The high crystallinity (typically 55–80%) makes it relatively stiff and brittle. The glass transition temperature (*T*g) of PHB lies between 5 and 9° C, and the melting point (*T*m) lies between 173 and 180° C. PHB polymer is decomposed at approximately 200° C, which is close to its melting temperature. Mechanical properties such as the Young's modulus (3.5 GPa) and the tensile strength (43 MPa) of PHB material are close to those of isotactic polypropylene. However, the extensions to break (5 %) for PHB is significantly lower than that of polypropylene (400 %). Therefore, PHB is stiffer and more brittle plastic material as compared to polypropylene [3].

The incorporation of other monomer units into PHA structure can markedly improve its mechanical properties. The flexibility of the material is changed when, for instance, 3-hydroxyvalerate units are incorporated into the polymer, resulting in a decrease of Young’s

modulus below 0.7 and a decrease of tensile strength below 30 MPa. The elongation to break also increases as the co-monomer fraction increases. The melt temperature is greatly depressed, down to 130° C, dependent on the 3-hydroxyvalerate content. This property generates a relatively wide window of conditions that allow thermal processing as a melting of polymer without thermal degradation of the material. Because of its mechanical properties, copolymer of 3-hydroxybutyrate and 4-hydroxybutyrate seems to be very promising material. Its monomers can not form isomorphic crystals due to the extent of the structural differences between the monomer units. The influences of monomer composition on SCL-PHA mechanical properties are summarized in **Tab. 1** [1, 3].

Tab. 1 *The mechanical properties of some of SCL-PHA and polypropylene [1]*

	P(3HB)	P(3HB/3HV) 90:10	P(3HB/3HV) 20:80	P(4HB)	P(3HB/4HB) 90:10	P(3HB/4HB) 10:90	PP
Melting point (°C)	179	150	135	53	159	50	170
Young's modulus (GPa)	3.5	1.2	0.8	149	-	100	1.7
Elongation to break (%)	3	20	100	1000	242	1080	400

P(3HB) – poly(3-hydoxybutyrate), P(3HB/3HV) – poly(3-hydroxybutyrate-co-3-hydroxyvalerate), P(4HB) – poly(4-hydroxybutyrate), P(3HB/4HB) – poly(3-hydroxybutyrate-co-4-hydroxybutyrate), PP – polypropylene

MCL-PHA have significantly lower melting point temperature as compared to SCL-PHA and, moreover, this value is also strongly dependent on thermal history of material. Melting point temperature varies about 39-61° C. The glass transition temperature is usually below room temperature, ranging from -43 to -25° C and crystallinity of MCL-PHA achieves about 25 % [3].

2.2.2 Structure of intracellular PHA granules

As was mentioned above, PHA are accumulated in cells in the form of intracellular granules. Nuclear magnetic resonance (NMR) spectroscopy of various bacteria has clearly demonstrated that the polyester in the cells occurs in a metastable amorphous state and granule morphology is under kinetic rather than thermodynamic control [4]. The density of PHB granules is about 1.18 – 1.24 $g.cm^{-3}$. The isolated granules consist of polyester, proteins and phospholipids. The composition of PHB granules was determined for granules of *Bacillus megaterium*, which consist of 97.7 % polyester, 1.87 % proteins and 0.46 % lipids or phospholipids [1].

In native PHA granules, polyester material is surrounded by a phospholipids layer in which proteins are incorporated. Because of strongly hydrophobic nature of PHA, phospolipids with proteins present intermediate layer between cytoplasm and polyesters [1].

Many proteins have been discovered in the lipid layer of native PHA granules, nevertheless, function of some of them has not been identified so far. First of all, PHA polymerases and depolymerases are present. In addition, granule associated proteins also

called phasins are embedded in the phospholipid layer. Generally, phasins are low molecular weight proteins that are assumed to form a close protein layer at the surface of the granules, providing the interface between the hydrophilic cytoplasm and the hydrophobic core of the PHA inclusion [5]. Furthermore, phasins are considered to be involved in regulation of PHA synthesis, but mechanism has not been clearly understood [6, 7]. Another important protein called GA24 was identified in *Cupriavidus necator*. Mutants lacking the GA24 protein synthesized only one large PHB granule per cell in contrast to 10–30 granules as found in the wild type. The amino acid sequence of the GA24 protein revealed two closely related stretches consisting exclusively of non-hydrophilic amino acids at the C-terminal region, which are presumably involved in the binding of GA24 to the granules [8].

Recently, Uchino et al. surprisingly reported the presence of other enzymes responsible for PHB synthesis and mobilization in native PHB granules structure. These enzymes were expected to be present only in cytoplasm [9].

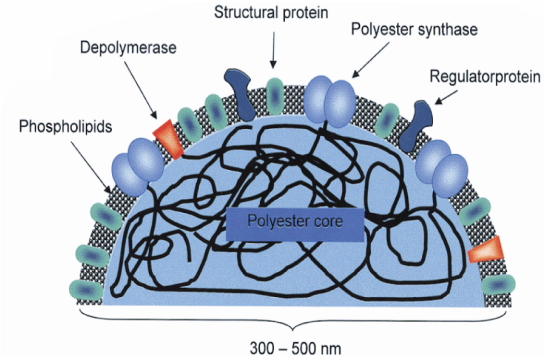


Fig. 2 *Structure of PHA granule [5].*

Gerngross and Martin *in vitro* experiments demonstrated that for synthesis of PHB and self-assembly of granules only PHA synthase and substrate are needed [10]. On the other side, biosynthesis of PHA granules *in vivo* is described by two models. The first - micelle model - is based on the observation of PHA synthesis *in vitro,* whereas the second - budding model - is supported by observation of membrane-like material surrounding PHA granules and by the fact that early stage granules are not randomly distributed in the cytoplasm but are situated close to the membrane [11].

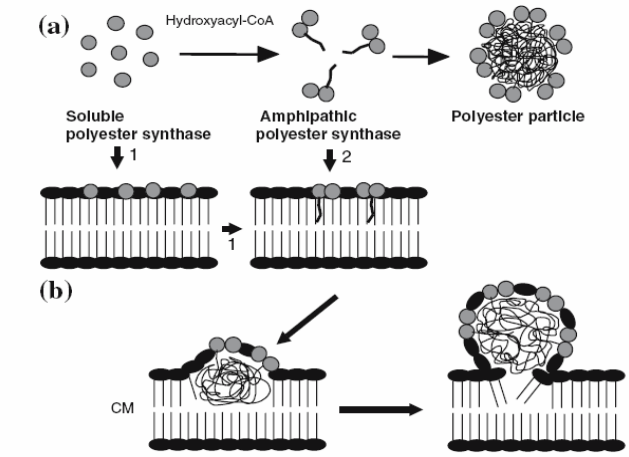


Fig. 3 *Models for polyester granule self-assembly. a) In vitro assembly process b) in vivo assembly and its two possible routes 1 and 2 [12].*

2.3 Biosynthesis of polyhydroxyalkanoates

2.3.1 SCL-PHA biosynthetic pathway

The biochemistry of SCL-PHA biosynthesis is well studied [1, 3, 4, 6]. 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 [6].

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, 3].

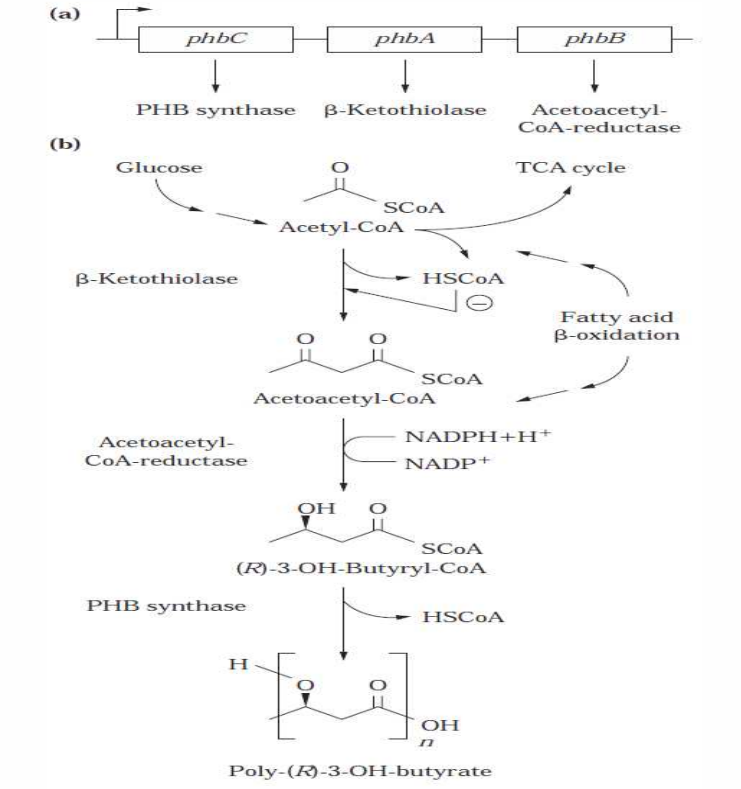


Fig. 4 *PHB biosynthesis in C. necator [1].*

Another type of PHA biosynthesis pathway is exhibited by the *Pseudomonas* which can synthesize MCL-PHA from various alkanes, alkanols or alkanoates. In contrast to *C. necator*, most *Pseudomonas* sp. generally do not synthesize PHA containing SCL monomers. Hence, the range of monomers incorporated into MCL-PHA synthesized by these *Pseudomonas* sp. is much wider. Bacteria in this group, derives the 3-hydroxyacyl-CoA substrates from the intermediates of fatty acid β-oxidation pathway, de novo fatty acid biosynthesis pathway and alkane oxidation pathway [3].

The intermediate precursors of MCL-PHA are ketoacyl-CoA, *S*-3-hydroxyacyl-CoA, enoyl-CoA, or *R*-3-hydroxyacyl-acyl carrier protein (ACP). Regarding the similarity between the SCL-PHA synthases and the MCL-PHA synthases, it seems likely that the ultimate substrate for polymerization is the *R*-form of the CoA activated 3-hydroxy fatty acid intermediates. Because the monomeric units in MCL-PHA, like those in SCL-PHA, are in the *R*-form, and the SCL-PHA synthases and MCL-PHA synthases are homologous, the intermediates in fatty acid metabolism must presumably be converted to the *R*-form of 3-hydroxyacyl-CoA before transesterification. The MCL-PHA precursors are generally considered to be available in a much lower in situ concentration than the PHB precursors because they are intermediates of ongoing fatty acid metabolic cycles [1, 3]. The general overview of metabolic pathway involved in SCL and MCL is provided in **Fig. 5**.

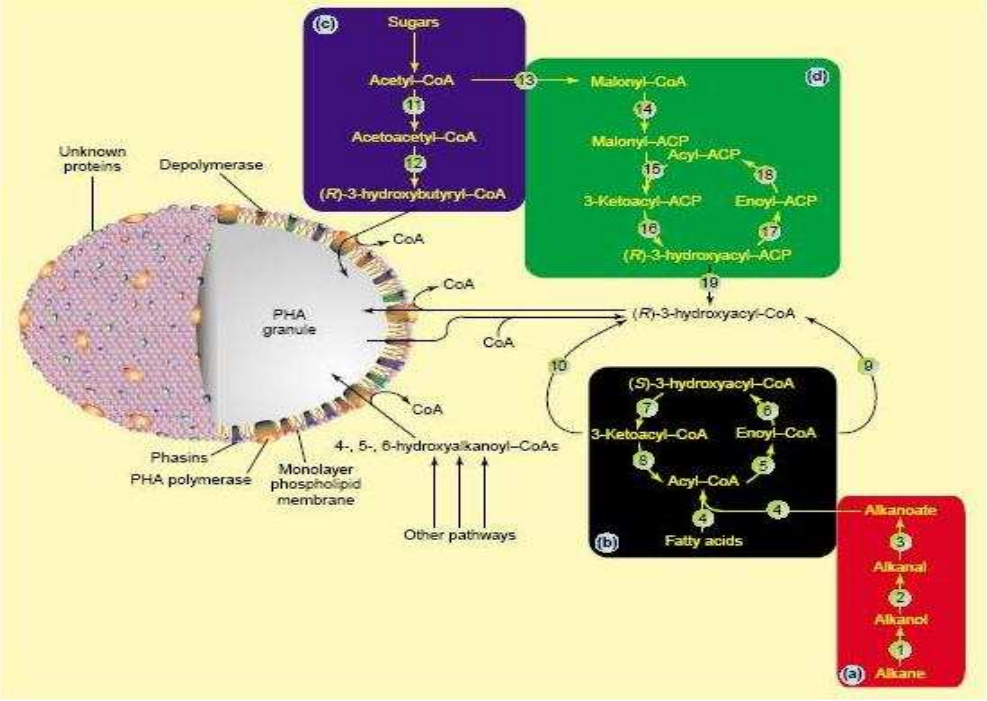


Fig. 5 *Structure of PHA granule and metabolic interconnections between different pathways involved in the biosynthesis of SCL and MCL-PHA. (a) Alkane oxidation pathway. (1) Alkane 1-monooxygenase, (2) alcohol dehydrogenase, (3) aldehyde dehydrogenase (b) Fatty-acid β-oxidation. (4) acyl–CoA ligase, (5) acyl–CoA dehydrogenase, (6) enoyl–CoA hydratase, (7) 3-hydroxyacyl–CoA dehydrogenase, (8) 3-ketothiolase, (9) R-enoyl–CoA hydratase, (10) 3-ketoacyl–CoA reductase. (c) Biosynthesis from carbohydrates. (11) β-ketothiolase (12) NADPH-dependent acetoacetyl–CoA reductase. (d) De-novo fatty acid synthesis. (13) acetyl–CoA carboxylase, (14) ACP-malonyltransferase (15) 3-ketoacyl-ACP synthase, (16) 3-ketoacyl-ACP reductase, (17) 3-hydroxyacyl-ACP reductase, (18) enoyl-ACP reductase, (19) 3-hydroxyacyl-ACP–CoA transacylase [13].*

2.3.2 PHA synthases and their catalytic mechanism

Some authors consider PHA synthases to be the key enzymes of PHA biosynthesis [5]. As was mentioned above, PHA synthases catalyse the stereo-selective conversion of *R*-3-hydroxyacyl-CoA substrates into PHA with concomitant release of CoA.

Thus far, the nucleotide sequences of 59 PHA synthase genes from 45 different bacteria have been obtained. With respect to the primary structures deduced from these sequences, the substrate specificities of the enzymes and the subunit composition, four major classes of PHA synthases can be distinguished. PHA synthase which belong to class I and class II are enzymes consisting of only one type of subunit (PhaC) with molecular masses between 61 kDa and 73 kDa. According to their *in vivo* and *in vitro* substrate specificity, class I PHA synthases (for instance that of *C. necator*) preferentially utilize CoA thioesters of various *R*-3-hydroxy fatty acids comprising 3 to 5 carbon atoms, whereas class II PHA synthases (e.g. in *Pseudomonas aeruginosa*) preferentially utilize CoA thioester of various *R*-3-hydroxy fatty acids comprising 6 to 14 carbon atoms [5].

Class III PHA synthases (e.g. *Allochromatium vinosum*) comprises enzymes consisting of two different types of subunits, the PhaC subunit (molecular mass of approx. 40 kDa) exhibiting amino acid sequence similarity of 21–28% to class I and II PHA synthases and the PhaE subunit (molecuar mass of approx. 40 kDa) with no similarity to PHA synthases. These, class III PHA synthases, similarly to class I synthases, prefer CoA thioesters of *R*-3-hydroxy fatty acids comprising 3 to 5 carbon atoms. PHA synthases belonging to the recently identified Class IV (e.g. in *Bacillus megaterium*) resemble the class III PHA synthases, but subunit PhaE is replaced by PhaR (molecular mass of approx. 20 kDa) [5].

Extensive comparison of the 59 PHA synthases from various bacteria revealed that these enzymes exhibit strong similarity (up to 96 % of identical amino acids). With respect to amino acid sequence regions with stronger similarity, six conserved blocks could be identified. The N-terminal region is highly variable whereas C terminus, containing high number of hydrophobic amino acids, seems to be more conservative [5]. Site-directed mutagenesis demonstrated that the cysteine$_{319}$ residue is required for class I PHA synthase activity in *C. necator*. Furthermore, the enzyme requires posttranslational modification by phosphopantetheine to provide a second thiol group per PHA synthase subunit [1].

In general, proposed catalytic mechanism of PHA synthases is based on mechanism of lipases. The similarity between PHA synthase and lipase can be mechanistically informative for number of reasons. First, ester bonds formation by lipases is 100-fold accelerated if enzyme is bound to micelle, generally, lipases serve as a paradigm for interfacial catalysis. Similarly, PHA synthases are bound to the surface of PHA granules, which is extensively covered with monolayer of lipids. Second, lipases are members of the α/β superfamily proteins and atomic resolution structures are available. A threading model for both class I and III PHA synthase has been generated using sequence alignments and lipase structures. Finally, the mechanism of lipases is very well understood and involves covalent catalysis using an active site serin that is activated for nucleophilic attack by a histidine [14].

These information have facilitated formulation of potential mechanisms for PHA synthesis, however, many other mechanisms are possible. Two of proposed mechanisms are shown in **Fig. 6** [14]. Mechanism A involves an active site located at the interface of two protein monomers. According to this proposal, two cysteines (one provided by each

monomer) are involved in the covalent catalysis. In each monomer, histidine residue activates the cysteine residue for nucleophilic attack. In this model, the growing chain of polymer remains covalently attached during polymer elongation and switches from one monomer to the other [15]. An alternative mechanism (Fig. 6 B) involves covalent catalysis with single cysteine. In this case, the second 3-hydroxybutyryl-CoA binds non-covalently to the synthase. The second mechanism is partially based on the mechanism of several polyketide synthases rather than that of lipases, however, some authors prefer this one [16].

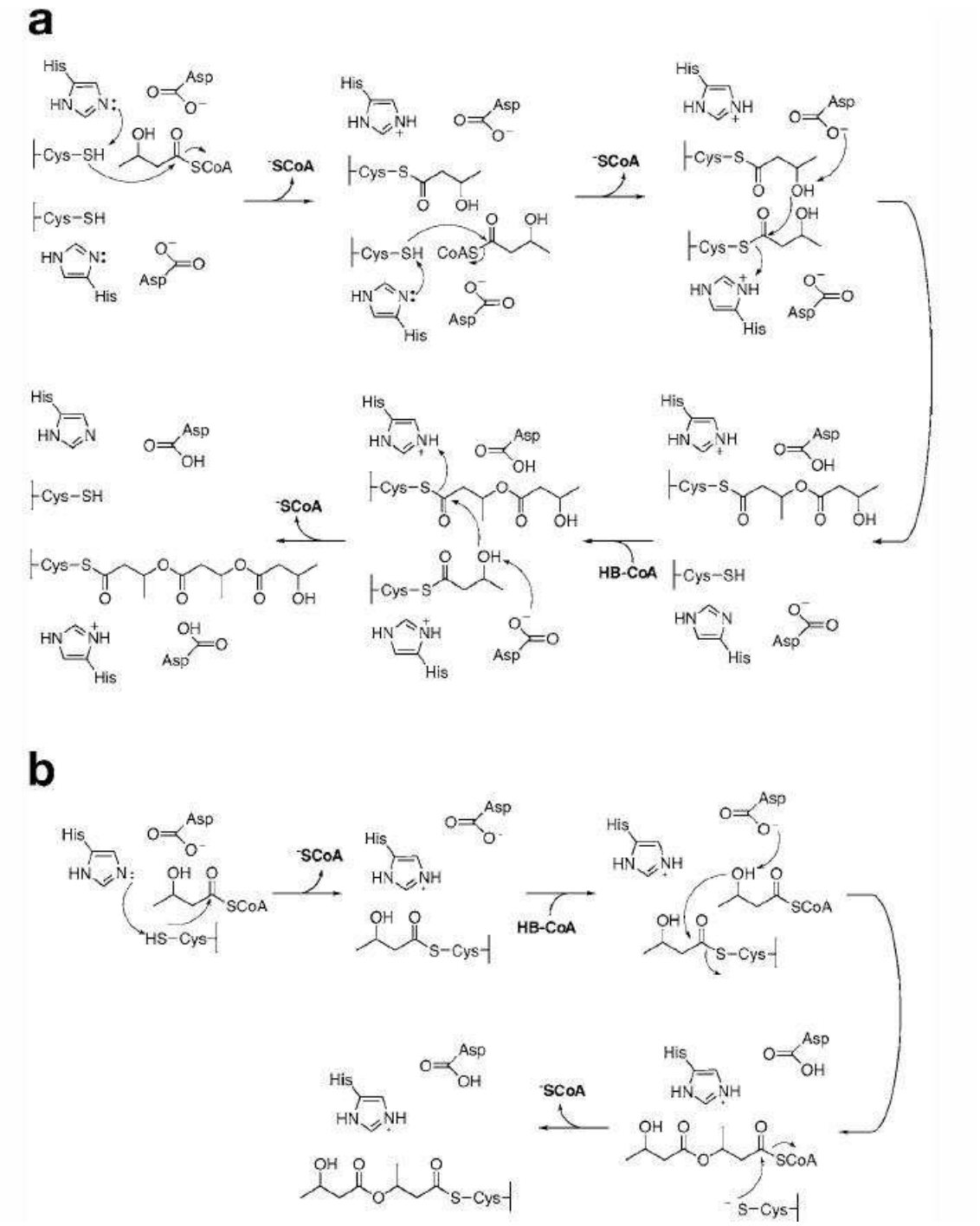


Fig. 6 *Two proposed mechanisms for class I and class III synthases involving two (a) or one (b) covalent intermediates [14].*

2.4 Regulation of PHA biosynthetic pathway

2.4.1 Regulation at enzymatic level

As could be expected, biosynthesis of PHA is strongly regulated at enzymatic level. The regulation is described in details for *C. necator* as a model bacterial strain. The concentrations of acetyl-CoA and free CoA are the key factors regulating activity of β-ketothiolase which catalyze the first step of polymer synthesis. In addition, it has been shown that PHB biosynthesis is strongly stimulated by both high intracellular concentrations of NAD(P)H and high ratios NAD(P)H/NAD(P)$^+$ [17]. Furthermore, citrate synthase and citrate dehydrogenase are significantly inhibited by coenzymes NAD(P)H, which consequently enhances flux of acetyl-CoA into PHB biosynthetic pathway instead of TCA cycle [18]. This statement was supported by experiment of Park and Lee who observed that isocitrate dehydrogenase-leaky mutant of *C. necator*, which exhibited lower activity of TCA cycle, produced PHA at a faster rate as compared to wild type [19]. According to Jung et al. the rate of PHA biosynthesis is controlled by β-ketothiolase and acetoacetyl-CoA reductase, whereas the content of PHB is controlled by PHA synthase [20].

In the cyanobacterium *Synechococcus* sp. MA19, it has been found that PHA synthase is exclusively presented in membrane fractions of cells cultivated under nitrogen limitation conditions. It is proposed that PHB synthase is posttranslationally activated by acetyl-phosphatase. Furthermore, a second enzyme involved, phosphotransacetylase that converts acetyl-CoA to acetyl phosphate is regulated by the acetyl-CoA concentration as well as by the carbon/nitrogen ratio. Therefore, acetyl phosphate could be the signal of carbon/nitrogen balance affecting PHA metabolism [21].

2.4.2 Regulation at transcription level

In various bacterial strains, regulatory proteins involved in PHA biosynthesis have been either experimentally identified or postulated based on the sequence upstream of *pha* genes (Tab. 2).

Tab. 2 *Regulatory proteins involved in the PHA metabolism [6].*

Regulatory protein	Regulator family	Organism
PhaF	Histone H1-like	*Pseudomonas oleovorans* *Pseudomonas aeruginosa* *Pseudomonas putida*
$PhaR_{Ps}$	AraC/XylS	*Pseudomonas* sp. 61-3
$PhaR_{Pd}$	?	*Paracoccus denitrificans*
PhaS	Two component systems	*Pseudomonas putida*
GacS	Two component systems	*Azotobacter vinelandii*
NtrB/NtrC	Two component systems	*Azospirillum brasilense* Sp7
LuxR	Quorum senser	*Vibrio harveyi*

For instance, promoter induced under conditions of nitrogen starvation was identified in *Acinetobacter* sp. [22]. Moreover, *ntrB* and *ntrC* genes, which are involved in nitrogen regulation in various bacterial strains, are also connected with regulation of PHB biosynthesis

in *Azospirilum brasilence* during ammonium starvation [23]. It has been found that the LuxR regulatory protein controls not only the bioluminescence system, but also PHB biosynthesis in *Vibrio harveyi*. Generally, LuxR is activated on growth of cells to a high density due to the accumulation of an excreted N-acyl homoserine lactone into the media. This is probably the reason why *V. harveyi* accumulates PHB in stationary phase. *V. harveyi* LuxR negative mutants were found to be unable of PHB accumulation [24].

Many other regulation factors were described in *Psedomonas*. For instance a transcriptional regulator called $PhbR_{Ps}$ was found in strain *Pseudomonas* sp. 61-3. This strain is unique due to its ability to produce PHB homopolymer as well as a random copolymer containing units from four to twelve carbon atoms. The $PhbR_{Ps}$ is only involved in expression of genes which are required for PHB homopolymer biosynthesis. Interestingly, transcription of the $phbR_{Ps}$ genes in a recombinant *C. necator* PHB negative mutant strain was independent of the $PhaR_{Ps}$ regulator encoding gene, suggesting either another functional promoter upstream of the genes or a regulator of *C. necator* which can substitute $PhaR_{Ps}$ [25].

For *Pseudomonas aeruginosa* it has been shown, that for PHA accumulation from gluconate, a functional RpoN sigma factor is required, whereas PHA accumulation was only reduced when culture was cultivated on fatty acids. Generally, RpoN is an alternative *sigma* factor of the RNA polymerase, which is involved in growth phase depending activation of certain ”non-house-keeping” promoters. However, it is still not clear, why this *sigma* factor is required for PHB biosynthesis from carbohydrates *via* de novo fatty acid synthesis and not from fatty acid *via* β-oxidation [26]. Recently, it was observed in *Pseudomonas putida*, that *sigma* factor RpoS, which controls the expression of several genes involved in survival under adverse conditions, is indirectly involved in the regulation of genes encoding for PHA synthase (*phaC*) and PHA depolymerase (*phaZ*). Actually, RpoS negative mutants exhibited higher *phaC* and *phaZ* gene expression which, surprisingly, caused lower PHA content in cells [27].

Furthermore, protein PhaF was observed to be involved in regulation of genes of PHA biosynthetic pathway and to its regulation function. In addition, PhaF is also bound to PHA granules. This bifunctional character of PhaF is probably due to its two domains showing homology to histone-like DNA associated proteins and PHA granule associated proteins, respectively. According to the suggested model, in the absence of substrate for PHA biosynthesis PhaF is bound to DNA and inhibits expression of genes of PHA biosynthetic pathway. When PHA granules are formed, DNA binding is reduced PhaF binds to PHA granules and *pha* genes are expressed [28]. Similar regulation protein called PhaQ was found in *Bacillus megaterium*. Also this protein is bound to either PHA granules or DNA. If PhaQ is bound to DNA, it serves as s transcriptional regulator that negatively controls both *PhaQ* and *PhaP* genes [29]. McCool and Canon proved that phasin protein PhaP, in addition to its structural function in PHA granules, serves as amino acids storage protein. Moreover, in *Bacillus megaterium*, PhaP is probably involved in regulation of expression of spore specific storage protein SspD, because expression of both genes is mutually exclusive [30].

2.4.3 Regulation of PHA molecular weight

Molecular weight of PHA is an important index determining mechanical properties of PHA. Generally, many factors such as producing strain, medium composition and cultivation conditions influence molecular weight of polyesters. Effect of different growth conditions

(pH, temperature, aeration, carbon substrate etc.) on molecular weight of PHB was studied in *Azotobacter chroococum* 7B. It was observed that conditions optimal for PHB production, in the terms of PHB content in cells, resulted in maximal molecular weight as well [31].

In spite of the fact that the mechanism of PHA chain termination has not been clearly understood yet, PHA synthase is generally considered to be the enzyme responsible for control of PHA molecular weight. For instance Sim et al. reported that, in recombinant *E. coli* harboring *phaCAB* operon of *C. necator*, an increase in PHA synthase activity resulted in lower molecular weight of PHB [32]. Similar results were obtained by Gernoss and Martin who demonstrated that PHB molecular weight decreased with an increase of PHB synthase activity *in vitro* [33]. On the contrary, Kichise et al. observed that molecular weight of produced PHB is independent of PHB synthase activity in *C. necator* [34]. Generally, it is proposed that in bacterial strains naturally producing PHA, chain-transfer agent is generated, which reacts with propagating polyester chain to regulate the chain length. However, such an agent has not been isolated or identified so far [35]. Nevertheless, mentioned theoretical model of chain-transfer agent is supported by work of Mantzaris et al., who made up a mathematical model describing the dynamics of molecular weight distribution in PHA producing cells. According to this model the termination rates increase up to a critical molecular weight and then subsequently decrease. This could be due to the action of proposed chain-transfer agent [36].

Moreover, it was reported that the molecular weight of PHB produced by *C. necator* decreases during the course of polymer accumulation in batch culture. It is probably partially due to the intracellular degradation of polyesters [37]. Because in recombinant cells harboring only PHA synthetic genes enzymes responsible for PHA degradation are missing, these strains have potential to accumulate PHA of a high molecular weight. This strategy of production of materials of supra molecular weight was used in *E. coli* harboring *Azohydromonas lata* PHA biosynthetic genes [38].

2.5 Biodegradation of PHA

2.5.1 Intracellular PHA degradation

In general, it is assumed, that intracellular biosynthesis and degradation are highly regulated. According to this presumption, most of bacterial strains repress PHA depolymerase in presence of a soluble carbon source and after exhaustion of nutrients, the synthesis of PHA depolymerases is derepressed [39]. However, in contradiction to this theory are findings that the inhibition of protein synthesis did not influence PHA degradation in *P. oleovorans* [40]. According to these results, PHA depolymerase is always present and active and, therefore, PHA biosynthesis and degradation are occurred simultaneously. These suggestions are supported by results of Doi et al. who worked with *C. necator* [41].

Similarly to regulation of PHA biosynthetic pathway, the system of intracellular biodegradation is the best described in *C. necator*. The first PHA depolymerase, PhaZa, was identified and characetized by Seaguza et al. [42]. According to gene sequence, the enzyme consists of 419 amino acids with a predicted molecular mass of 47 kDa. The enzyme was capable of digesting amorphous PHB, it means form of PHB which occurs in cells (see chapter 2.5.2), while oligomers of 3-hydroxybutyrate were released. Enzyme was not able to hydrolyze crystalline PHB and the deduced amino acid sequence lacked sequence

corresponding to a classical lipase box Gly-X-Ser-X-Gly [42]. Next two intracellular PHA depolymerases, PhaZb and PhaZc, were discovered by York et al. [43]. According to their study, PhaZa was being presented in cell during whole cultivation, whereas PhaZb was expressed only under conditions of PHA degradation. The role of PhaZb was clarified by Kobayashi et al [44]. The purified enzyme degraded amorphous PHB in rate similar to PhaZa, but, moreover, it hydrolyzed linear and cyclic 3-hydroxybutyrate oligomers as well. Because of ability to hydrolyze cyclic oligomers, the enzyme appears to be endo-type of hydrolase. PhaZb was found as bound to PHB granules as well as a soluble enzyme in cytoplasm. In contrast, PhaZa is exclusively bound to granules. In the next paper, Kobayashi et al. [45] focused on PhaZc depolymerase. Most of PhaZc was observed in cytoplasm, while only small amount was bound to PHA inclusion bodies. PhaZc revealed high specific activity for various 3-hydroxybutyrate oligomers and low specific activity for amorphous PHB. Native PHB granules and crystalline PHB were not degraded at all [45]. Another PHA depolymerase of *C. necator*, PhaZd, was identified by Abe et. al. [46] The position of catalytic triad (Ser^{190}-Asp^{266}-His^{330}) and oxyanion hole (His^{108}) were similar to extracellular PHB depolymerase of *Ralstonia pickettii.* The specific activity of PhaZd toward artificial amorphous granules was significantly higher than that of the other known intracellular depolymerases of *C. necator*. Interestingly, PhaZd distributed nearly equally between PHB inclusion bodies and the cytoplasm.

On the bases of finished genome sequence of *C. necator*, it is assumed that as many as nine PHB depolymerases and 3-hydroxybutyrate oligomer hydrolases are presented in *C. necator*. Five putative PHB depolymerases isoenzymes (PhaZa1 to PhaZa5), two 3-hydroxybutyrytic acid oligomer hydrolases PhaZb and PhaZc and two isoenzymes of putative PHB depolymerase (PhaZd1 and PhaZd2) [47].

Novel PHA depolymerase PhaZ1 was recently identified in *Bacillus megaterium.* PhaZ1 had strong affinity towards native PHB granules which were rapidly degradated by the enzyme. Almost exclusive products of the enzyme activity were 3-hydroxybutyric acid monomers. Unlike other intracellular polymerases, PhaZ1 was also able to hydrolyze semicrystalline PHB with the mutual generation of monomers [48].

Egenio et al. [49] characterized interesting intracellular MCL-PHA depolymerase of *Pseudomonas putida*. The enzyme is located in PHA granules and it hydrolyzes specifically MCL-PHA containing some aliphatic and aromatic monomers. The enzyme behaves as a serine hydrolase that is inhibited by phenylmethylsuplhonylfluoride (PMFS).

Generally, it is assumed, that bacterial strains which are able to accumulate PHA dispose with systems of enzymes allowing hydrolysis and following utilization of PHA under conditions of carbon substrate starvation. These enzymes can be divided according to their substrate specificity to (i) PHA depolymerases that are usually bound to PHA inclusion bodies (PhaZa and PhaZd in case of *C. necator*) and hydrolyze polymeric chain to oligomers. The next group of enzymes is (ii) oligomer hydrolases present in cytoplasm, which consequently hydrolyze oligomers to monomers (PhaZb and PhaZc in *C. necator*). This very quick and effective mechanism of PHA mobilization was observed in many bacterial strains including *C. necator, Ralstonia pickettii* T1 and *Acidovorax* sp. [50] or *Rhodospirillum rubrum* [51].

From biotechnological point of view, intracellular PHA depolymerases negative mutant strains could be interesting candidates for industrial PHA production. For instance,

Cai et al. significantly improved MCL-PHA production in *Pseudomonas putida via* knock-out of PHA depolymerase [52].

2.5.2 Extracellular PHA degradation

In living cells, PHA granules are formed by polyester material in metastable amorphous state which is often called native (nPHA). It was proposed that *in vivo* the polymer morphology is under kinetics rather than thermodynamic control [4]. After cell lysis and exposition of polyester material to surrounding conditions, cover layer of phospholipids and proteins is quickly damaged and polymer chains transform from amorphous to partially crystalline state which is often called denaturated (dPHA). Next state of PHA material is called artificial amorphous state (aPHA). PHA in artificial amorphous state could be prepared when PHA are solubilised in appropriate solvent (trichlormethane, acetone), added to detergent solution in water and emulsified. After evaporation of solvent, stable latex of artificial PHA granules is obtained [53].

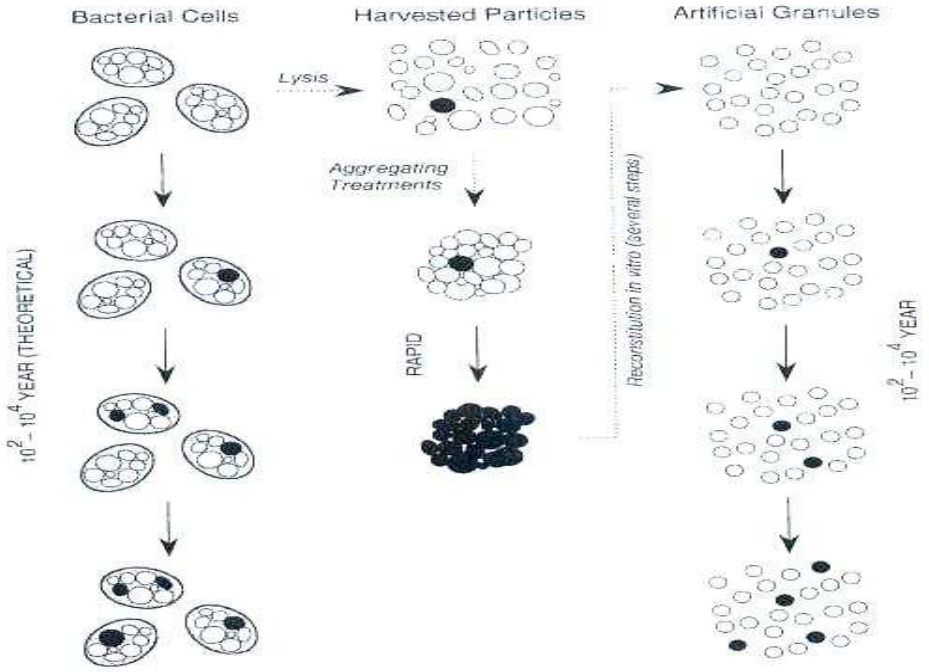


Fig. 7 *Process of transformation of PHA states (black – crystalline form, white – amorphous) [54].*

The ability to degrade extracellular PHA is widely distributed among microorganisms and depends upon the secretion of extracellular PHA depolymerases which hydrolyze the water insoluble polymer to water soluble monomers and/or oligomers. It is important to note, that enzymes that hydrolyze PHA are often specific for one of the form of PHA. For example, extracellular depolymerases hydrolyze dPHA, whereas intracellular PHA depolymerases are specific for nPHA and possibly for aPHA. More than 30 extracellular depolymerases, most of them were of prokaryotic origin, were characterized in the last two decades [53, 54].

Many extracellular depolymerases were purified from different microorgnaism and characterized. The purified PHA depolymereases consisted of a single polypeptide chain and

their molecular weights are in ranges of 37 000 – 60 000 Da. Analysis of the structural genes for extracellular PHA depolymerases has shown that all enzymes are comprised of an N-terminal catalytic domain, a C-terminal substrate-binding domain, and a linker region connecting the two domains. The presence of both catalytic and binding domains has been found in many depolymerizing enzymes such as cellulase, xylanase and chitinase, which hydrolyze water-insoluble polysaccharides [3]. The catalytic domain contains a lipase box pentapeptide (Gly-X-Ser-X-Gly) as an active site which is common for serine hydrolase. The active site serine forms a catalytic triad with an aspartate and a histidine, and a transient tetrahedral intermediate of the substrate carbonyl carbon is stabilized by NH groups around the histidine residue (oxyanion hole) [1].

2.6 PHA and stress response of bacteria

2.6.1 Stress response of bacteria from general point of view

Bacteria have successfully colonized every niche on the planet, from the soil-dwelling Gram-pozitive (e.g. *Bacillus subtilis*) to Gram-negative (e.g. *Escherichia coli*), found in the lowest intestines of mammals, to *Deinococcus radiodurans*, which persist in nuclear reactors and can survive radiation doses sufficient to kill all other living forms. In this vast range of different environments, bacteria are exposed to wildly fluctuating environmental stresses including changes in temperature, pH, osmolarity, radiation, toxins and nutrient starvations. To ensure survival in the face of these adversaries, bacteria developed sophisticated system of different strategies. Bacteria may move by „swimming“ using their molecular motor, the flagellum, to more favorable locations, or they may adapt to changes in their immediate vicinity by responding to the imposed stress. The stress response is complex process involving changes at all the levels – from changes in morphology and cell structure to activation of particular metabolic pathways which is accompanied by enhanced accumulation of some metabolite(s) and degradation of another. Nevertheless, the most important ones are changes at transcription level. Expression of genes, which products are required to combat deleterious nature of the stress, is enhanced, while expression of other genes is inhibited [55].

The up-regulation of the transcription of stress responsive genes is achieved by the activation of transcription factors that interact with RNA polymerase to co-ordinate gene expression. One family of transcription factors with a role of stress resistance is a subunit of RNA polymerase, the *sigma* factor, which is essential for transcription initiation by playing the key role in promoter recognition. Each of several *sigma* factors in the cell is required for the transcription of specific subset of genes/operons within their “regulon” [55]. In the **Tab. 3**, there are summarized *sigma* factors of *Ralstonia metallidurans* CH34 and their functions. This bacterial strain is able to endure high concentration of heavy metals and is closely related to *Cupriavidus necator* – model organism for polyhydroxyalkanoates metabolism [56].

Sigma factor RpoS (also called σ^s, σ^{38}) is considered to be responsible for general response to wide variety of stress conditions such as starvation, high osmolarity, acidic or alcalic pH, low or high temperature, UV radiation and presence of many toxic substances (including ethanol) [56]. Studies comparing RpoS depending promoters revealed a potential -10 motif for RpoS-dependent RNA polymerase holoenzyme TG(N)0-2CCATA(c/a)T. By standard genetic and molecular biology methods, more than 80 RpoS controlled genes have been identified in *E. coli* [57]. Nevertheless, also other *sigma* factors are involved in stress

response to particular stress conditions, for instance RpoH (heat shock, chromate tolerance), RpoE (extreme heat shock) or RpoN (nitrogen limitation, toxic substances) [56].

Tab. 3 *Sigma factors in Ralstonia metallidurans strain CH34 and their functions [56].*

Name	Contig	Cluster	Related proteins	Possible function
RpoD	629	ICF	RpoD_Ecoli	Main housekeeping sigma factor
RpoS	533	ICF	RpoS_Ecoli	Starvation and other cytoplasmic stress conditions
RpoH	660	ICF	RpoH_Ecoli	Reponse to protein denaturation in the cytoplasm, heat shock
Sig345	345	(ICF)	None	Sequencing artifact?
FliA	706	MDF	FliA_Ecoli	Synthesis of the bacterial flagellum
Sig605	605	(ECF)	SigH_Bsub	Transition from exponential to stationary phase of growth?
Sig538	538	(ECF)	SigH_Bsub	Transition from exponential to stationary phase of growth?
Sig588	588	ECF:RpoE	CnrH	Another metal resistance?
CnrH	(pMOL28)	ECF:RpoE	(NccH)	Nickel resistance
RpoE	534	ECF:RpoE	RpoE_Ecoli	Reponse to protein denaturation in the periplasm and cell wall
Sig562	562	ECF:FecI	None	Cell wall integrity?
Sig681	681	ECF:FecI	None	Cell wall integrity?
Sig679	679	ECF:FecI	None	Cell wall integrity?
Sig680	680	ECF:FecI	FecI_Ecoli	Metal (iron?) homeostasis?
Sig697	697	ECF:FecI	FecI_Ecoli	Uptake of citric iron
Sig611	611	ECF:FecI	FecI_Ecoli	Uptake of citric iron
RpoN	669	RpoN	RpoN_Ecoli	Tight control of operons, e.g., nitrogen assimilation, hydrogenase synthesis, degradation of xenobiotics and aromatic compounds
(ORF)	480	None	TrpF	No sigma factor
(ORF)	593	None	None	No sigma factor

2.6.2 Involvement of PHA in stress resistance 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 [58, 59]. 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 [60]. 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 [61-72]. 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 [61]. 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 [62, 63, 64]. 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 [65].

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 [66, 67]. Natarajan et al. reported that NaCl stress resulted in the accumulation of PHB in *Rhizobium* DDSS-69 cultures grown under unbalanced growth conditions [68]. Positive effect of ethanol stress on PHB production was observed in genetically modified *Pichia pastoris*. Ethanol formed under initial phase of oxygen limited growth was further utilized for PHB production [69]. Interesting contribution was given by Wang et al. [70] 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*. It was observed that PHA degradation is accompanied with an increase of ATP and guanosin tetraphosphate (ppGpp) levels in cells [71]. 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 [72]. Thus, PHA seem to be involved in stress response under conditions of carbon starvation through RpoS, which consequently enhancew 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 [27]. 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 [73].

To conclude this short review on involvement of PHA in stress response of bacteria, it should be stated that despite many observations of enhanced stress resistance in PHA producing strains, there are still many questions. It was observed that presence of PHA granules in cytoplasm improves stress durability, but what is the reason? Moreover, intracellular mobilization of PHA even enhances this effect. Is it the main role of PHA in

stress response as many authors suggested? Furthermore, it was observed and it can be expected as well, that there are some connections between PHA and RpoS sigma factors in both directions. First, degradation of PHA seems to stimulate RpoS expression and, second, PHA synthetic and degradation genes could be partially under control of RpoS. But what are the consequences of such a connection for metabolism? Is this effect general for all PHA producing strains or is it only among *Pseudomonas* strains? The answers to these questions would be interesting not only from scientific point of view but, moreover, could bring many practical biotechnological outcomes - for instance in fields of PHA production as well as in bioremediation, waste water treatments and other environmental processes during which bacteria are exposed to adverse conditions.

2.7 Modern trends in industrial production of PHA

Polyhydroxyalkanoates have been in attention of many companies as biodegradable and biocompatible alternative to synthetic polymers for very long time. In 1976, Imperial Chemical Industries (ICI Ltd., UK) recognized the potential applicability of PHB to replace some of the oil-derived commodity plastics. Although the bacterially produced PHB was relatively expensive compared to petrochemical plastics, it was expected that the price of crude oils would rise to high levels, making the production of PHB economically feasible. Because no rise in oil prices occurred, PHB came to be seen as a biodegradable and biocompatible plastic material. Mainly because of their high price, PHB and P(HB-co-HV) known under the trademark Biopol® have only rarely found their way to the market. In 1990, the German company Wella released a new shampoo packaged in bottles made of Biopol®. Early in 1996, Monsanto (USA) bought the Biopol® business from ZENECA BioProducts, a daughter company of ICI. Monsanto tried to employ transgenic plants (i.e., soybean and rapeseed) for agricultural production of PHB and P(HB-co-HV), which was expected to result in a significantly lower price of the final product [1]. Nevertheless, Monsanto never started commercial PHA production and in the year 1999 sold Biopol® to Metabolix. According to Metabolix webpages (www.metabolix.com) plant for large scale fermentation production of PHA using recombinant *E. coli* was opened in December 2009. Moreover, Metabolix plans to produce PHA using metabolically engineered perennial grass - Switchgrass (*Panicum virgatum*).

2.7.1 PHA production from waste or inexpesive substrates employing bacterial monoculture

Traditionally, polyhydroxyalkanoates are produced using sugars (for instance glucose or fructose) as carbon sources employing bacterial monoculture of PHA producing strain. Many bacteria have been screened to produce PHA. However, only a few of them could be used for biotechnological production of these polyesters on a large scale. The suitability of a bacterium for PHA production depends on many different factors such as stability and safety of the organism, growth and accumulation rates, achievable cell densities and PHA contents, extractability of the polymer, molecular weights of accumulated PHA, range of utilizable carbon sources, costs of the carbon source and the other components of the medium, and occurrence of by-products [1].

As was mentioned above, the main restriction preventing large scale production of PHA is their price. Analysis and economic evaluation of the bacterial PHA productions suggested, that the cost of substrate (mainly carbon source) contributed the most significantly (up to 50%) to the overall production cost. Thus, cheap waste substrates attract attention of both scientific researches and industrial companies in order to reduce PHA production cost and make this environmental friendly product also more economically feasible [74]. Hence, this chapter is intended to be brief review focused on PHA production using wide variety of cheap waste substrates.

First, substrates containing oils and fatty acids are very promising carbon sources for PHA production in large scales. From metabolic point of view, fatty acids are energetically advantageous substances because their complete β-oxidation generates more chemical energy equivalents in the form of ATP. In addition, the theoretical yield coefficient of PHA production from fatty acids (for instance 0.65-0.9 $kg.kg^{-1}$ from butyric acid) is considerably higher than that from sugars (for instance 0.32-0.48 $kg.kg^{-1}$ from glucose) [75]. PHA production using soybean oil was tested by Kahar et al., who employed *Cupriavidus necator* H16 and its PHA synthase negative mutant strain harboring PHA synthase of *Aeromonas caviae*. They achieved high yields with wild strain (PHB 95.8 $g.l^{-1}$) and as well as with recombinant strain (P(HB-co-HHx) 102.1 $g.l^{-1}$) [76]. Further, Alias and Tan isolated a Gram-negative bacterium, FLP1, from palm oil mill [77]. The isolate was tentatively identified as closely related to *Burholderia cepacia*. FLP1 produced PHB when grew on the crude palm oil and palm kernel oil. Supplementation of odd-numbers fatty acids (i.e. valerate and propionate) resulted in the synthesis of P(HB-co-HV).

Second, the dairy industry is an important part of the agricultural sector. Cheese whey is the major by-product coming from the manufacture of cheese and casein, representing 80-90% of the volume of transformed milk. Cheese whey is rich in fermentable nutrients such as lactose, lipids and soluble proteins. A total cheese production in European Union corresponds to approximately 40 462 000 tons of whey per year. A major part is used for production of lactose and feeding, but an annual amount of 13 462 000 tons of whey per year containing about 619 250 tons of lactose constitutes a surplus product. That is why cheese whey is promising substrate for cheap production of PHA in large amounts [78].

Although biotechnological production of PHA from different sugars *via* condensation of acetyl-CoA units stemming from hexose catabolism is well described, only limited number of bacterial strains directly converts lactose into PHA. Few reports are available on PHB production from lactose and whey by recombinant *Escherichia coli*. Lee et al. reported that PHB can be produced from cheese whey employing recombinant strains of *E. coli* harboring PHA synthetic genes of *C. necator* [79]. Park et al. studied pilot plant-scale, fed-batch fermentation production of PHB from cheese whey using genetically engineered *E. coli* expressing *Azohydromonas lata* PHA synthetic genes [80]. Also production of PHA using non-recombinant strain was reported. Yellore and Desai described the isolation of *Methylobacterium* sp. ZP24 that grew on cheese whey and produced PHB [81]. Subsequently, Nath et al. performed scale up studies on PHB production from whey using *Methylobacterium* sp. ZP24 and optimization of cultivation conditions which significantly enhanced PHB yields [82]. Marangoni et al. reported production of copolymer P(HB-co-HV) with *C. necator* using cheese whey as a basal medium supplemented with invert sugar and with periodic feeding of propionic acid [83]. Also thermophillic strain *Thermus thermophilus* HB8 is able to accumulate PHA during its growth on cheese whey up to 35 % of biomass content [84].

Dhanasekar and Viruntagiri mathematically modeled and experimentally examined the batch production of PHB using *Azotobacter vinelandii* using sucrose and whey as a substrate [85]. Finally, *Pseudomonas hydrogenovora* was used for PHA production from cheese whey, but this bacterial strain was not able to utilize lactose directly and, therefore, lactose in whey must have been hydrolyzed prior cultivation [86].

Molasses is a sugar-rich co-product stream generated by crop refining industries. Sugar beet and sugar cane refining plants are the major sources for sugar molasses that contain high sucrose content. Depending on the grades and sources, sugar molasses can not be further used in foods and feeds and is, thus, ideal for consideration as an inexpensive carbon source for fermentative processes [87]. Page et al. reported the production of P(HB-co-HV) with yields about 19-22 $g.l^{-1}$ by *Azotobacter vinelandii* UWD grown on beet molasses supplemented with valerate [88]. Further, Celik and Beyatli screened collection of *Pseudomonas* spp. isolates and reported the synthesis of PHB from one of the strains identified as *P. cepacia* G13. The PHB content in cells was about 70 % when culture was cultivated on 3 % molasses [89].

Bio-fuels, such as ethanol or biodiesel, are manufactured using agricultural products as raw materials. The production of biodiesel results in high quantities of co-product stream rich in glycerol. One potential use of glycerol is in industrial fermentation where it can be employed as a carbon substrate for microorganisms [87]. For instance, Borman and Roth used *Methylobacterium rhodesianum* and *C. necator* DSM 11348 to produce PHB in medium containing casein as a nitrogen source and glycerol as a carbon source [90]. Also *Psedomonas oleovorans* disposes with ability to utilize glycerol and produce PHB. The study showed that increasing concentration of glycerol in the medium resulted in the synthesis of lower molecular weight PHB due to glycerol end-capping [91].

Homopolymer PHB and copolymer P(HB-co-HV) were produced by *Azotobacter chroococcum* strain H23 when growing in culture media amended with alpechin (wastewater from olive oil mills) as the sole carbon source. *A chroococcum* formed PHA up to 80 % of the cell dry weight, when grown on NH_4^+ medium supplemented with 60 % alpechin [92]. Other strain of *Azotobacter* family, *Azotobacter vinelandii* UWD was used by Cho et al. to produce copolymer P(HB-co-HV) from swine waste water supplemented with glucose [93].

Very interesting application comes from O´Connor et al. who employed bacterial strain *Pseudomonas putida* in order to turn polystyrene foam waste into PHB. The idea is to heat polystyrene foam to convert it to styrene oil, distillate pure styrene oil and use it as a feed solution for fermentative production of PHB [94].

2.7.2 PHA production from waste substrates employing mixed bacterial culture

Because traditional PHA production using bacterial monoculture has its limitation in range of utilizable substrates and, moreover, all procedures preventing contamination during fermentation process are high in cost, mixed bacterial cultures have attracted attention of many researches. The idea of PHA production using mixed culture arose from recognition of the PHA role as a metabolic intermediate in microbial processes for wastewater treatment. Biological wastewater treatment usually occurs under dynamic conditions. Activated sludge, a well-known mixed culture, is able to store PHA as a carbon and energy storage material under unsteady conditions arising from an intermittent feeding regime and variation in the presence of an electron acceptor. The microorganisms involved experience rapidly changing conditions

of availability of nutrients and can adapt continuously to change in substrate. Microorganisms which are able to quickly store available substrate and consume the storage to achieve a more balanced growth have a strong competitive advantage over organisms without the capacity of substrate storage. Activated sludge accumulates PHA to around 20 % of dry weight under anaerobic conditions. The PHA content of activated sludge can be increased to 62 % in a microaerophilic–aerobic sludge process [95].

However, for PHA production using mixed cultures, new fermentation strategies have to be performed in order to achieve high PHA content and PHA yields. Recently, much research has concentrated on the production of PHA by mixed cultures when exposed to a transient carbon supply. Activated sludge processes are highly dynamic with respect to the feed regime. The biomass subjected to successive periods of external substrate availability (feast period) and no external substrate availability (famine period) experiences what in the literature is often called an unbalanced growth. Under dynamic conditions, growth of biomass and storage of polymer occur simultaneously when there is an excess of external substrate. When all the external substrate is consumed, stored polymer can be used as carbon and energy source. In these cases, storage polymers are formed under conditions that are not limiting for growth. The storage phenomenon usually dominates over growth, but under conditions in which substrate is present continuously for a long time, physiological adaptation occurs, and growth becomes more important. The ability to store internal reserves gives to these microorganisms a competitive advantage over those without this ability, when facing transient substrate supply. Among the mentioned systems for industrial production of PHA, the feast and famine approach is the most promising because of high PHA accumulation. This approach promotes the conversion of the carbon substrate to PHA and not to glycogen or other intracellular materials [96]. Under the successive periods of external substrate availability (feast phase) and no external substrate availability (famine phase), it appears that in feast phase, 66–100% of the substrate consumed is used for storage of PHB, and the remainder is used for growth and maintenance. The growth rates in the feast and famine phases are similar, but growth in the feast phase is higher relative to the famine phase [97].

Generally, employment of mixed bacterial cultures is very promising way to produce PHA using waste streams for instance waste waters coming from agriculture and food industry (see **Tab. 4**). Moreover, the use of mixed microbial cultures facilitates the use of mixed substrates. Cost for these processes can be sustainably reduced because cheap substrates and non-sterile reactors are used and little process control is needed [97].

Tab. 4 *Overview of waste streams suitable for PHA production using mixed cultures [97]*

Substrate source	Flow ($m^3 h^{-1}$)	Availability (months/year)	COD (kg COD/m^3)	Capacity (ton COD/m^3)	Production (ton PHA/year)
Potato starch production process (AVEBE)	300	12	2.5	6750	2431
Innuline production process (Cosun)	60	5	14.0	3066	1134
Sugar beets process (CSM)	3750	3	1.9	15,604	5773
Brewery wastewater (Heineken)	300	12	2.8	7358	2723
Vegetable, fruit and garden (VAM)	90	12	15	11,774	4356
Household garbage (OWF)	30	12	50	13,333	4933

In calculations, the yield is assumed to be 0.37 kg PHA/kg COD.

2.7.3 Modern trends in down-stream processing of PHA

Besides cost of carbon source, one of the most important factors contributing to production price of PHA is the cost of down-stream process. Therefore, a lot of afford have been made to improve such a process. Generally, the major step of PHA isolation is extraction of polyester granules. Nevertheless, step involving cell disintegration could be added in order to improve the recovery of extraction and, if high purity of PHA is expected, purification step is needed [98].

PHA content of the produced biomass strongly affects the efficiency and price of the down-stream process. For example, Lee and Choi reported that a relatively low PHB content in cells – 50 % results in a high recovery cost of 4.8 $\$.kg^{-1}$ PHB. On the other hand, the recovery cost for a process with 88 % PHB cell content was only 0.92 $\$.kg^{-1}$ PHB. A lower PHB content clearly resulted in a high recovery cost. This is mainly due to requirement of large amounts of digesting agents for breaking the cell walls and to the increased cost of waste disposal [99].

The use of solvents to recover PHA is one of the oldest methods. The action of solvent can be divided in two steps. First, solvent modifies the cell membrane permeability and, second, it solves PHA. Usually, chlorinated hydrocarbon solvents such as chloroform, 1,2-dichlorethan, methylene chloride or some cyclic carbonates like propylene and ethylene carbonates were used. Consequently, the separation of PHA from the solvent was performed by solvent evaporation or by precipitation in non-solvent [98]. Solvent extraction has some advantageous as compared to other methods. A solvent extraction results in polyesters of a high purity and it does not degradate the polymer. Moreover, it was observed that extraction of PHB from Gram-negative bacterial strain reduced level of endotoxines in material, which could be useful in medical applications [100]. Nevertheless, solvent extraction is very expensive procedure and it presents hazards for the operators as well as for the environment. Therefore, solvent extraction is widely used in laboratories but not in large-scale processes [101].

Polyhydroxyalkanoates can be isolated using digestion methods. First, surfactants, such as sodium dodecyl sulphate, can be used to disrupt cells by incorporating itself into the lipid bilayer membrane. As more surfactant is added, more of it enters the membrane to increase the volume of the cell envelope until it is saturated. Further addition breaks the membrane to produce micelles of surfactants and membrane phospholipids, which leads to release of PHB into the solution. Furthermore, another function of surfactant is solubilization. Surfactant is able to solubilize not only cell proteins, but also other non-PHA cellular material [102]. Nevertheless, surfactant alone can not give a high purity PHA and other agents such as hypochlorite and NaOH are needed. Furthermore, a high surfactant dose increses the cost of down-stream processing and causes problems in wastewater treatment [98]. Secondly, other digestion method is based on application of sodium hypochlorite. Within this method high purity of PHA can be achieved, but sodium hypochlorite causes severe degradation of PHA resulting in up to 50 % reduction of molecular weight [103]. In order to overcome this adverse effect, hypochlorite treatment is combined with solvent extraction [104] or with surfactant [105].

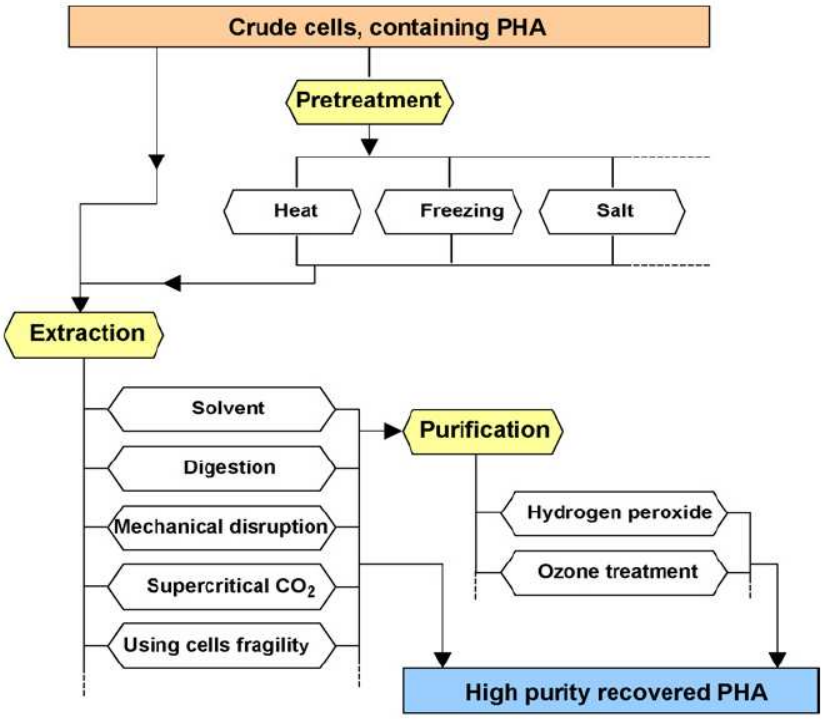


Fig. 8 *Down-stream processing strategies for PHA [98].*

Alternative digestion method could be based on application of enzymes facilitating solubilization of non-PHA cell materials. Kapritchkoff et al. tested enzymatic recovery of PHB produced by *C. necator* [106]. Among six tested enzymes (papain, bromelain, bovine tripsyn, bovine chymotrypsin, lysozime and cellulaze) 2 % (enzyme mass per biomass) bromelain was the most efficient one. In order to reduce price of potential process, pancreatin, which is three times cheaper, could be used instead of bromelain. Also lytic enzymes of *Cytophaga* sp. can be used to achieve complete lysis of *C. necator* cells in order to isolate PHA [107]. According to Yasotha et al. combination of commercially available enzymes Alcalase (Novozymes) and lysozyme with SDS and EDTA to recover PHA produced by *Pseudomonas putida* resulted in high purity and recovery of PHA. Alcalase had the most significant effect on treatment process and contributed to about 71.5 % in terms of process factor importance [108].

Supercritical fluid extraction could be potentially used for isolation of PHA from bacterial cells. Heijazi et al. studied recovery of PHB from *C. necator* using supercritical CO_2 with methanol as polar modifier. The recovery obtained by such a procedure was 89 % which is similar to the other methods [109].

Very efficient methods of PHA recovery are based on the fact, that some bacterial strains such as *Azotobacter vinelandi*i or recombinant *E. coli* become fragile after accumulation of large amount of PHB. Simple treatment of *A. vinelandii* cells with 1 M NH_4OH at 45° C for 10 minutes resulted in high purity and recovery of PHA produced [110]. PHB recovery from recombinant *E. coli* is also suggested to be simple. Strain developed by Fideler and Dennis was able to accumulate PHB up to 95 % of dry cell weight and, moreover, strain harbored mediated lysis system called phage lysis that allowed the PHB granules to be released gently and efficiently [111].

Spontaneous liberation of produced PHA is promising strategy for cheap PHA purification. Jung et al. reported spontaneous liberation of PHB into cultivation media in

recombinant *E. coli* MG1655 harboring *phbCAB* operon of *C. necator*. Autolysis of cells was reached under controlled cultivation conditions [112]. Hori et al. constructed self disruptive strain *Bacillus megaterium* which released accumulated PHB when glucose in cultivation medium was depleted. This system allowed cell disruption immediately after the PHB content in cells had reached its maximal level [113].

2.8 Applications of PHA

Wide range of PHA applications in industry is impressive. For instance, PHA latex can be used to cover paper or cardboard to make water-resistant surfaces as opposed to the combination of cardboard with aluminium that is currently used and which is non-biodegradable. This also works out to be cost-effective since a very small amount of PHA is required for this purpose. Furthermore, it is also possible to use PHA to make the following articles due to their piezoelectric nature: pressure sensors for keyboards, stretch and acceleration measuring instruments, material testing, shock wave sensors, lighters, gas lighters; acoustics: microphone, ultrasonic detectors, sound pressure measuring instruments; oscillators: headphones, loudspeakers, for ultrasonic therapy and atomization of liquids. The gas barrier properties of PHA are useful for applications in food packaging and for making plastic beverage bottles. The same property can be exploited to make coated paper and films which can be used for coated paper milk cartons. PHB or other copolymers can be used to make the non-woven cover stock and the plastic film moisture barriers in nappies and sanitary towels along with some special paramedical film applications in hospitals [114].

Moreover, very important field of PHA application is medicine, therefore, the medical applications of PHA have been explored extensively in recent years [114, 115]. PHA have been used to develop devices including sutures, nerve repair devices, repair patches, slings, cardiovascular patches, orthopedic pins, adhesion barriers, stents, guided tissue repair/regeneration devices, articular cartilage repair devices, nerve guides, tendon repair devices, bone-marrow scaffolds, tissue engineered cardiovascular devices and wound dressings. So far, various tests on animal models have shown polymers, from the PHA family, to be compatible with a range of tissues. Nevertheless, pyrogenic contaminants copurified with PHA often limit their pharmacological application rather than the monomeric composition and thus the purity of the PHA material is critical for medical application [115].

PHA have been used as mulch films for agricultural purposes. Because of their biodegradable nature, these films can be used for controlled release of pesticides. Such an application was tested by Holmes. Insecticides were integrated into PHA pellets and sown along with the farmer’s crops. The insecticide would be released at a rate related to the level of pest activity since the bacteria breaking down the polymer would be affected by the same environmental conditions as that of the soil pests [116].

PHA have a wide variety of applications, among which the medical applications seem to be the most economically practical area. With the currently increased interest level and the extensive research being carried out in this area, PHA are potentially emerging as the next generation of environmentally friendly materials with a wide range of applicability [114].

2.9 Characterization of selected bacterial strains naturally producing PHA

2.9.1 Cupriavidus necator H16

The Gram-negative, faculcative chemolithoauthotrophic bacterium *Cupriavidus necator* H16 has been intensively investigated for almost 50 years. Nowadays, *C. necator* H16 serves as a model organism for PHA research and, moreover, has become an important prokaryotic strain for several biotechnological applications. Due to the affiliation to different taxa, the name of this bacterium changed frequently in the past and confusing different names are still being used in parallel. It was isolated as a member of the genus *Hydrogenovorans* in 1961. In 1969 it was transferred to the genus *Alcaligenes*, and the new name became *Alcaligenes eutrophus* H16. In 1995 the name was changed to *Ralstonia eutropha* H16, which was valid until 2004 when it changed first to *Wautersia eutropha* H16 and later in the same year to *Cupriavidus necator* H16 [117].

As facultative chemolithoautotrophic 'Knallgasbakterium' *C. necator* H16 assimilates in the autotrophic growth mode CO_2 *via* the Calvin-Benson-Basham cycle but is also able to grow heterotrophically utilizing fructose, gluconic acid, various other organic acids and even aromatic compounds as carbon and energy sources. The central carbon metabolism of *C. necator* H16 is quite well understood. Fructose and gluconic acid are catabolized *via* the Entner-Doudoroff pathway with 2-keto-3-desoxy-6-phosphogluconate aldolase as the key enzyme. The Embden-Meyerhoff-Parnass pathway is incomplete because the key enzyme fructose-1,6-bisphosphate (FBP) aldolase is lacking. However, glycolysis is established if FBP aldolase from *Escherichia coli* is expressed in *C. necator* [117]. In the absence of oxygen, the organism can respire by denitrification [118]. Nevertheless, *C. necator* H16 secrets typical fermentation products into the medium if the cells are cultivated under conditions of limited oxygen [117].

Recently, the whole genome of *C. necator* H16 was sequenced, and all genes were annotated. The genome comprises 7,416,678 bp with 6,626 coding sequences (CDS) identified. It turned out that chromosome 1 (4,052,032 bp, 3,651 CDS) contains almost all essential genes for the metabolism and for other essential cell functions and that the megaplasmid and chromosome 2 (2,912,490 bp, 2,555 CDS) encode a repertoire of genes that extend the range of substrates and terminal electron acceptors (e.g. H_2 oxidation, CO_2 fixation, denitrification) and contain only very few – if any – indispensable genes [119].

C. necator H16 is able to accumulate polyhydroxyalkanoates up to 80-90 % of dry cell weight. Details related to PHA metabolism in *C. necator* are described in chapter 2.3.1. Aside its PHA production potential, *C. necator* H16 has been promising bacterial strain for number of biotechnological processes. Thanks to its ability of authotropic growth, *C. necator* H16 was considered as a candidate for single cell protein (SCP) production for animal feeding and also for humans in orbit-based space vessels [120]. Recently an investigation demonstrated the potential application of the O_2-tolerant, CO-resistant, membrane-bound hydrogenase of *C. necator* H16 for the construction of biological fuel cells and for designing light-driven H_2 production complexes [121]. Another promising experimental approach documented the usefulness of *C. necator* H16 hydrogenase for the construction of an H_2-sensing device [122].

Moreover, *C. necator* H16 could be used for bioremediation of aromatic compounds. Traditionally, strains of the genus *Ralstonia* have played a key role in research on microbial degradation of aromatic compounds. Although *R. eutropha* strain JMP134 was used in several

of these studies, it has been known for many years that *C. necator* H16 can grow on a similar spectrum of aromatic compounds. It is, therefore, not surprising that the genomic sequence contains an impressive array of genes related to the metabolism of aromatics [119, 123].

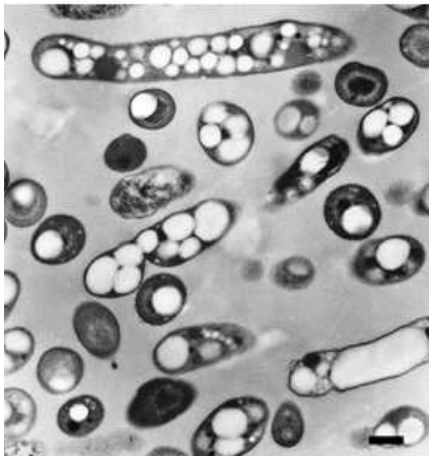


Fig. 9 *Cells of Cupriavidus necator H16 with PHB granules, bar 0.5 μm [124].*

2.9.2 Bacillus megaterium

Similarly to *Cupriavidus necator*, also *Bacillus megaterium* has been intensively investigated and industrially used for more than 50 years, as it possesses some very useful and unusual enzymes and high capacity for the production of exoenzymes. It is also a desirable cloning host for the production of intact proteins, because it does no possess external alkaline protease and can stably maintain a variety of plasmid vectors [125].

Bacillus megaterium is a Gram-positive, mainly aerobic spore-forming bacterium found in widely diverse habitats from soil to seawater, sediment, rice paddies, honey, fish, and dried food. It can grow in simple media on more than 62 carbon sources out of 95 tested, including all tricarboxylic acid cycle intermediates, formate, and acetate. In 1884, De Bary named *Bacillus megaterium* "big beast" because of its large size with a volume approximately 100 times larger than *Escherichia coli* (see Fig. 10). It has been ideal model for studies of cell structure, protein localization, sporulation, and membranes [126].

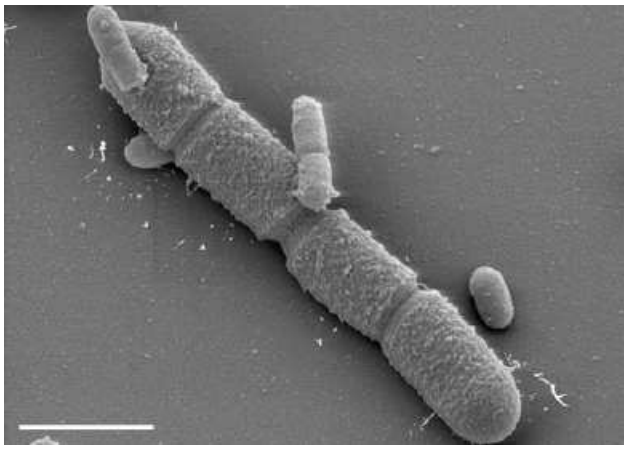


Fig. 10 *Electron microscope image of Bacillus megaterium and Escherichia coli vegetative cells. B. megaterium cells grow up to a volume more than 60 μm^3 (2.5 x 2.5 x 10). Compared to the E. coli volume of 0.5 μm^3 (0.5 x 0.5 x 2) [124].*

Bacillus megaterium played important role in history of PHA research. In 1927, it was *B. megaterium* from which Lemoigne at the Pasteur Institute (France) using chloroform isolated unknown substance and proved that the material was a polymer of 3-hydroxybutyryc acid [114]. Since that, *B. megaterium* PHA synthetic metabolic pathway and its regulation were well understood. Generally, metabolic pathway is rather similar to that of *C. necator*, nevertheless, different PHA synthase (class IV) is involved in PHA synthesis and other protein, called phaQ, regulates expression of PHA synthetic genes (see chapters 2.3.2 and 2.4.2) [126]. Despite the fact that PHA content in *B. megaterium* cells is rather low (about 50 %) as compare to *C. necator*, thanks to wide range of utilizable carbon sources, *B. megaterium* is promising candidate for PHA production from different waste substrates such as sugarcane molasses, corn steep liquor [127] or dairy waste [128].

Currently, *B. megaterium* is intensively used in biotechnological and pharmaceutical industry for enzymes, vitamin B_{12} or oxetanocin production (see **Tab. 5**) [124]. Besides that, *B. megaterium* is widely used as a host cell for heterelogous protein production, because heterologous gene expression in *B. megaterium* seems to be an interesting alternative system in contrast to *E. coli*. It has a number of favorable features as an expression host, including low protease activity, structural and segregational stability of plasmids, and the ability to grow on a wide variety of substrates. Highly efficient expression of homologous and heterologous genes was reported in the 1980s and was becoming popular in the 1990s. Plasmids with the promoter of the xylose-operon are most frequently used for inducing high-level expression of heterologous genes. Genes are 130- to 350-fold induced by using 0.5 % xylose. Induction is strongly inhibited by adding glucose because it bounds to XylR and thereby acts as an anti-inducer [129].

Tab. 5 *Industrial products of B. megaterium and their applications [124]*.

Product/use	**Comment**
α-Amylase	Can replace pullulanases
β-Amylases	Bread industry
Chitosanasen	Yeast cell wall lysis
Glucose dehydrogenase	Generator of NADH/NADPH, glucose blood test, biosensors
Neutral protease	Leather industry
Oxetanocin production	Inhibits HIV, hepatitis B virus, cytomegalovirus, herpes virus
Penicillin amidase	Construction of synthetic penicillins
Toxic waste cleanup	Herbicides, C-P bond lysis
Vitamin B_{12} production	Aerobic and anaerobic Vitamin B_{12} producer

2.9.3 Azotobacter vinelandii

Azotobacter vinelandii is a gamma-proteobacterium belonging to the family *Pseudomonadaceae* [130]. Over the years, *A. vinelandii* has served as a model for biochemical analyses due to the extraordinary yields and quality of enzymes that can be isolated from this organism. Most recently, these studies have been focused on the ability of *A. vinelandii* to fix diatmospheric nitrogen using three distinct nitrogenase systems under free-living conditions, a process that occurs in the presence of oxygen levels that typically inactivate the nitrogenase enzyme. *A. vinelandii* is also unusual in that, for reasons which are not clear, it increases the copy number of its chromosome up to 50-100 times during late log phase. In addition, it provides an ideal system in which to investigate the processes of cyst

formation and xenobiotic degradation [130]. Because of all these scientific reasons, project on *A. vinelandii* genome sequencing and annotation is currently ongoing.

Azotobacter vinelandii produces two industrially interesting products. First, linear polysaccharides called alginates, which are composed of variable amounts of (1–4)-β-D-mannuronic acid and its epimer, α-L-guluronic acid. Alginates present a wide range of applications, acting for example as stabilizing, thickening, gel or film-forming agents, in various industrial and pharmaceutical fields [131].

Second, under unbalanced growth conditions this bacterium accumulates PHA. Metabolic pathway of PHA synthesis is again similar to that of *C. necator*, nevertheless, its regulation seems to be a little bit different, because other regulatory systems are involved. Transcription of the *phbBAC* biosynthetic operon is initiated from two overlapping promoters, pB1 and pB2. PhbR, encoded by *phbR*, activates transcription of the PHB biosynthetic operon from the pB1 promoter, whereas transcription from pB2 is dependent on the sigma factor RpoS and increases during the stationary phase of growth. Transcription of *phbR* itself also starts from two promoters, pR1 and pR2. Transcription from pR2 is also induced during the stationary phase and is dependent on RpoS, although probably in an indirect manner [132]. Thanks to their good PHA production parameters, some mutants of *A. vinelandii* are considered to be potential candidates for industrial production of PHA [131].

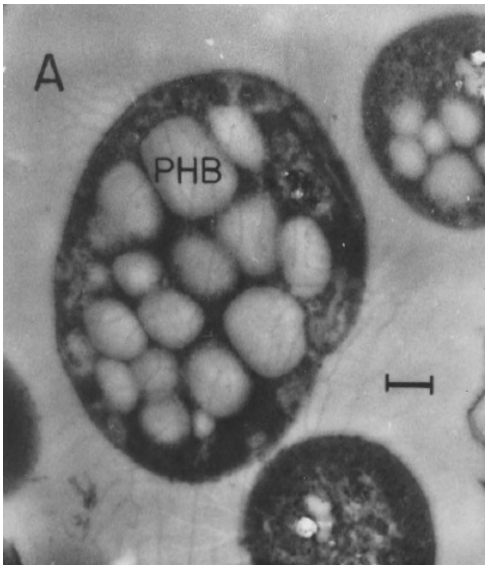


Fig. 11 *A. vinelandii cells with PHB granules, bar 0.4 μm [133].*

2.9.4 Halomonas boliviensis

Halomonas boliviensis is modarate halophilic bacterial strain belonging to family *Halomonadaceae* [134]. *Halomonas boliviensis* was islotaed from a soil sample around Laguna Colorada (Bolivia), a large shallow hypersaline lagoon that is located 4300 m above sea level. It is Gram-negative bacteria with a size 0.4 x 1.1 x 0.6-3.4 μm. Cells occur singly or in pairs and show a wide size distribution during exponential phase, but uniform size at the end of their growth. It is moderately halophilic, alkalitolerant and psychrophylic strain. Growth occurs at 0-25 % of NaCl in medium, with an optimum about 5 %. This bacterium is heterotrophic with ability to utilize various carbohydrates as a carbon source [134, 135].

Halomonas boliviensis attracts attention because of the ability to produce ectoines and PHB. Ectoines belong to the family of compative solutes, which are accumulated in halophilic bacterial strains in order to maintain osmotic equilibrium with respect to the salts in surrounding environment. Ectoines have gained much attention in biotechnology as protective agents for enzymes, DNA and whole cells against stresses such as freezing, drying and heating. The cryoprotective properties of these compounds make them interesting for increasing the stability and freshness of food. Moreover, ectoines also find applications as moisturizers in cosmetics and skin care products [135]. PHB is accumulated in *H. boliviensis* up to 75 % of its cell dry weight and also cheap carbon substrates such as starch hydrolysate [136], wheat bran or potato waste [137] can be turned into bioplastics. Moreover, economically interesting co-production of ectoines and PHB is possible [135].

2.10 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 [138]. 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 [138, 139].

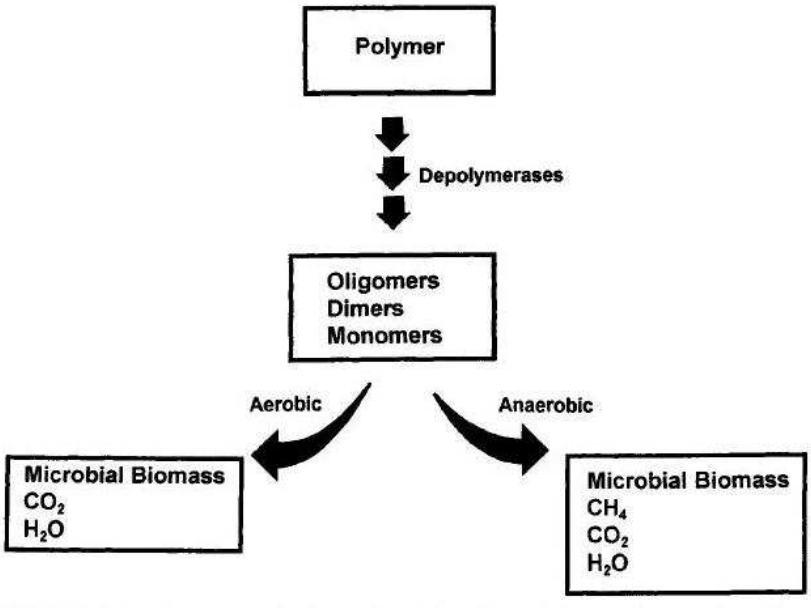


Fig. 12 *Schema of polymer biodegradation [139].*

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 [139]. 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 [138, 139].

The rate of biodegradation is influenced by many factors such as environmental conditions or presence of microorganisms, nevertheless, among the parameters affecting process of biodegradation, properties of polymeric materials are probably the most important ones. That is why some of them are summarized in **Tab. 6** [140].

Tab. 6. *Properties of polymeric materials influencing biodegradation process [140]*.

Material property	Comment
Structural uniformity	Monomeric units having different chemical structures require several enzymes to degrade them.
Structural similarity	Certain functional groups found in natural polymers, such as amide, ester, ether, etc., are more likely to be degraded by the existing microorganisms.
Molecular weight	Increasing molecular weight decreases biodegradation rate. In most cases, microorganisms do not degrade synthetic polymers which have a molecular weight higher than 20 000.
Cross-linking	Reduces crystallinity but in the same time increases packing density which inhibits the penetration of enzymes.
Chain flexibility	Increases the probability of biodegradation by facilitating formation of complexes of enzyme-substrate that require a certain configuration.
Functional groups	Hydrophillic groups increase the probability of enzymatic reactions because they either increase polymer solubility or at least attract water (water is needed in most biodegradation reactions).
Crystallinity	Amorphous regions are preferentially degraded. Crystalline material can only be degraded on a lamellar surface, because enzymes can not easily penetrate inside densely packed crystallites.
Structure porosity	Increased surface area increases the probability of contact and concentration of enzyme-substrate complexes which are rate-controlling.

2.10.1 Polyurethanes and their environmental impact

Polyurethane (PUR) is the general term used for a polymer derived from the condensation of polyisocyanates and polyols having intramolecular urethane bonds (-NHCOO-). PUR has been in use since the 1940s and is now widely used as a base material in

various industries. PUR can adopt various forms (from soft to hard) depending on the chemical structures of the polyisocyanates and polyols (functional group number or molecular mass) and therefore can be used as foams, elastomers, paints, adhesives, elastic fibers or artificial leathers [141].

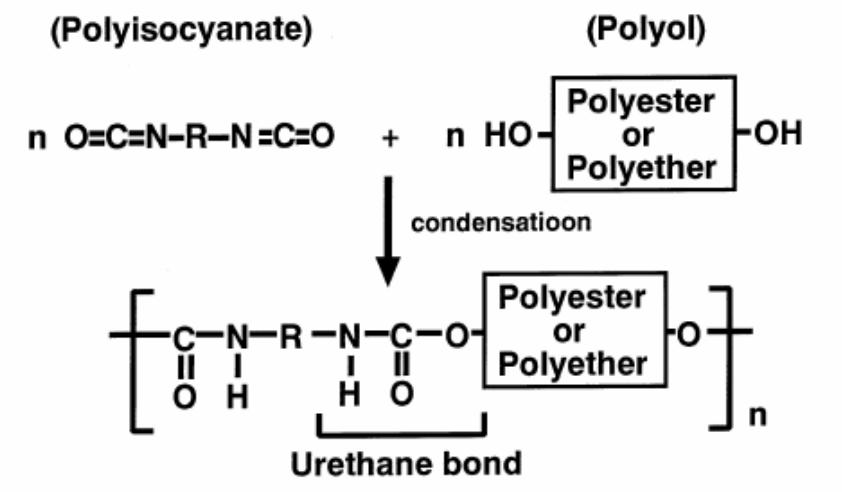


Fig.13 *Structure of polyurethane [141].*

Thanks to their mechanical properties and wide range of possible applications, world production capacities of PUR are still growing. Leading exporters are the USA, Germany and Italy, covering over 55 % of worldwide PUR exports [142].

Tab. 7 *World production capacities of PUR [142]*

Year	1998	1999	2000	2001	2002	2003	2004	2005
Production (10^3 tonnes)	7201	7553	8027	8223	8387	8567	8714	8821

As could be expected, such a high amounts of polymeric materials production has serious environmental consequences. Enormous quantities of used PUR materials (as well as other synthetic polymer materials) are disposed of in landfills, which results in a serious accumulation of solid waste. Of a current world production of more than 9 Mt per year PUR materials, about 1/3 is consumed in Europe, roughly distributed over PUR foams and other products. Only 150 kt per year is recycled while the rest of material is disposed of in landfills or incinerators. Moreover, main part of PUR waste stream is consisted of polyether-polyol based PUR materials that are extremely durable against environmental conditions and microorganisms action (see chapter 2.10.2) [141, 142]. For these reasons, process of PUR biodegradation of PUR materials has attracted attention of many scientists. Better knowledge in this field could result in development of biodegradable and environmental friendly PUR materials.

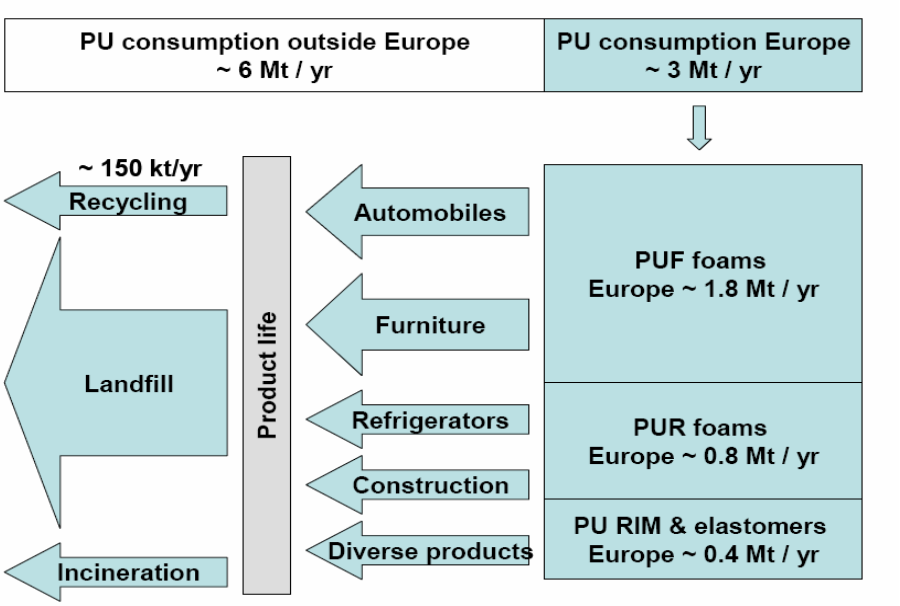


Fig.14 *PUR products consumption in Europe and post-consumer disposal. (PUF – flexible polyurethane foam, PUR – rigid polyurethane foam, RIM – reaction injection moulding) [142].*

2.10.2 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 [143].

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. 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 [144]. Similarly, Jansen et al. reported that some kinds of polyether based PUR were degraded by *Staphylococcus epidermis* KH11, but the degradation progressed very slowly [145]. 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. The experimental results have shown that the polyether PUR foam is likely not biodegradable under anaerobic conditions [146]. 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 [147].

Polyester PUR possesses many ester bonds that are vulnerable to enzymatic hydrolysis. Hence, it is proposed, that degradation of polyester PUR is mainly due to the hydrolysis of ester bounds. In regard to the biodegradation of polyester PUR, fungi seem to be very effective. Crabbe et al. isolated four fungi species, *Curvularia senegalensis*, *Fusarium solani*, *Aerobasidium pullulan*s and *Chladosporium* sp. that were able to growth on polyester PUR as the sole energy and carbon source. *Curvularia senegalensis* was observed to have very high PUR degrading activity and, therefore, subsequent analysis of this fungal isolate was carried out. An extracellular polyurethanase displaying esterase activity was purified from this organism. The protein has a molecular mass of 28 kDa, is a heat stable at 100° C for 10 minutes and it is inhibited by PMFS [148].

Among bacterial strains, both Gram-positive and Gram-negative bacteria have been reported as PUR degraders. In large-scale test of bacterial activity against polyester PUR, Kay et al. investigated the ability of 16 bacterial isolates to degraded PUR when the media was supplemented with yeast extract. Two isolates, *Corynebacterium* sp. and *Pseudomonas aeruginosa*, could degradate PUR in presence of basal media. However, none of the isolates grew on PUR alone. Physical tests of degraded PUR revealed different but significant decreases in tensile strength and elongation for each isolate [149, 150].

Bacterial strain *Pseudomonas chloraphis* was observed to be able to degrade polyester PUR and three enzymes involved in PUR hydrolysis were purified and characterized [151, 152]. Gautam et al. investigated ability of *Pseudomonas chlororaphis* ATCC 55729 to biodegrade waste polyester PUR foam obtained from an automotive industry. Ammonia nitrogen, pH and diethylene glycol (DEG) concentrations were found to increase steadily during biodegradation test. Furthermore, scanning electron photomicrographs of foam pieces also showed evidence of biodegradation [153].

Comamonas acidovorans strain TB-35 was isolated from soil samples thanks to its ability to degrade polyester PUR. Solid cubes of polyester PUR were synthesized with various polyester segments. The cubes were completely degraded after 7 days incubation when they were supplied as the sole carbon source and degraded 48% when they were the sole carbon and nitrogen source. Analysis of the breakdown products of the PUR revealed that the main metabolites were from the polyester segment of the polymer. Further analysis of strain TB-35 revealed that the degradation products from the polyester PUR were produced by an esterase activity. Strain TB-35 possesses two esterase enzymes, a soluble, extracellular and one membrane-bound [154, 155]. The membrane-bound enzyme was found to catalyze the majority of the polyester PU degradation. Therefore, the membrane-bound enzyme was purified and characterized. The protein has a molecular mass of 62 kDa, it was heat stable up to 65◦C and inhibited by PMSF. The structural gene, *pudA*, for the PUR esterase was cloned in *Escherichia coli*. Upon nucleotide sequencing of the open reading frame (ORF), the predicted amino acid sequence contained a Gly-X-Ser-X-Gly motif characteristic of serine hydrolases. This enzyme reacted with solid polyester PUR to hydrolyze the ester bonds of PUR. PUR degradation by the PUR esterase was strongly inhibited by the addition of 0.04% deoxy-BIGCHAP, a kind of surfactant. On the other hand, deoxy-BIGCHAP did not inhibit the activity when p-nitrophenyl acetate, a water-soluble compound, was used as a substrate. These observations indicated that this enzyme degrades PUR in a two-step reaction: hydrophobic adsorption onto the PUR surface was followed by hydrolysis of the ester bonds of PUR. Thus, the PUR esterase was considered as having a hydrophobic-PUR-surface-

binding domain and a catalytic domain, and the surface-binding domain was considered to be essential for PUR degradation [156].

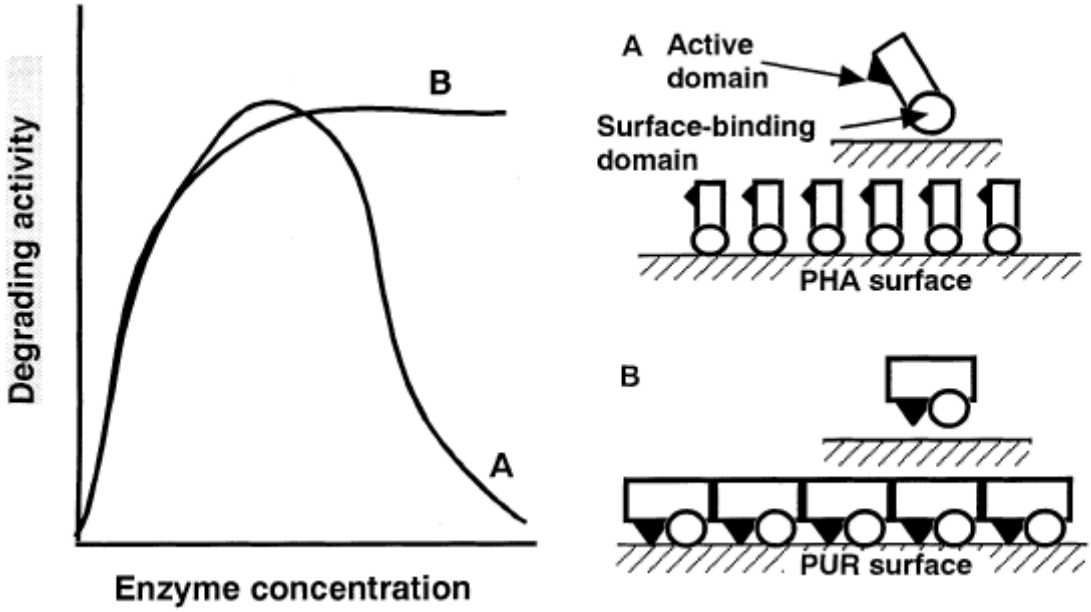


Fig.15 *Effects of PHA depolymerase (A) and polyurethane esterase (B) concentration on the degradation of solid substrates and kinetic models of these enzymes [141].*

This unique structure observed in PUR esterase has also been reported in PHA depolymerase, which degrades PHA. In PHA depolymerase, the existence of a hydrophobic PHA-binding domain has already been determined by the analysis of its amino acid sequence and its various physicochemical and biological characteristics. PUR esterase and PHA depolymerase are similar, because both enzymes have a hydrophobic surface-binding domain in addition to their catalytic domain, but their three-dimensional structures are considered to be different. The PHA-binding domain in most PHA depolymerases is located at the C terminus of the polypeptide chain, and the active and surface-binding domains are linked by a flexible linker, which is a threonine-rich region, or a module resembling fibronectin type III. Owing to this structure, the degradation activity of PHA depolymerase is decreased in the presence of an excess of the enzyme. On the other hand, in the case of PUR esterase, the degradation activity did not decrease but rather remained constant when an excess of the enzyme was present. On the basis of this observation it was inferred that the surface-binding site and the catalytic site of the PUR esterase existed in three-dimensionally close positions, unlike those in PHA depolymerase. In this model, the catalytic domain can gain access to the PUR surface even if the PUR surface is saturated with enzyme molecules. However, since the number of adsorbable enzyme molecules per unit surface area of the PUR is fixed, the PUR-degradation activity also remains constant (see **Fig. 15**) [141].

3. THE AIMS

The first goal of proposed thesis is to study biotechnological production of selected biomaterials, particularly polyhydroxyalkanaotes, employing selected bacterial strains. Further, the second part of the thesis 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.

4. DISCUSSION PART

This chapter is intended to be detailed discussion for the results which were already published in papers or which have been recently submitted for publication in international journals. All papers and manuscripts are attached to this work as supplements (the list of supplements is provided in Chapter 8., supplements themselves are attached in Chaper 9). Of course, supplied papers and manuscripts also contain detail descriptions of methods and results. For better demonstration of time course of experiments also 2 contributions at international conferences are introduced.

Proposed thesis was focused on controlled production and biodegradation of selected biomaterials. The first part of this work was aimed at production of bacterial storage polymers – polyhydroxyalkanoates usable as biodegradable plastics. This part includes study on production of polyhydroxyalkanoates from various carbon sources and application of stress conditions to control process yields and properties of produced materials. Papers, manuscripts and abstracts related to this part of work are attached as Supplements I-VII.

The second part of the thesis was focused on the study of biodegradation of polyether polyurethanes modified by some substituted biopolymers, mostly based on cellulose. Particular papers and manuscripts are shown as Supplements VIII-XI.

More detailed links to particular paper, manuscript or abstract are always provided in the text of this chapter. Complete list of author publications can be found in Chapter 11.

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 (see **Fig. 16** picturing growing interest in polyhydroxyalkanoates) 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 [157].

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 and are supplied as **Supplement I** ***(Obruca et al., Chemicke Listy 102 (2008) p. 1255-1256)***.

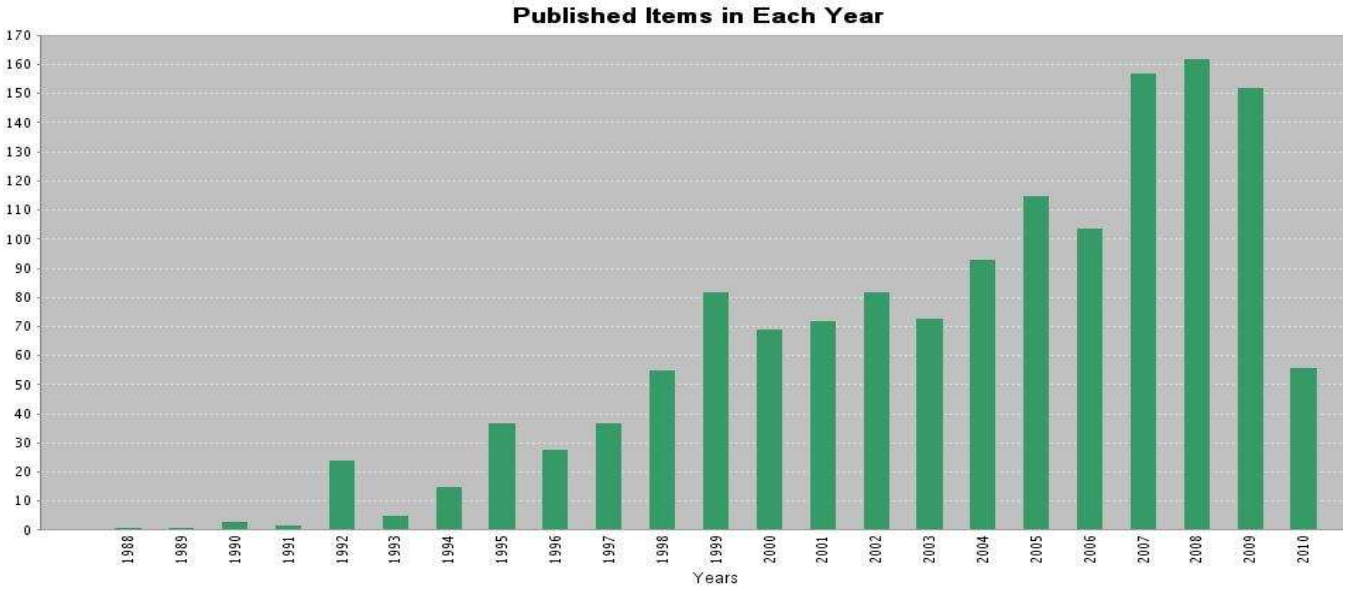


Fig.16*. Published items per year according to Web of Knowledge (23.5.2010), key word used: polyhydroxyalkanoates.*

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 [158] proline [159] or ectoines [160], but to our knowledge only Natarajan et al. used controlled osmotic pressure to increase PHB yields in *Rhizobium* DDSS-69 [68]. 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 [126] 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 Analysis of PHA

Generally, PHA can be analyzed using several methods. For instance, the presence of PHA in cells can be screened by staining of microbial colonies with Nile Blue or Sudan Black [161]. Basic quantification of PHA can be done by gravimetric analysis of PHA isolated using solvent extraction [162]. A more specific analysis was developed by Law and Slepecky [163], who treated polymer with concentrated sulfuric acid to convert PHB into crotonic acid which can be analyzed spectrophotometrically at 235 nm. However, according to our experiences, due to necessary dilution of real sample with sulfuric acid, this method is time consuming. Manipulation with concentrated sulfuric acid is dangerous and, finally, spectrophotometric determination of PHA does not allow analysis of monomer composition of polyester. Unsaturated acids resulting from sulfuric acid treatment of PHA can be analyzed by HPLC/UV-VIS as well [164]. Introduction of separation step improves sensitivity of analysis (see **Tab. 8**) and allows determination of monomer unit composition, but the dilution and the work with concentrated sulfuric acid, the main disadvantages of spectrophotometric method, are still necessary.

Therefore, for our purposes gas chromatography (GC) analysis of PHA according to Brandl et al. was the method of choice [165]. Lyophilized cells were treated in the mixture of chloroform and 15 % sulfuric acid in methanol. Resulting methyl esters of hydroxyacids were further analyzed by using GC with mass spectrometry (MS) or flame ionization detector (FID). The main advantage of MS detection was the possibility to identify or to confirm presence of various monomers in PHA structure and retention times. Moreover, MS analysis was very sensitive especially in Selected Ion Monitoring (SIM) mode. On the other side, calibration was linear up to concentration of 0.5 mg PHA per ml. Because concentration of typical samples was in range of 0.7-2.0 $mg.ml^{-1}$, GC/MS analysis required further dilution which was labor-intensive and impractical. Oppositely, GC/FID analysis was linear in range of 0.01 – 4 $mg.ml^{-1}$ and, therefore, no additional dilution was needed after sample preparation. Parameters of all the methods for PHA analysis tested in this work are compared in **Tab. 8**.

Analytical part of this work was presented at 14th International Symposium on Separation Science, summary of our contribution is shown as **Supplement II** ***(Obruca et al., New Achievements in Chromatography, 2008)***.

Tab 8. *Comparison of different methods for quantitative determination of PHB*

Method	**Calibration curve ($mg.ml^{-1}$)**	**Regression coefficient R^2**	**Range of linear calibration ($mg.ml^{-1}$)**
Direct UV-VIS method*	$y = 13.81x$	0.9720	0.02 – 0.1
HPLC/UV-VIS	$y = 5.9\ 10^4 x$	0.9904	0.001 – 0.01
GC/MS**	$Y = 2.10^9 x$	0.9982	0.001 – 0.5
GC/FID	$y = 2.5\ .10^7\ x$	0.9992	0.01 – 4

* method does not allow analysis of monomer composition

** calibration for SIM mode (fragments 43, 74, 87, 103)

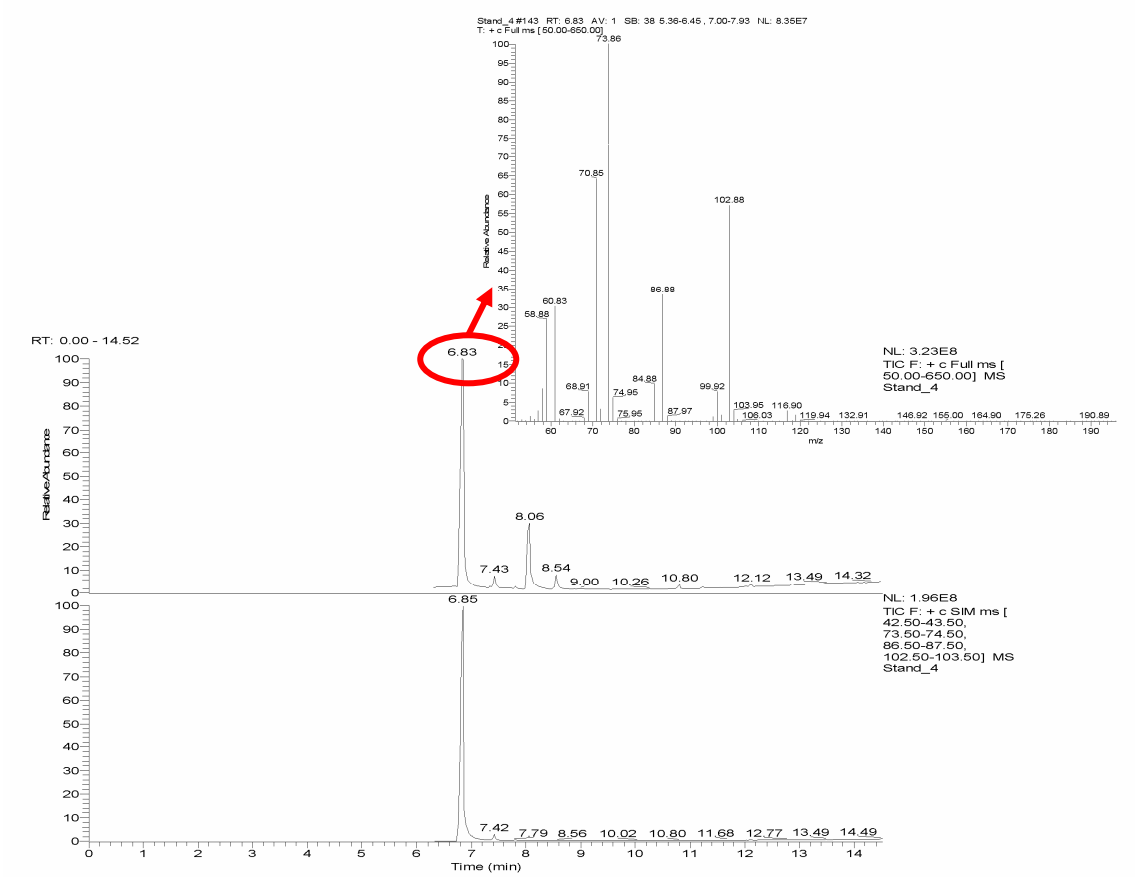


***Fig.17**. Chromatograms of GC/MS analysis of methyl ester of 3-hydroxybutyrate (retention time 6.83 min) (full scan and SIM mode) and full scan spectrum.*

4.3 The influence of exogenous stress on PHA production in *Cupriavidus necator* H16

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 (purchased from Czech Collection of Microorganism, CCM 3726). As was noticed in Chapter 2.9.1, *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, attached as **Supplement III *(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.

Because the highest PHB yields were obtained, if stress factor was applied at the beginning of stationary phase, when nitrogen was depleted, it was assumed that stress application even enhanced positive effect of nitrogen limitation.

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. [65].

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 **Fig. 17**). 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 (see Chapter 2.7.3) [99]. 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.

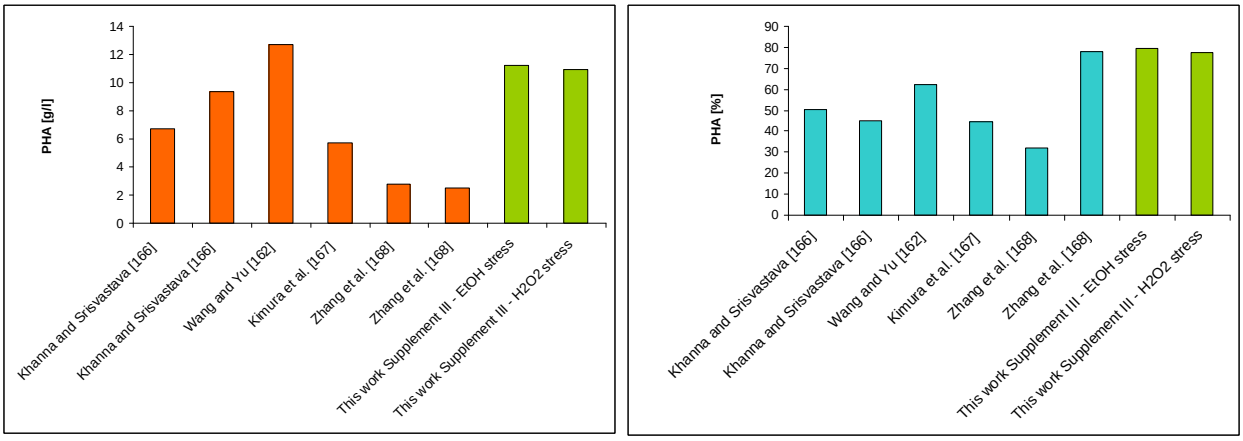


Fig.17*. Comparison of PHB yields and PHB contents in cells obtained in our work and those published in literature for Cupriavidus necator in batch culture.*

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 paper attached as **Supplement IV *(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)$^+$ [169]. It was the reason why exposition of *Escherichia coli* [170] and *Saccharomyces cerevisiae* [171] 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 [60]. 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. 17**.

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 [172] as well as Senior and Dawes [173]. 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 [174]. 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.

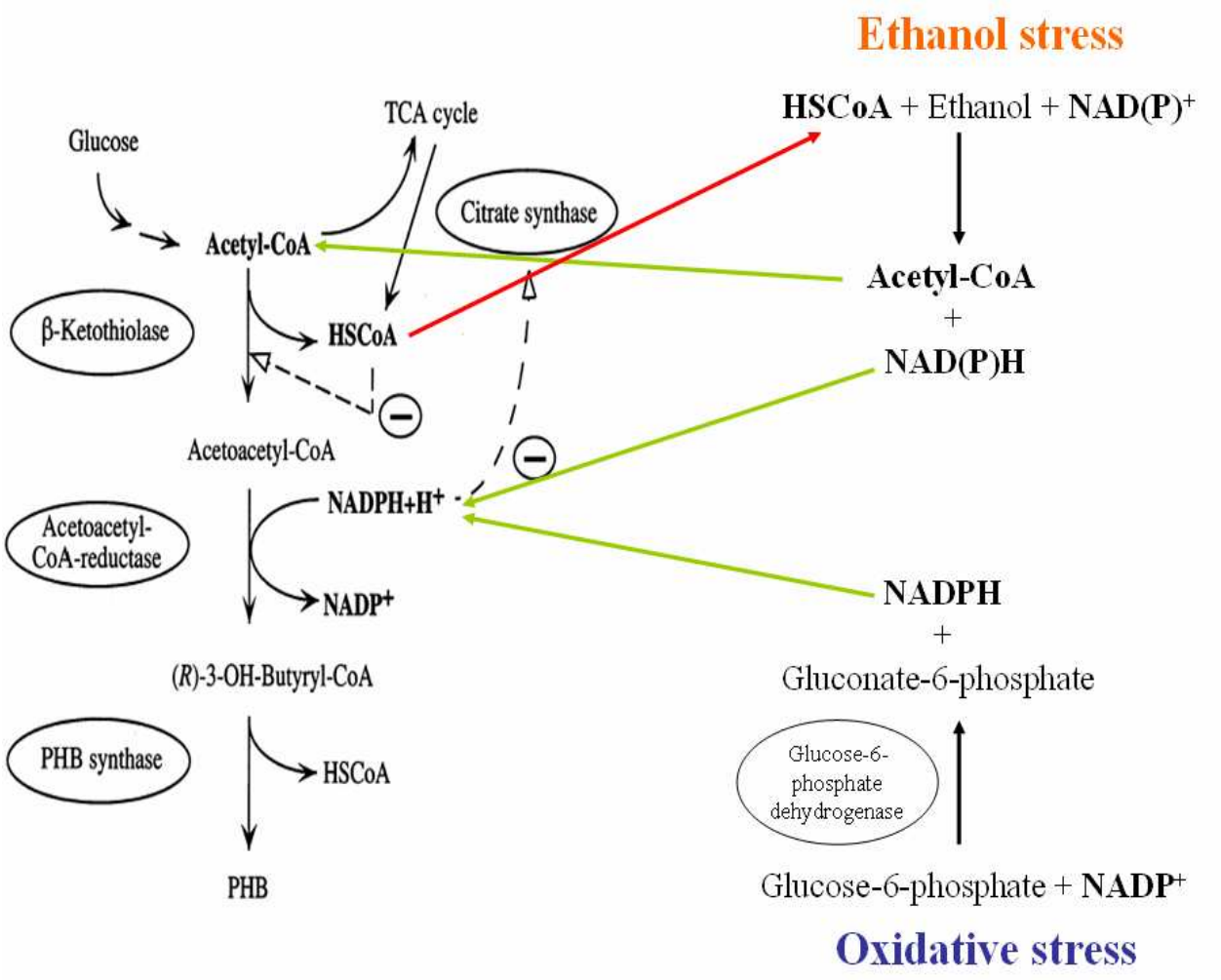


Fig.17*. Schema of interaction between stress response (ethanol and oxidative pressure) and PHB biosynthesis.*

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 **(Supplement IV)** 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 [33]. 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 [35]. 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 **Supplement VII** ***(Obruca et al. Biotechnol. Letters, submitted)*** 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. In our work attached as **Supplement VII** 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* [175]. 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 [176]. 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. 18**.

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.

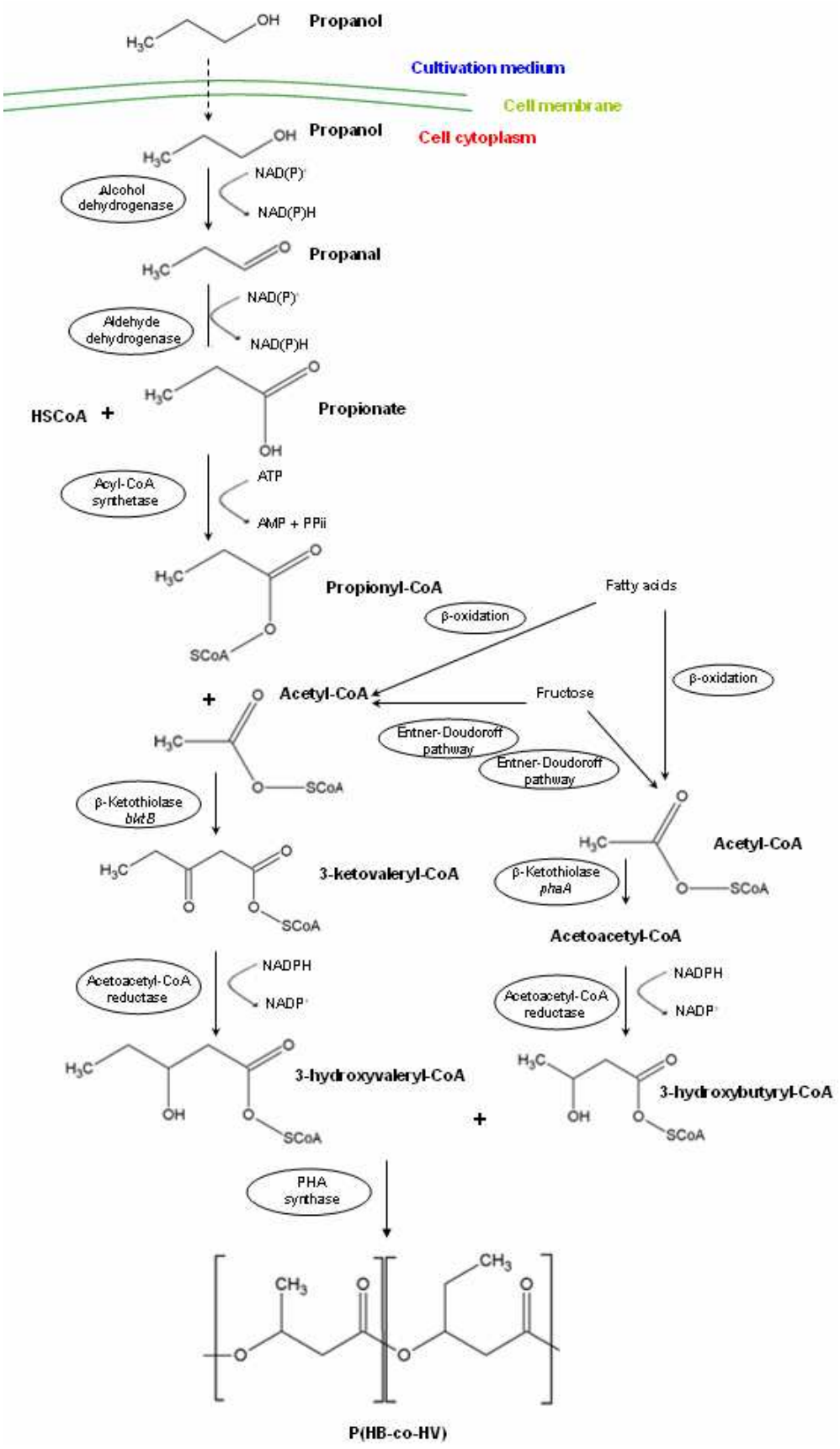


***Fig.18**. Proposed mechanism of metabolization of propanol by C. necator H16 and its incorporation into P(HB-co-HV) copolymer structure.*

4.4 Production of PHA from cheap/waste substrates

As we reported above (Chapter 4.1), *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 (these reported in paper attached as **Supplement I** were rather low).

Optimization of cheese whey 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 **Supplement VI**).

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 as poster at 14th European Congress on Biotechnology, abstract of our contribution is supplied as **Supplement V** ***(Obruca et. al., New Biotech. 25 (2009) p. S257)*** and also manuscript (**Supplement VI**) ***(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 [177].

For PHA production from plant edible oils we employed bacterial strain *C. necator* H16 **(Supplement VII)** ***(Obruca et al. Biotechnol. Letters, submitted)***. 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.

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 [178]. 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 $g.l^{-1}$ [178]. 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 [179]. Finally, Mas et al. cultivated *Pseudomonas aeruginosa* on waste frying oil and reached MCL-PHA yields 3 $g.l^{-1}$ [180]. 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 (see **Supplement VII**).

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 $\$.kg^{-1}$ [74]. 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. 9** 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.

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.

Tab 9. *Basic economical consideration of PHA production based on results obtained in our work (Supplements III and VII) (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 [74].

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

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 (see **Supplement VII**). Surprisingly, application of all stress factors at the 24th 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. The mechanisms of connections of stress responses to ethanol and propanol with PHA biosynthetic pathway are discussed in details above (see Chapter 3.3) and also in papers attached as **Supplement III** ***(Obruca et al., Folia Microbiol 55 (2010) p. 17-23)***, **Supplement IV** ***(Obruca et al., World J. Microbiol. Biotechnol. 26 (2010) p. 1261-1267)*** and **Supplement VII** ***(Obruca et al. Biotechnol. Letters, submitted).***

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 18th 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 30th 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. **Tab. 10** summarizes PHA production from oils and other fatty substrates reported in literature and our yields belong among the best ones. 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.9**, 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. The part of our work dealing with PHA production from oils has been recently submitted for publication, manuscript is shown as **Supplement VII** ***(Obruca et al. Biotechnol. Letters, submitted).***

Tab 10. *Overview of yields and other parameters of PHA production from oils and other fatty substrates reported in literature.*

Oil	Strain	Cultivation mode	PHA	Biomass [g.l^{-1}]	PHA [%]	PHA [g.l^{-1}]	Yield[a] [g.g^{-1}]	Ref
Palm kernel oil	*Cupriavidus necator*	Batch, E. flasks	P(HB-co-HV-co-HHx)	8.0	80.0	6.4	-	[181]
Rice oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	12.9	55.8	7.2	0.72	[182]
Safflower oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	17.6	49.8	8.8	0.88	[182]
Soya oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	13.8	42.4	5.9	0.59	[182]
Olive oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	12.3	45.6	5.6	0.56	[182]
Linseed oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	9.6	48.7	4.7	0.47	[182]
Peanut oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	12.6	35.1	4.4	0.44	[182]
Corn oil	*Chromobacterium sp.*	Batch, E. flasks	PHB	10.1	47.0	4.7	0.47	[182]
Olive oil	*Cupriavidus necator**	Batch, E. flasks	P(HB-co-HHx)	3.5	76.0	2.7	-	[183]
Corn oil	*Cupriavidus necator**	Batch, E. flasks	P(HB-co-HHx)	3.6	77.0	2.8	-	[183]
Palm oil	*Cupriavidus necator**	Batch, E. flasks	P(HB-co-HHx)	3.6	81.0	2.9	-	[183]
Palm kernel oil	*Cupriavidus necator*	Batch, E. flasks	P(HB-co-HV)	3.0	73.0	2.2	-	[184]
Palm kernel oil	*Cupriavidus necator*	Batch, E. flasks	P(HB-co-4HB)	3.3	68.0	2.2		[184]
Palm olein	*Burkholderia cepacia*	Batch, E. flasks	PHB	5.1	43.1	2.2	-	[185]
Waste sesame oil	*Cupriavidus necator*	Batch, E. flasks	PHB	7.4	62.0	4.6	0.46	[178]
Palm oil	*Pseudomonas aeruginosa*	Batch, Ferm.	MCL-PHA	2.2	36.0	0.8	0.11	[186]
Peanut oil	*Pseudomonas aeruginosa*	Batch, E. flasks	MCL-PHA	4.0	3.8	0.2	-	[179]
Olive oil	*Pseudomonas aeruginosa*	Batch, E. flasks	MCL-PHA	4.5	6.0	0.3	-	[179]
Waste oil	*Pseudomonas aeruginosa*	Batch, E. flasks	MCL-PHA	5.4	5.7	0.3	-	[179]
Rapeseed oil	*Alcaligenes sp. AK 201*	Batch, E. flasks	PHB	2.5	44.0	1.1	0.40	[187]
Olive oil	*Alcaligenes sp. AK 202*	Batch, E. flasks	PHB	3.1	47.0	1.5	0.49	[187]
Lard	*Alcaligenes sp. AK 203*	Batch, E. flasks	PHB	2.4	31.0	0.7	0.25	[187]
Waste frying oil	*Pseudomonas aeruginosa*	Batch, E. flasks	MCL-PHA	8.1	37.0	3.0	0.08	[180]
Soybean oil	*Cupriavidus necator*	Fed-Batch, Ferm.	PHB	126.0	76.0	95.8	0.76	[188]
Soybean oil	*Cupriavidus necator**	Fed-Batch, Ferm.	P(HB-co-HHx)	138.0	74.0	102.1	0.72	[188]
Waste rapeseed oil	***Cupriavidus necator***	**Fed-Batch, Ferm.**	**P(HB-co-HV)**	**138.5**	**75.9**	**105.1**	**0.83**	**This work**

***** - recombinant strain of *C. necator*; [a] – yields g-PHA per g-oil; **E. flasks** – Erlenmeyer flasks; **Ferm.** - Fermentor

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.

4.5 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 [188]. 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 [189] and further published in paper (**Supplement VIII**) ***(Obruca et al., Chemicke Listy 102 (2008) p. 1219-1220)*** and another manuscript has been recently submitted for publication (**Supplement IX**) ***(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 (**Supplement X**) ***(Obruca et al., Chemicke Listy 102 (2008) p. 1257-1258)*** and the second one has been submitted for publication (**Supplement XI*) (Obruca et al., Environ. Technol., submitted)***.

To understand the biodegradation of PUR elastomeric films, it is necessary to comment also the results obtained in PUR foams. As was noticed above, PUR foams were modified by

selected biopolymers by partial (usually 10 %) replacement of polyether polyol. These samples were exposed to bacterial culture. The first tested culture was mixed thermophillic aerobic bacterial culture which was originally used for waste water treatment and which was obtained from sludge of waste water treatment in Bystřice pod Hostýnem. The second tested culture was monoculture of *Arthrobacter globiformis* purchased from Czech Collection of Microorganisms (CCM 193). Samples were placed into Erlenmeyer flasks containing medium, which were afterwards inoculated by bacterial culture. During incubation of samples with culture we analyzed growth of bacterial culture, in most cases we also monitored Chemical Oxygen Demand (COD). Typically, cultivation lasted for 300 hours and, after that, the degradation of material was determined as a weight loss of sample and also surface changes were observed using optical microscope.

The results of these experiments revealed that degradation of PUR foams consisted of two subsequent steps. Because weight losses of samples were independent of presence of bacterial culture, the bacterial culture did not contribute to direct degradation of PUR foams and the mechanism of PUR degradation was of abiotic origin. On the other side, presence of PUR foams in the cultivation media supported growth of both bacterial cultures. We assumed that bacterial culture is capable of utilization of the products of abiotic degradation of PUR foams as additional carbon and/or nitrogen sources, which supported their growth in comparison with control. Thereby, both these facts indicate that the mechanism of PUR foams degradation involves two steps. The first one, abiotic degradation of PUR foams is followed by assimilation of abiotic degradation products by bacterial culture. In the **Fig. 19**, there is displayed the comparison of both mechanisms – biotic degradation and abiotic degradation of polymer [190].

The mixed thermophillic culture seemed to be more effective in utilization of PUR foams than monoculture of *Arthrobacter globiformis*. The growth of mixed culture was more pronounced in presence of PUR foam in medium and also the value of elimination of COD was always higher in mixed culture. It is likely to be facilitated by a wider range of enzymatic activities present in mixed culture and also by relations of individual strains in mixed culture such as synergism or metabiosis.

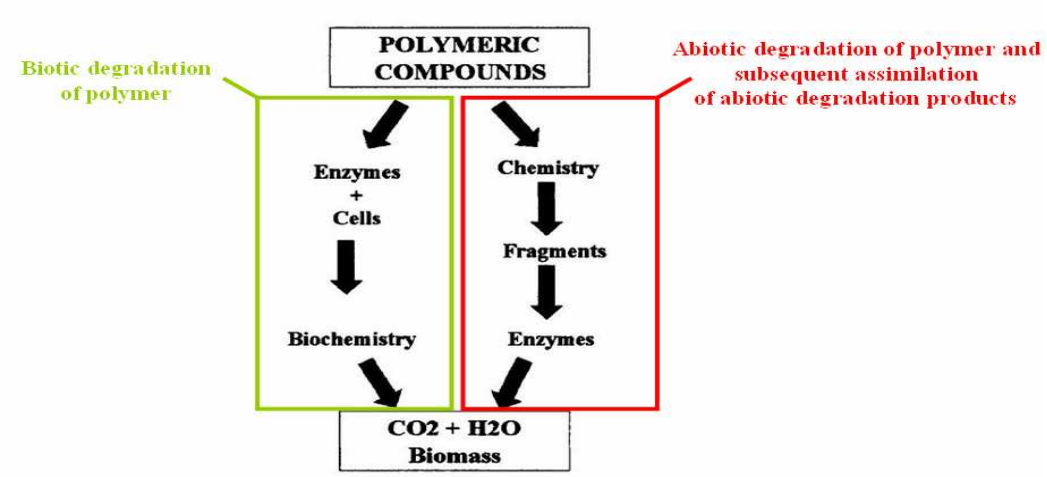


***Fig.19**. Comparison of biotic and abiotic mechanism of degradation of polymeric material [190].*

The measure of weight losses of samples was dependent on the type of biopolymer used for modification of PUR. The highest mass losses were observed in PUR foams modified by carboxymethyl- and hydroxyethylcellulose. Because both cellulose derivatives are well water-soluble, it could be expected that mass losses were predominantly caused by solubilization of biopolymer.

We also performed the test of bioavailability of PUR foams as the sole carbon and nitrogen source for thermophillic mixed culture. Surprisingly, all tested foams served as the sole nitrogen source supporting growth of mixed culture. On the other side, bacterial culture was able to utilize only foams modified by acetylated starch and hydroxyethylcellulose as the sole carbon source (**Supplement VIII and IX**). It is necessary condition of successful biodegradation of PUR material in environment. Therefore, modification of resistant polyether based PUR foams by biopolymer seems to be promising strategy for reduction of amount of solid waste of PUR origin [189].

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 **(Supplement X)** ***(Obruca et al., Chemicke Listy 102 (2008) p. 1257-1258)*** **(Supplement XI)** ***(Obruca et al., Environ. Technol., submitted)***

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, the presence of PUR elastomeric films in the cultivation media strongly inhibited the growth of bacterial culture for unusually long period **(Supplement XI)**. 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 **(Supplement XI)**. 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. Microbial degradation of polyethers, such as polyethylene glycol, progresses very slowly because of exo- type of its depolymeration, but the growth characteristics of involved microbes are not strongly changed by low concentration of polyether [191]. Also biodegradation of dibutyltin compounds by bacterial cultures was already reported, but none extra long lag-phase was observed during the process [192].

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 [193]. 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.

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 **(Supplement XI)**.

5. CONCLUSIONS

The aim of the Ph.D. thesis was to study production and biodegradation of selected biomaterials including control mechanisms of both processes. The first part of this work was focused on production of bacterial storage polymers – polyhydroxyalcanoates, which are usable as biodegradable bioplastics. The next part was aimed at study of biodegradation of polyether polyurethanes modified by partial replacement of polyol by biopolymers. The results are presented in the form of comments to 5 published papers and 4 submitted manuscripts, these papers are attached in supplements (Chapter 9).

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.

6. FUTURE PERSPECTIVES

The results and experiences, which we obtained in experiments presented in this thesis, brought up new questions and goals that we would like to explain and reach.

Here is the brief list of them:

- Up-scale PHA production from waste oils in fed-batch mode. Up-scale study should be performed in larger laboratory (5 or 10 l) fermentors and in semi-industrial fermentors.

- Screen other alcoholic precursors that can be used as stress agents as well as precursors of other monomer units. For instance, application of 1,4-butadiol could result in incorporation of 4-hydroxybutyrate into PHA.

- Develop down-stream process for recovery of polyhydroxyalkanoates based on enzymatic solubilization of other cell components than PHA.

- Test mechanical and chemical properties of produced materials.

- Use various PHA copolymers for development of controlled delivery systems for selected drugs and other active substances.

- Test stability and efficiency of developed delivery systems and activity of transported biomolecules.

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9. SUPPLEMETS

Supplemet I

Chem. Listy, *102*, s265–s1309 (2008) Polymers & Polymer Composites

P08 STRATEGIES FOR ENHANCING POLY(3-HYDROXYBUTYRATE) PRODUCTION IN SELECTED BACTERIAL STRAINS

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Introduction

Polyhydroxyalkanoates (PHAs) are a group of hydroxyacid polyesters that are producted and accumulated in the form of intracellular granules by a wide variety of bacterial strains. These strains use PHA as carbon, energy and reducing power storage material[1]. Of the big family of PHAs, a homopolymer of 3-hydroxybutyrate, poly(3-hydroxybutyrate) (PHB), is the most widespread in nature and the best characterised. PHB aroused much interest in industry and research thanks its biocompatible, biodegradable, thermoplastic and piezoeletric properties. PHB appears to find many pottentional applications in medical, industrial and agricultural fields[2].

Generally, the main problem of PHB is high cost in comparison with traditional plastics from petrochemical routes. That is why strategies for enhancing of PHB production are developed. Typical strategy, which is widely used for enhancing PHB production, is limitation with nitrogen, phosphorus or another element when excess amount of carbon source is present[3].

Among the factors restricting the economy of PHB production very important is the cost of carbon source. In PHB production, about 40 % of the total cost is for raw materials. Cheap waste substrates, for instance cheese whey or waste potato starch, are very attractive from this point of view. Effective utilization of waste subtrates should lead to reduction of PHB cost[4].

The positive influence of stress factors (such a peroxide stress, osmotic stress, ethanol stress or heavy metal stress) on accumulation of differents secondary metabolites were reported[5]. However, to this time only little is known about accumulation of PHA under exogenous stress condition.

The aim of this work was to compare different strategies that could enhance PHB production and reduce costs of PHB. Limitations with nitrogen and/or phosphorus source were tested. The influence of several exogenous stresses on PHB production was compared. Finally, production of PHB was studied in synthetic and organic medium as well as using cheese whey as a cheap waste substrate.

Experimental

Bacterial Strain

Cultivations were performed with culture *Bacillus megaterium* CCM 2037.

Cultivation Condition

Cultivations were carried out at 30°C under permanent shaking (150 rpm) in 100 ml of media. Several media were tested, as the first synthetic medium (SM) containing glucose as the only carbon source, KH_2PO_4 and Na_2HPO_4 (1 : 1) as the phosphorus source and $(NH_4)_2SO_4$ as the nitrogen source was used. SM was used for limitation experiments. Culture was transferred (centrifugation 20 min., 6,000 rpm) into limited medium (medium without nitrogen or/and phosphorus source) after 20 hours of growth on complete medium. After following 15 hours, biomass and PHB concentration were determined. SM was also used for stress experiments. The second tested medium was *Bacillus* medium (BM) containing peptone and beef extract. The last medium was cheese whey obtained from cheese manufactury Pribina Přibyslav. Thermal denaturation and sulphuric acid were used for precipitation and removing of whey proteins before cultivation. Whey was tested with and/or without addition of salts according to SM.

Analytical methods

Biomass concentration was analyzed spectrophotometrically at 630 nm after suitable dilution with distilled water. Relationship between absorbance A 630 nm and dry cell mass was evaluated. Before PHB determination biomass was lyophillized. Dryied cell material was hydrolyzed and derivatized in a mixture of 0.8 ml acidified methanol (15 % v/v H_2SO_4) and 1 ml chloroform at 100 °C for 140 min. Resulting methyl esters of 3-hydroxybutyric acids were determined using gas chromatography with MS or FID detection. Commercial PHB (Fluka) was used as the external standard[6].

Results

First, the effect of nitrogen and/or phosphorus source limitation was tested. All tested limitations led to enhancing PHB content in bacterial cell biomass in comparison with cultivation without limitation. However, it seems that for *Bacillus megaterium* limitation with nitrogen source is probably the best stimulating factor for PHB production (see Table I).

Table I
PHB production under limitation (35 hours)

Limitation	PHB content [% dry wieght]	PHB yield [g dm^{-3}]
Without limitation	14.164 ± 1.248	0.234 ± 0.025
N limitation	24.035 ± 1.406	0.360 ± 0.029
P limitation	14.857 ± 0.826	0.177 ± 0.009
N + P limitation	17.648 ± 0.691	0.237 ± 0.018

Also some other tested exogenous stress factors influenced positively the productivity of culture in comparison with control. Ethanolic stress as well as Na_2SO_4 supported PHB accumulation.

Mainly ethanolic stress resulted in improving PHB production. This effect could be caused by the fact, that ethanol

is metabolised *via* acetyl-coenzym A, which is starting metabolite for PHB production. It is not clear if enhanced PHB production was casued by ethanolic stress or by utilization of ethanol alone. Both factors could be involved. The culture could utilize ethanol with the aim to remove toxic substance from medium. Increase of acetyl-coenzym A concentration led then to enhanced PHB accumulation.

Table II
PHB production under exogenous stress (35 hours)

	Biomass [g dm^{-3}]	PHB content [%]	PHB yield [g dm^{-3}]
H_2O_2	0.047 ± 0.001	*	*
NaCl	0.134 ± 0.039	6.864 ± 0.082	0.016 ± 0.001
EtOH	1.812 ± 0.029	20.793 ± 0.358	0.377 ± 0.007
$NiCl_2$	0.053 ± 0.000	*	*
Citrate	1.739 ± 0.025	14.620 ± 0.054	0.254 ± 0.001
Na_2SO_3	1.744 ± 0.050	19.881 ± 0.612	0.345 ± 0.011
Control	1.762 ± 0.009	17.030 ± 0.179	0.300 ± 0.003

* no PHB production was observed

Table III
PHB production on different media (45 hours)

	Biomass [g dm^{-3}]	PHB content [%]	PHB yield [g dm^{-3}]
BM	1.288 ± 0.015	0.050 ± 0.011	0.001 ± 0.000
BM + glucose	2.602 ± 0.050	44.720 ± 3.290	1.164 ± 0.086
BM + starch	2.150 ± 0.110	21.054 ± 0.711	0.453 ± 0.012
Whey	0.389 ± 0.033	*	*
Whey + salts	1.612 ± 0.033	15.464 ± 0.181	0.249 ± 0.003

* no PHB production was observed

Addition of Na_2SO_3 led to higher PHB production too. This effect could be caused by reducing properties of Na_2SO_3, but the effect on PHB production was relatively low.

Other stress factors inhibited the growth of culture and/or PHB accumulation. Because of relationship between stress factor concentration and its biological effect further experiments will be needed. Detailed analysis of concentration effects was not included into this pilot study.

Because of relatively low PHB amount (14–20 % of total biomass) other media then SM were tested. Culture growing on BM medium itself did not contain almost any PHB after 45 hours of growth. However, when glucose was added, production of relatively high PHB amount was observed. This is probably caused by the fact, that culture depleted nitrogen and phosphorus sources of BM medium, but carbon source (sugar) was still present. Culture was able to utilize sugar for the growth as well as for PHB accumulation.

Finally, cheese whey was tested as a component of medium for PHB production. Low growth and no PHB production were observed when cultivations were performed on whey itself. Addition of salts (according to SM) led to the strong increase of growth and production properties of culture.

Conclusions

All strategies tested in this work could be used for enhancing PHB production in *Bacillus megaterium* CCM 2037. Limitation with nitrogen seems to be a strong stimulating factor for PHB production.

Positive effect of two exogenous stress factors on PHB production was observed. Ethanolic stress and Na_2SO_3 enhanced PHB production. However, the mechanism and optimal concentration of stress factors have to be clarified.

Culture was able to utilize organic substrates (BM, cheese whey) and product high amount of PHB. The highest yield of PHB (1.16 g dm^{-3}) was obtained during growth in BM medium with glucose. *Bacillus megaterium* is also able to use cheap cheese whey or starch as a carbon source. That should lead to reducing of PHB cost. However, further otimization studies are needed.

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Supplement II

P – 41

Analysis of Poly-(3-hydroxybutyrate) Produced by *Bacillus megaterium* and *Wautersia eutropha* Grown under Physiological Stress

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Polyhydroxyalkanoates (PHAs) are a group of hydroxyacid polyesters that are produced and accumulated in the form of intracellular granules by a wide variety of bacterial strains. These strains use PHA as carbon, energy and reducing power storage material. Of the big family of PHAs, a homopolymer of 3-hydroxybutyrate, poly-(3-hydroxybutyrate) (PHB), is the most widespread in nature and the best characterized. PHB aroused much interest in industry and research thanks its biocompatible, biodegradable, thermoplastic and piezoelectric properties. PHB appears to find many applications in medical, industrial and agricultural fields. Generally, the main problem of PHB is high cost in comparison with traditional plastics from petrochemical routes. That is why strategies for enhancing of PHB production are developed. Simultaneously, methods for accurate determination of produced PHB in bacterial cells including effective isolation procedures are developed.

The aim of this work was to compare different methods for isolation and analysis of PHB in bacterial cells to evaluate some strategies that could enhance PHB production and reduce costs of PHB. Limitations with nitrogen and/or phosphorus source as well as other stress types were tested. Finally, production of PHB on some waste substrates was studied.

For PHB accumulation two bacterial strains were used: *Bacillus megaterium* CCM 2037 and *Wautersia eutropha* CCM 3726. Cultivations were carried out at 30°C under permanent shaking. Several media were tested: 1) Synthetic glucose medium (SM); 2) *Bacillus* medium (BM) containing peptone and beef extract and 3) cheese whey obtained from cheese manufactory Pribina Přibyslav. SM was used for limitation experiments. Biomass concentration was analyzed spectrophotometrically at 630 nm. Before PHB determination biomass was lyophilized. Dried cell material was used for PHB analysis by GC/FID, GC/MS and LC/MS/UV-VIS. Hydrolyzed and derived in a mixture of 0.8 ml acidified methanol (15% v/v H_2SO_4) and 1 ml chloroform at 100°C for 140 min. Resulting methyl esters of 3- hydroxybutyric acid were determined using gas chromatography with MS or FID detection. Commercial PHB (Fluka) was used as the external standard. For LC/MS/UV-VIS analysis, PHB from dried cells was turned to crotonic acid (CA) using 96% H_2SO_4 (100°C, 1 hour). CA was determined using LC. Samples (20 µl) were injected into the RP-18 column (Restek C18, 5 µm, 250 mm x 4.6 mm). CA was eluted isocratically (water: methanol 1:1 v/v) at a flow rate 0.4 $ml.min^{-1}$ and detected using MS/ESI (Mass spectrometer LCQ Advantage Max) in negative mode and UV-VIS (220 nm). Crotonic acid (Aldrich) was used as standard.

In both strains most of strategies tested in this work led to enhancing of PHB production. Limitation with nitrogen seems to be a strong stimulating factor for PHB production. Positive effect of two exogenous stress factors - ethanol and Na_2SO_3 was observed in *B. megaterium*. However, the mechanism and optimal concentration of stress factors have to be clarified. Cultures were able to utilize organic substrates (BM, cheese

whey) and produce high amount of PHB. That should lead to reducing of PHB cost. However, further optimization studies are needed.

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Supplemet III

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Use of Controlled Exogenous Stress for Improvement of Poly(3-hydroxybutyrate) Production in *Cupriavidus necator*

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ABSTRACT. The PHB production by *Cupriavidus necator* H16 depends on the type and concentration of stress factors and on the time of stress application. Hydrogen peroxide and ethanol significantly enhanced PHB accumulation in *C. necator* cells. Improved yields (10.9 g/L PHB) were observed after exposure of bacterial culture to 0.5 mmol/L H_2O_2 at the beginning of cultivation and to additional peroxide stress (5 mmol/L H_2O_2) after 60 h of cultivation (beginning of the stationary phase). Production was then ≈28 % higher than in control (8.50 g/L PHB). The highest yields (11.2 g/L PHB) were observed when ethanol (0.5 %) was applied at the beginning of stationary phase. An application of exogenous stress could thus be used as a simple strategy for a significant improvement of PHB production in *C. necator*.

Abbreviations

NB	nutrient broth (agar, medium)	PHA	polyhydroxyalkanoates
MS	mineral salt medium	PHB	poly(3-hydroxybutyrate)

Polyhydroxyalkanoates are a group of hydroxyacid polyesters that are accumulated in the form of intracellular granules by a wide variety of bacterial strains that use PHA as carbon and energy storage material. Most bacteria accumulate PHA when carbon source is provided in excess and another nutrient element, such as nitrogen, phosphate *etc.* is limiting (Kessler and Wilholt 1999).

Of the large family of PHA, a homopolymer of 3-hydroxybutyrate, poly(3-hydroxybutyrate) (PHB), is the most widespread in nature. PHB aroused much interest in industry and research thanks to its biocompatible, biodegradable, thermoplastic and piezoelectric properties. PHB appears to find many possible applications in medical, industrial and agricultural fields (Reddy *et al.* 2003).

Cupriavidus necator is frequently studied due to its ability to accumulate large amount of PHA using different carbon sources, such as fructose, glucose, alcohols, amino acids, protein hydrolysates or CO_2–H_2 mixture. The pathway and regulation of PHB synthesis in *C. necator* were studied in detail by Kessler and Wilholt (1999).

Generally, it is assumed that PHA production and degradation ability improves bacterial survival under stress conditions but the mechanisms have not yet been fully understood. However, the relation of the presence of intracellular PHB in bacteria to stress endurance was demonstrated in many studies (Kadouri *et al.* 2003; Ruiz *et al.* 2003; Ayub *et al.* 2004; Zhao *et al.* 2007). In most cases, stress response was accompanied by PHB degradation. Breedveld *et al.* (1993) reported that *Rhizobium leguminosarum* and *R. meliloti* respond to exposure of osmotic stress by augmenting the cellular trehalose content whose synthesis proceeds simultaneously with the breakdown of PHB.

The above results indicate that exposure of a culture to stress conditions leads to a degradation of PHA. On the other hand, support of PHB accumulation by stress has been already reported; *e.g.*, in strain *Azospirillum brasilense* Sp7 'heavy metals' induced accumulation of PHB (Kamnev *et al.* 2007). Natarajan *et al.* (1995) found that NaCl stress results in enhanced production of PHB in *Rhizobium* DDSS-69.

We investigated the influence of exogenous stress (represented by some stress factors in different concentrations and various time-periods of the treatment) on PHB production in cultures of *C. necator* H16. The ratio of PHB/biomass was evaluated.

MATERIALS AND METHODS

Microorganism. C. necator strain H16 (CCM 3726) (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha*, *Wautersia eutropha*) from *Czech Collection of Microorganisms* (Brno, Czech Republic) was used. The culture was maintained on NB agar at 5 °C and subcultured monthly.

Media. NB medium consisted of (in g/L) peptone 10, beef extract 10, NaCl 5. MS medium (Kimura *et al.* 2008) was used for all production experiments and contained (in g/L distilled water) $(NH_4)_2SO_4$ 3.0, KH_2PO_4 1.0, $Na_2HPO_4 \cdot 12H_2O$ 11.1, $MgSO_4$ 0.2, and 1 mL microelement solution (in g/L: $FeCl_3$ 9.7, $CaCl_2$ 7.8, $CuSO_4 \cdot 5H_2O$ 0.156, $CoCl_2$ 0.119, $NiCl_2$ 0.118, $CrCl_2$ 0.062 in 1 L of 0.1 mol/L HCl). Fructose, salt solution and microelement solution were sterilized separately (25 min, 121 °C) and then aseptically reconstituted at room temperature prior to inoculation. pH was adjusted to 7.0 using 2 mol/L NaOH and 2 mol/L HCl.

Cultivations. Growth characteristics. MS medium with 10 g/L fructose was used for inoculum. The microorganism was cultivated with agitation (150 rpm) for 2 d at 30 °C in 300-mL Erlenmmeyer flasks containing 100 mL of medium. For production cultivation (150 rpm, 30 °C), 100 mL of medium with 20 g/L fructose in 300-mL Erlenmmeyer flasks was used and inoculated with 5 mL of inoculum. Samples were analyzed for biomass, PHB content and residual nutrients.

Stress experiments. The production of PHB under stress conditions was studied in MS medium. Fructose (10 g/L) was used for inoculum (150 rpm, 2 d, 30 °C), 300-mL flasks contained 100 mL of media. For producing cultivation (150 rpm, 30 °C), 50 mL MS media with 20 g/L fructose in 100-mL flasks were inoculated with 2.5 mL of inoculum. Stress factors (applied aseptically) were: ethanol 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 3.00, 10.0 % (*W/W*); H_2O_2 0.5, 1.0, 3.0, 5.0, 7.0, 9.0, 11.0 mmol/L; NaCl 0.5, 2.0, 5.0 % (*W/W*), $NiCl_2$ 0.02, 0.1, 0.2 mmol/L, $CoCl_2$ 0.02, 0.1, 0.2 mmol/L. All cultivations (including controls) were done in triplicate; each sample was also analyzed in triplicate.

Analytical methods. Cell growth was monitored by measuring the absorbance of culture broth at 630 nm on Helios Alpha (*Unicam*, UK). Dry cell mass was estimated after centrifugation (8000 rpm, 10 min) and drying at 105 °C to constant mass. Cell biomass was determined by standard plot of relationship between A_{630} and dry cell mass (g). The supernatant obtained after centrifugation (8000 rpm, 10 min) was used for residual substrate analysis; fructose was estimated by Somogyi–Nelson method (Deng and Tabatabai 1994); ammonia nitrogen was determined using Nessler reagent (A_{436} of a diluted sample was measured). PHB content was determined according to Brandl *et al.* (1988) by gas chromatography (*Finnigan* Trace GC Ultra; DB-WAX 30 m × 0.25 mm) with mass spectrometry detection (*Finnigan* Trace DSQ).

RESULTS AND DISCUSSION

PHB, biomass and nutrient changes during cultivation. Biomass, PHB, fructose and diammonium sulfate concentrations during cultivation are shown in Fig. 1 (phosphate limitation was not expected; because of its high concentration in medium, therefore, phosphate concentration was not analyzed).

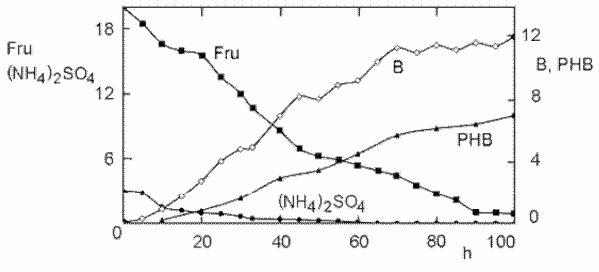

Fig. 1. The PHB, biomass and nutrient development during cultivation of *C. necator*; *circles* – diammonium sulfate, *squares* – fructose (Fru), *rhombs* – biomass (B), *triangles* – PHB (all in g/L).

Cultures accumulated PHB from the beginning of cultivation. After 60 h of cultivation, ammonium was depleted while fructose was still present. After 100 h of cultivation, fructose was almost exhausted and cultivation was stopped. Final concentration of PHB was 6.99 g/L which represented 57.8 % of biomass content.

Effect of stress factor application time. Stress factor (1 % ethanol) was applied at different time periods (0, 30, 60 and 90 h) of cultivation. Biomass and PHB content were analyzed after 100 h of cultivation

(Table I). When the stress factor was applied at the beginning of cultivation, culture growth was inhibited. Because PHB is an intracellular storage material, the growth inhibition negatively influenced the total PHB yield. Later application of ethanol did not exhibit any negative effect on biomass production. Highest total PHB yields were obtained when the effector was applied after 60 h of cultivation. At this time, nitrogen source was depleted and, as was reported previously by Kessler and Wilholt (1999), nitrogen limitation naturally enhanced the PHB synthesis. The addition of the ethanol as the second stress factor probably even supported this effect. Therefore we decided to add stress factors in all stress experiments at 60th h of cultivation.

Table I. Biomass and PHB production[a] by *Cupriavidus necator* in the presence of 1 % EtOH

Addition, h	Biomass, g/L	PHB, g/L	PHB, %
0	10.8 ± 0.28	7.21 ± 0.34	66.6
30	14.2 ± 0.17	7.71 ± 0.14	54.1
60	14.3 ± 0.24	10.2 ± 0.04	71.0
90	14.5 ± 0.35	9.31 ± 0.09	64.4
Control	14.3 ± 0.23	9.34 ± 0.55	65.4

[a]Means ±SD.

Effect of stress factor type and concentration (Table II). Both types of stress factor were also expected to influence bacterial growth and PHB production. The culture response was markedly influenced by stress factor concentration. Culture exposure to a mild stress factor (0.5 % EtOH, all concentrations of H_2O_2, 0.5 %, NaCl, 0.02 mmol/L $CoCl_2$) resulted in unaffected biomass formation and enhanced PHB accumulation. The addition of mild stress factors probably increased natural PHB biosynthesis under nitrogen starvation. Higher stress level inhibited bacterial growth, which subsequently caused lower PHB yields. Decrease of PHB contents in cells exposed to higher NaCl concentrations could be explained by inhibition of PHB biosynthetic pathway and activation of other metabolic processes related to osmotic stress response. Moreover, partial degradation of PHB could occur in order to endure high osmotic press (*see* Breedveld *et al.* 1993).

Table II. Biomass and PHB yields[a] induced by stress factor application after 60 h

Stress factor	Concentration	Biomass, g/L	PHB, g/L	PHB, %
EtOH, %	1	13.4 ± 0.19	9.64 ± 0.09	72.1
	3	11.4 ± 0.19	7.76 ± 0.30	68.0
	10	9.4 ± 0.14	6.31 ± 0.14	67.0
H_2O_2, mmol/L	0.5	13.1 ± 0.09	8.86 ± 0.18†	67.9
	1	12.8 ± 0.25	10.5 ± 0.31	81.7
	5	12.6 ± 0.26	9.89 ± 0.18	78.3
NaCl, %	0.5	12.7 ± 0.23	9.37 ± 0.29	73.7
	2	10.1 ± 0.33	6.36 ± 0.07	57.8
	5	9.3 ± 0.26	5.16 ± 0.15	55.3
$NiCl_2$, mmol/L	0.02	12.3 ± 0.22	8.43 ± 0.32†	68.5
	0.1	12.5 ± 0.18	8.42 ± 0.32†	67.6
	0.2	12.5 ± 0.45	8.04 ± 0.30†	64.3
$CoCl_2$, mmol/L	0.02	12.8 ± 0.13	9.18 ± 0.20	71.8
	0.1	12.4 ± 0.20	8.36 ± 0.13†	67.4
	0.2	10.8 ± 0.07	6.51 ± 0.18	60.1
Control		13.0 ± 0.15	8.50 ± 0.14	65.2

[a]Means ±SD.
†Not statistically significant (cultivation under stress *vs.* control; *t*-test, $p > 0.05$, confidence level = 95 %).

The use of mild stress resulted in PHB over-production which can be used for improvement of its production; hydrogen peroxide and ethanol were the most effective. When either 1 or 5 mmol/L H_2O_2 and 1 % EtOH were added into cultivation media, PHB production increased to 10.5, 9.89 and 9.64 g/L, res-

pectively. Thus, the addition of appropriate amount of a cheap substance can be considered to improve the polymer production about 20 % compared to control (8.50 g/L PHB).

Optimization of stress factor concentration. We assumed that ethanol as a toxic compound is metabolized in the cell primarily according to the following simplified equation.

$$CH_3CH_2OH + 2NAD^+ (NADP^+) + CoA \longrightarrow CH_3CO\text{-}CoA + 2NADH\ (NADPH)$$

Final product of ethanol oxidation, acetyl-CoA, is a key substrate for PHB synthesis. Moreover, during ethanol metabolism reduced coenzymes NADH (NADPH), both important for PHB synthesis, are formed, and a free CoA, which inhibits PHB synthesis, is built into acetyl-CoA. This can explain why ethanol supports PHB synthesis in *C. necator*.

The highest PHB productions were observed when 0.25 and 0.50 % ethanol was used (Table III); the PHB yields were then 11.1 and 11.2 g/L, respectively, which is ≈32 % higher than in control. Higher ethanol concentration exhibited a negative effect on biomass formation (which resulted in lower total PHB yields). Nevertheless, even cells exposed to 10 % ethanol contained ≈67 % of PHB, which was still more than control (65 %).

Table III. Biomass and PHB yields[a] induced by ethanol application after 60 h of cultivation

EtOH, %	Biomass, g/L	PHB, g/L	PHB, %
0.25	14.2 ± 0.21	11.1 ± 0.22	77.9
0.50	14.2 ± 0.15	11.2 ± 0.13	79.3
0.75	14.0 ± 0.07	10.5 ± 0.12	74.5
1.00	13.5 ± 0.16	9.72 ± 0.10	72.0
1.25	13.2 ± 0.19	9.66 ± 0.14	73.2
1.50	13.0 ± 0.14	9.99 ± 0.18	76.7
2.00	12.6 ± 0.36	9.25 ± 0.18	73.1
3.00	11.4 ± 0.10	7.76 ± 0.15	68.0
10.0	9.42 ± 0.10	6.31 ± 0.07	67.0
Control	13.0 ± 0.15	8.50 ± 0.14	65.2

[a]Means ±SD.

Hydrogen peroxide also positively influences PHB production but the mechanism leading to PHB overproduction has not been clarified, *e.g.*, hydrogen peroxide may enhance NAD(P)H/NAD(P)$^+$ ratio in cells (Izawa *et al.* 1998). We observed the highest yields when the cells were exposed to low concentration of hydrogen peroxide since the beginning of cultivation and the main stress dose was applied at 60th h of cultivation (Table IV).

Table IV. Biomass and PHB yields[a] induced by H_2O_2 application at zero time and after 60 h of cultivation

H_2O_2, mmol/L		Biomass, g/L	PHB, g/L	PHB, %
0 h	60 h			
0.5	–	12.9 ± 0.26	8.90 ± 0.36[†]	69.1
0.5	0.5	13.3 ± 0.09	9.07 ± 0.13	68.0
0.5	1.0	13.8 ± 0.18	10.1 ± 0.16	73.2
0.5	3.0	13.7 ± 0.05	10.6 ± 0.18	77.4
0.5	5.0	14.3 ± 0.08	10.9 ± 0.15	76.2
0.5	7.0	11.6 ± 0.19	9.58 ± 0.22	82.3
0.5	9.0	11.1 ± 0.08	8.08 ± 0.26[†]	73.1
0.5	11.0	11.7 ± 0.24	7.98 ± 0.07	68.5
Control		13.4 ± 0.15	8.50 ± 0.14	65.2

[a]Means ±SD.

[†]Not statistically significant (cultivation under stress *vs.* control; *t*-test, $p > 0.05$, confidence level = 95 %).

Time course of PHB accumulation after stress factor application (Fig. 2). Control culture slowly accumulated PHB until fructose was depleted (100 h); then the culture started to utilize intracellular PHB. The exposure of cells to either ethanol or hydrogen peroxide resulted in very intensive PHB accumulation. The best yields were observed for 0.5 % ethanol (11.2 g/L), PHB production in the presence of 0.5 mmol/L peroxide was slightly lower (10.9 g/L).

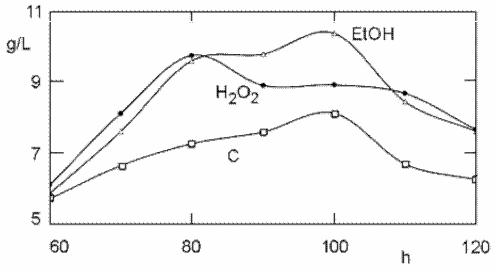

Fig. 2. PHB yields (g/L) induced by stress factor applied after 60 h of cultivation; *squares* – control (C), *circles* – 5 mmol/L H_2O_2, *triangles* – 0.5 % ethanol (EtOH).

Table V. Biomass and PHA production of *C. necator* in batch cultures

Substrate/effector[a]	Biomass, g/L	PHA, g/L	PHA, %	Reference
Fructose (40)	13.4	6.75	50.4	Khanna and Srivastava 2005
Fructose (40)[b]	20.7	9.35	45.1	*ditto*
Glucose (40)	20.5	12.7	62.0	Wang and Yu 2007
L-Thr (15), butyric acid (5)	13.0	5.73	44.3	Kimura *et al.* 2008
Glucose (20)[c]	8.78	2.80	31.9	Zhang *et al.* 2004
Glucose (20)[d]	3.22	2.50	77.8	*ditto*
Fructose (20), ethanol stress	14.2	11.2	79.3	*this study*
Fructose (20), peroxide stress	14.3	10.9	77.4	*this study*

[a]Concentration (in g/L) in parentheses. [b]Cultivated in a fermentor. [c]Undiluted medium. [d]Diluted medium.

The PHB yields obtained in this work, particularly under mild stress conditions, are significantly higher than yields obtained in other studies (Table V).

The main factor preventing the large-scale PHB production are high production costs (compared with those of plastics based on petrochemicals; Khanna and Srivastava 2005). Our results indicate that the use of mild stress can be used for improvement of the PHB production. However, introducing the stress factors into large aerated and stirred bioreactor can lead to a different response of cultures.

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ORIGINAL PAPER

Effect of ethanol and hydrogen peroxide on poly(3-hydroxybutyrate) biosynthetic pathway in *Cupriavidus necator* H16

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Abstract Exposition of *Cupriavidus necator* to ethanol or hydrogen peroxide at the beginning of the stationary phase increases poly(3-hydroxybutyrate) (PHB) yields about 30%. Hydrogen peroxide enhances activity of pentose phosphate pathway that probably consequently increases intracellular ratio NADPH/$NADP^+$. This effect leads to stimulation of the flux of acetyl-CoA into PHB biosynthetic pathway and to an increase of enzymatic activities of β-ketothiolase and acetoacetyl-CoA reductase while activity of PHB synthase remains uninfluenced. During ethanol metabolisation, in which alcohol dehydrogenase is involved, acetyl-CoA and reduced coenzymes NAD(P)H are formed. These metabolites could again slightly inhibit TCA cycle while flux of acetyl-CoA into PHB biosynthetic pathway is likely to be supported. As a consequence of TCA cycle inhibition also less free CoA is formed. Similarly with hydrogen peroxide, activities of β-ketothiolase and acetoacetyl-CoA reductase are increased which results in overproduction of PHB. Molecular weight of PHB produced under stress conditions was significantly higher as compared to control cultivation. Particular molecular weight values were dependent on stress factor concentrations. This could indicate some interconnection among activities of β-ketothiolase, acetoacetyl-CoA reductase and PHB molecular weight control in vivo.

Keywords Polyhydroxyalkanoates · Poly(3-hydroxybutyrate) · *Cupriavidus necator* · Stress conditions

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Introduction

Polyhydroxyalkanoates (PHA) are a group of hydroxyacid polyesters that are produced and accumulated in the form of intracellular granules by a wide variety of bacterial strains that use PHA as carbon, energy and reducing power storage material (Kessler and Wilholt 1999). Of the big family of PHA, a homopolymer of 3-hydroxybutyrate, poly(3-hydroxybutyrate) (PHB), is the most widespread in nature and the best characterised. PHB aroused much interest in industry and research thanks to its biocompatible, biodegradable, thermoplastic and piezoelectric properties. PHB appears to find many potential applications in medical, industrial and agricultural fields (Kadouri et al. 2005).

The Gram-negative, facultative chemolithoautotrophic β-proteobacterium *Cupriavidus necator* (formerly *Alcaligenes eutrophus*, *Ralstonia eutropha* and *Wautersia eutropha*) is often used as a model organism for PHB metabolism because of detailed study of PHB biosynthetic pathway and its regulation in this bacteria (Reinecke and Steinbuchel 2009). PHB is synthesised in the three step reaction. The entering substrate is acetyl-CoA. Two acetyl-CoA are coupled to form acetoacetyl-CoA in condensation reaction catalysed by β-ketothioloase (β-KT). Acetoacetyl-CoA is subsequently stereo-selectively reduced to (R)-3-hydroxybutyryl-CoA in a reaction catalysed by NADPH-dependent

acetoacetyl-CoA reductase (AACR). Finally, PHB is synthesised during polymerisation of (R)-3-hydroxybutyryl-CoA molecules catalysed by PHB synthase. Intracellular concentrations of acetyl-CoA, free CoA and NADPH are key factors influencing PHB synthesis on enzymatic level. Free CoA inhibits β-KT and NADPH is needed for reaction catalysed by AACR which is inhibited by oxidised coenzyme $NAD(P)^+$. Citrate synthase and isocitrate dehydrogenase activities are significantly inhibited by NADH and NADPH. Therefore, the concentration of NAD(P)H is considered to be the major regulatory factor determining the flux of acetyl-CoA to either TCA cycle or PHB biosynthetic pathway (Kessler and Wilholt 2001).

Generally, it is assumed that PHB production and degradation ability improves bacterial survival under stress conditions, but the mechanisms have been not fully understood yet. Ayub et al. (2004) reported that antarctic bacterium *Pseudomonas* sp. 14-3 shows high stress resistance in association with high PHB production. Stress tolerance of *Pseudomonas* sp. 14-3 was analysed in PHB accumulating and non-accumulating conditions and increased levels of stress resistance were observed when PHB was produced.

The connection of PHB with stress endurance was demonstrated in many other cases. Mutant strain of *Aeromonas hydrophila* 4AK4 unable to produce PHB and wild type were tested under stress conditions. It was found that PHB synthesis improved resistance against environmental stress factors such as heat and cold treatments, hydrogen peroxide, UV irradiation, ethanol and high osmotic pressure (Zhao et al. 2007). In another study, wild type of *Azospirillum brasilense* Sp7 was compared with PHB depolymerase mutant strain that was unable to degrade PHB. 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 higher than that of the mutant strain (Kadouri et al. 2003). Breedveld et al. (1993) also 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.

Studies mentioned above could indicate that exposition of culture to stress condition leads to mobilisation of PHB. On the other hand, an increase of PHB accumulation has been already observed in bacterial cells exposed to stress conditions. In non-endophyte strain *Azospirillum brasilense* Sp7 heavy metals induced enhanced PHB biosynthesis (Kamnev et al. 2007). Natarajan et al. (1995) reported that NaCl stress resulted in the accumulation of PHB in *Rhizobium* DDSS-69 cultures raised under unbalanced growth conditions.

Previously we observed that application of ethanol and hydrogen peroxide leads to the over-production of PHB in *Cupriavidus necator* (Obruca et al. 2010). This fact indicates that the stress factor application could be used biotechnologically as an effective and simple strategy for PHB production control. The objective of this work was to investigate the effect of both hydrogen peroxide and ethanol on the PHB biosynthetic pathway and its regulation on enzymatic level. Moreover, the influence of stress conditions on molecular weight of PHB was studied as well. In other words, this work tries to be insight into the connection between PHB biosynthesis and response of *C. necator* to exogenous stress.

Materials and methods

Microorganism

Cupriavidus necator H16 (CCM 3726) purchased from Czech Collection of Microorganisms, Brno, Czech Republic, was used in all experiments. The culture was maintained on Nutrient broth agar at 5°C and sub cultured monthly.

Media

Nutrient broth (NB) medium was used for maintaining of culture. NB consisted of 10 g l^{-1} pepton, 10 g l^{-1} beef extract, 5 g l^{-1} NaCl. Mineral salt (MS) medium described by Kimura et al. (2008) was used for all production experiments. MS medium contained 3.0 g l^{-1} $(NH_4)_2SO_4$, 1.0 g l^{-1} KH_2PO_4, 11.1 g l^{-1} $Na_2HPO_4 \cdot 12H_2O$, 0.2 g l^{-1} $MgSO_4$, 1 ml of microelement solution and 1,000 ml of distilled water. The microelement solution was composed of 9.7 g l^{-1} $FeCl_3$, 7.8 g l^{-1} $CaCl_2$, 0.156 g l^{-1} $CuSO_4 \cdot 5H_2O$, 0.119 g l^{-1} $CoCl_2$, 0.118 g l^{-1} $NiCl_2$, 0.062 g l^{-1} $CrCl_2$ in 1,000 ml of 0.1 M HCl. Fructose in concentration of 20 and 10 g l^{-1} was used as carbon source for production media and inocula development, respectively. Fructose, salt solution and microelement solution were sterilised separately at 121°C for 25 min and then aseptically reconstituted at room temperature prior to inoculation. The pH was adjusted to 7.0 using 2 M NaOH and 2 M HCl.

Cultivation

Mineral salt medium containing 10 g l^{-1} of fructose was used for inoculum development. The microorganism was cultivated at agitation speed of 150 rpm and 30°C for 48 h in a 300 ml Erlenmeyer flask containing 100 ml of medium described above. For production cultivation, 100 ml of

medium with 20 g l^{-1} fructose was taken into a 300 ml Erlenmeyer flask and inoculated with 5 ml of inoculum. The flasks were kept under constant shaking at 150 rpm and 30°C. Samples (each in triplicate) were withdrawn in regular intervals and analysed for biomass, content of PHB and enzymatic activities. Hydrogen peroxide (3%) and ethanol (96%) were applied at the 60th hour of cultivation in volumes needed to obtain desired final concentration of stress factors in medium (hydrogen peroxide 5 mM, ethanol 0.5%).

Measurement of biomass concentration

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.

PHB extraction

Polyesters were extracted from the lyophilized cells into chloroform by stirring for 24 h at 60°C. Solution was filtered in order to remove the cell material. Finally, pure PHB was obtained by non-solvent precipitation (five times the volume of chloroform) and filtration. The non-solvent used was a mixture of methanol and water (7:3 [vol/vol]) (Lee et al. 2003). Extracted PHB was used for determination of molecular weight.

PHB analysis

PHB content was determined by gas chromatography (Finnigan Trace GC Ultra; USA, column DB-WAX 30 m by 0.25 mm) with mass spectrometry detection (Finnigan Trace DSQ; USA) according to Brandl et al. (1988). Molecular weight data were obtained by gel-permeation chromatography [Agilent 1100 Series; refractive-index detector; USA column PLgel Mixed B (300 × 7.5 mm; 10 μm)] (Kusaka et al. 1997).

Measurement of enzyme activities

The cells were suspended in 50 mM phosphate buffer (pH 7) and disrupted by sonification at 4°C. The cytosol was separated by a centrifugation at 14,000 g for 10 min and stored at −20°C.

The activities of β-ketothiolase (β-KT) and acetoacetyl-CoA reductase (AACR) were analysed according to the method of Dorotina et al. (2008) based on measurement of absorbance of acetoacetyl-CoA at A_{303nm} and NADPH at A_{340nm} in crude cell extract. The activity of PHB synthase was determined using the method of Sharma et al. (2006) who measured the absorbance of CoA at A_{412nm}. The activity of glucose-6-phosphate dehydrogenase (G6PD) was analysed by measuring the released NADPH at A_{340nm} (Sagisaka 1972). Activity of NAD(P) dependent alcohol dehydrogenase (ADH) was measured according to Wales and Fewson (1994). Intracellular concentration of free CoA was determined according to Yamato et al. (1989), for protein concentration analysis the Bradford method was used (Bradford 1976). Enzyme activities are expressed as mmol of product formed or substrate used per minute per mg of protein in cell extract.

Results and discussion

Effect of stress factor application on biomass and PHB production

We observed that application of ethanol and hydrogen peroxide enhanced PHB production in culture of *Cupriavidus necator* considerably. However, the concentrations of stress factors in medium as well as the time of stress factor application have to be optimized in order to achieve the maximal PHB yields and to minimize the toxic effect of the stress factors on the culture growth. The best results were obtained when 0.5% ethanol and 5 mM hydrogen peroxide were applied at the beginning of the stationary phase (at the 60th hour of cultivation) (Obruca et al. 2010). In this work the time courses of biomass and PHB developments after stress application were measured. Exposition of culture to controlled stress significantly increased PHB content in cells while growth was not influenced (see Fig. 1).

Effect of stress factors application on enzymes of PHB biosynthetic pathway

In order to understand why ethanol and hydrogen peroxide caused over-production of PHB, activities of β-KT, AACR and PHB synthase were investigated in control and stressed cultures (see Fig. 2.).

Both ethanol and hydrogen peroxide significantly increased activities of β-KT (Fig. 2a) and AACR (Fig. 2b). On the contrary, PHB synthase activity seems to be independent of the exposition of the cells to the stress factors (Fig. 2c). These results indicate that for enhancement of PHB accumulation under stress conditions, the activities of β-KT and AACR are crucial. This observation is in consistence with the results of Oeding and Schlegen (1973) and Senior and Dawes (1971) who suggested β-KT as the

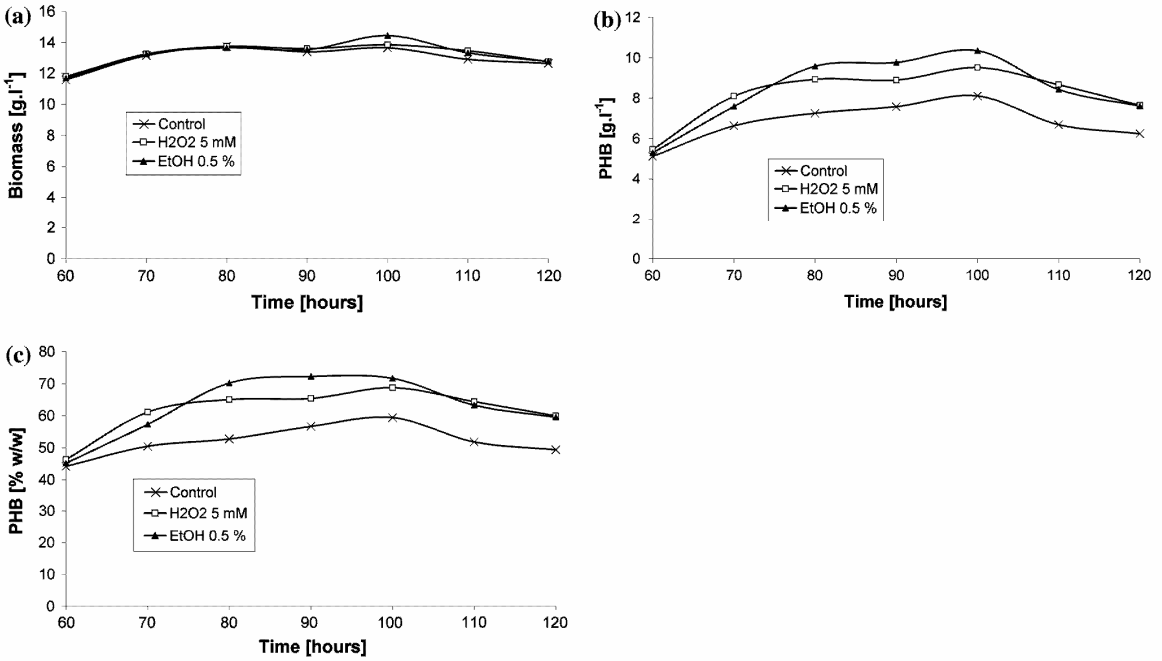

Fig. 1 Effect of stress factors application on cell growth (**a**), PHB total yields (**b**) and PHB content in cells (**c**). Stress factors were applied at the 60th hour of cultivation

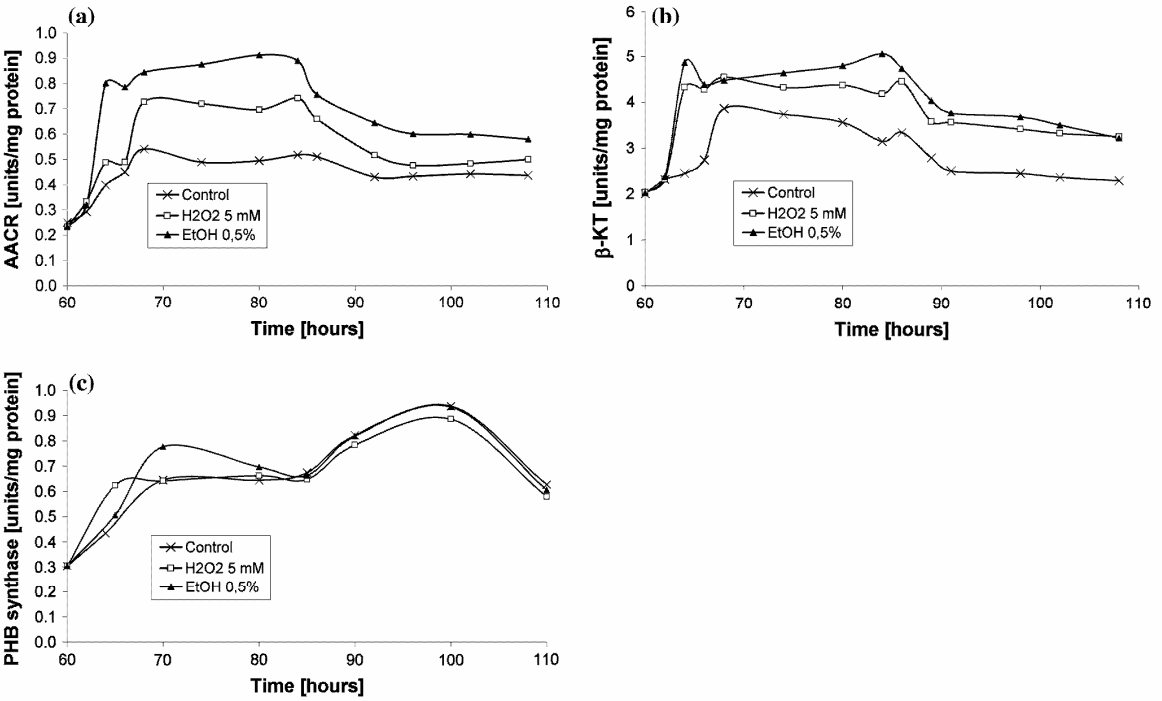

Fig. 2 Effect of stress factors application on activity of β-KT (**a**), AACR (**b**) and PHB synthase (**c**). Stress factors were applied at the 60th hour of cultivation

key enzyme regulating PHB biosynthesis. Doi et al. (1992) proposed AACR rather than PHB synthase as the main enzyme regulating PHB content in cells. The reasons why stress factors enhanced activities of β-KT and AACR are discussed bellow.

Response of bacterial culture to peroxide stress

Oxidative stress is phenomenon which is able to cause serious damage to cells. That is why practically all living organisms, including bacteria, developed sophisticated systems preventing them from damage caused by reactive oxygen species. Under oxidative stress, some molecules are constitutively present in cells and help to maintain intracellular reducing environment or to scavenge chemically reactive oxygen species. Among these molecules are non-enzymatic antioxidants such as NADPH and NADH, β-carotene, ascorbic acid, α-tocopherol, and glutathione (GSH). GSH, present at high concentrations, maintains a strong reducing environment in the cell. Its reduced form is regenerated by glutathione reductase using NADPH as a source of reducing power (Sigler et al. 1999; Cabiscol et al. 2000). Therefore, under oxidative stress cells need to maintain ratio NADPH/NADP$^+$ at high level. In most microorganisms including *Cupriavidus necator*, NADPH is generated in pentose phosphate pathway from the reactions catalysed by glucose-6-phospahate dehydrogenase (G6PD) and gluconate dehydrogenase (Romanova et al. 1970; Nenkov et al. 2008). It was observed that exposition of cells to oxidative stress leads to an increase of G6PD activity in *Escherichia coli* (Rowley and Wolf 1991) and *Saccharomyces cerevisiae* (Izawa et al. 1998).

If oxidative stress had increased activity of G6PD in *Cupriavidus necator*, resulting high ratio NADPH/NADP$^+$ could have been a strong stimulating factor supporting PHB accumulation. This was the reason why we decided to measure the activity of G6PD in cells exposed to stress factors (Fig. 3).

Application of hydrogen peroxide strongly enhanced activity of G6PD in comparison with both control culture and culture stressed by ethanol. It can be expected that one of the consequences of higher G6PD activity is the increase of intracellular ratio of NADPH/NADP$^+$, which controls the flux of acetyl-CoA to PHB biosynthetic pathway and slightly reduces activity of TCA cycle. Because of partial inhibition of TCA cycle, lower concentration of free CoA is presented in cells, which supports activity of β-KT (see Fig. 5). Furthermore, excess of NADPH could act as an electron donor in the AACR catalysed reaction transforming acetoacetyl-CoA into β-hydroxybutyryl-CoA. High level of NADPH/NADP$^+$ is also important stimulating factor for AACR activity (Kessler and Wilholt 2001). Coexistence of all these factors probably caused the over-production of PHB in culture of *Cupriavidus necator* exposed to hydrogen peroxide.

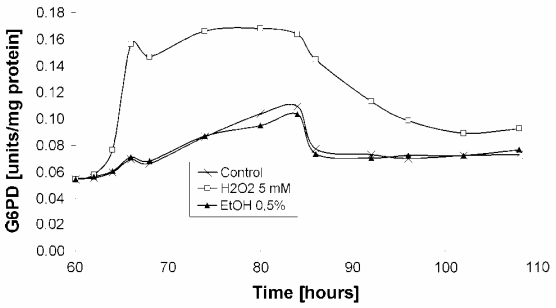

Fig. 3 Effect of stress factors application (the 60th hour) on activity of G6PD

Response of culture to ethanol stress

According to results of us, ethanol seems to be more effective factor supporting PHB biosynthetic pathway in comparison with hydrogen peroxide. It can be assumed that ethanol is metabolized in cell primarily according to following simplified equation.

$$CH_3CH_2OH + 2NAD(P)^+ + CoA + ATP \rightarrow CH_3CO - CoA + 2NADH(NADPH) + AMP + PPi$$

The first step of this reaction is catalyzed by alcohol dehydrogenase (ADH), therefore, we measured its activity in *Cupriavidus necator* cells after the stress factors application (see Fig. 4).

Activity of ADH in culture increased strongly after ethanol addition. This fact supports our theory about response of culture to ethanol stress. For PHB accumulation, it is probably very important, that the final product of ethanol oxidation, acetyl-CoA, is the key substrate of PHB biosynthetic pathway (Kessler and Wilholt 2001). Moreover, reduced coenzymes stimulating the activity of AACR

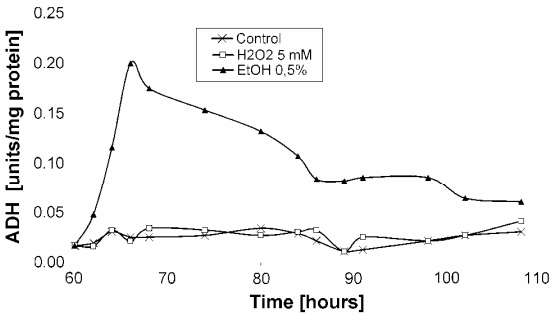

Fig. 4 Effect of stress factors application (the 60th hour) on activity of ADH

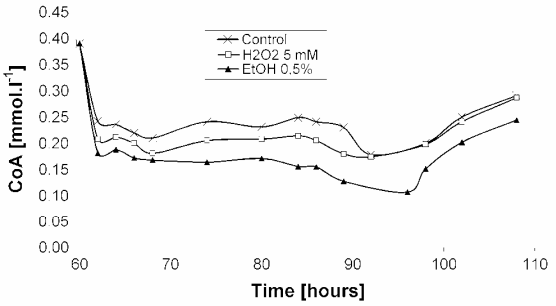

Fig. 5 Effect of stress factors application (the 60th hour) on intracellular concentration of free CoA

are formed during ethanol metabolisation and free CoA, which inhibits β-KT, is likely to be built into acetyl-CoA. In addition, reduced coenzymes also slightly inhibit TCA cycle (similarly with hydrogen peroxide) and the flux of acetyl-CoA into PHB biosynthetic pathway could be therefore supported. Also less free CoA is formed as a consequence of TCA cycle inhibition which supports activity β-KT (see Fig. 5). Metabolic effects described above could lead to even more significant influence of ethanol on PHB synthesis in *Cupriavidus necator* in comparison with hydrogen peroxide.

Effect of stress factor application on molecular weight of PHB

The potential effect of controlled stress on molecular weight of produced PHB was investigated as well. Final step of PHB biosynthesis is catalysed by PHB synthase which is often considered to be responsible for molecular weight of polyester chain (Sim et al. 1996) despite the fact that the mechanism of PHB molecular weight control has not been clearly understood yet and many other factors are probably involved.

Although activity of PHB synthase was observed to be independent of stress application (Fig. 2), the addition of stress factors into media surprisingly resulted in higher molecular weight of polymer (see Table 1). Our results are similar to those of Kichise et al. (1999) who reported that molecular weight of PHB is independent on PHB synthase activity. On the contrary, in vitro experiments of Gerngross and Martin (1995) demonstrated that molecular weight of PHB decreased with increase in the initial activity of PHB synthase. Kawaguchi and Doi (1992) 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.

According to our results (Table 1), under mild stress conditions longer PHB chains were formed, while increasing concentration of both stress factors led to a gradually decrease of PHB molecular weight. Higher molecular weight PHB, formed under mild stress conditions, could probably serve as a long-term storage material, while shorter-chain PHB is likely to be a result of a partial PHB mobilisation, that could be a mechanism activated as a response of cells to exposition to strong stress conditions.

The fact that stress conditions enhanced molecular weight of PHB as well as activities of β-KT and AACR indicate that activities of β-KT and AACR could be, in some way, connected with final molecular weight of PHB. A possible explanation might be that intracellular concentration of monomers is higher than in control culture as the result of increased activity of β-KT and AACR. It could partially stimulate PHB synthase or some unknown regulatory factor(s) to form longer chains of PHB. However, these hypotheses have to be confirmed in further experiments.

In general, the fact that stress responses of *Cupriavidus necator* to hydrogen peroxide and ethanol result in enhanced production of PHB could bring a practical biotechnological outcome. Simple addition of cheap substance into cultivation media intensified PHB yields about 30% (in case of 0.5% EtOH, Table 1). Moreover, resulting polymer has significantly higher molecular weight which indicates that stress factor application could be used to control molecular weight of produced PHB as well. However, strategy of stress application has to be optimised under conditions of large-scale fermentation, because the introduction of stress factor into aerated and stirred bioreactor might lead to different response of culture. Nevertheless, if

Table 1 Biomass, PHB yields and molecular weight (Mw) of PHB after stress factor application (the 60th hour) at the end of cultivation (the 100th hour)

	Biomass (g l^{-1})	PHB (g l^{-1})	PHB (% w/w)	Mw (Da $\times 10^5$)
EtOH 0.5%	13.66 ± 0.62	11.40 ± 0.05	83.45	9.749
EtOH 1.0%	12.77 ± 0.34	9.18 ± 0.13	71.90	8.932
EtOH 3.0%	11.45 ± 0.26	7.20 ± 0.01	62.93	7.539
H_2O_2 5 mM	13.33 ± 0.37	10.57 ± 0.07	79.30	9.054
H_2O_2 8 mM	13.44 ± 0.35	10.23 ± 0.16	76.12	8.049
H_2O_2 11 mM	13.29 ± 0.20	10.56 ± 0.42	79.49	6.876
Control	12.18 ± 0.29	8.63 ± 0.10	73.34	1.870

Results in form: mean ± standard deviation

the proposed strategy is successful, this will be important step helping to reduce production cost of PHB which is the main factor preventing its broader industrial production.

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Supplemet V

3.1.29

Production of polyester-based bioplastics by *Bacillus megaterium* grown on waste cheese whey substrate under exogenous stress

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Bacterial polyesters, polyhydroxyalkanoates (PHA), are a group of hydroxyacid polyesters that are accumulated in the form of intracellular granules by a wide variety of bacterial strains that use PHA as carbon and energy storage material. PHA have received much interest due to their biodegradable nature and mechanical properties which are very similar to plastics produced from petrochemical routes. This is the reason why PHA appears to find many potential applications in medical, industrial and agricultural fields. However, the main factor preventing the large-scale production of PHA is their high cost as compared with that of plastics based on petrochemicals. Among the factors restricting the economy of PHA production the most important is the cost of carbon source. In PHA production, about 40% of the total cost is for raw materials. Thus, cheap waste substrates, for instance cheese whey, are very attractive because of possibility to reduce PHA cost.

In this work PHA production in lactose-utilizing bacteria *Bacillus megaterium* grown in batch culture on cheese whey substrate was tested. The amounts of accumulated PHA were analyzed using gas chromatography with flame ionization detector. Activities of PHA biosynthetic enzymes were estimated too. After optimization of cheese whey medium composition and cultivation conditions relative high biomass yields were obtained using whey as the only carbon source, but the biomass contained only about 30% of PHA. Thus, in addition to nutritional stress exogenous ethanol and peroxide stress were applied to enhance PHA yields. Both these stress types seemed to be very effective stimulating factors improving PHA production. The response of culture to stress depended on the concentration of stress factor as well as on the time of stress factor application. According to our results, the best effect on PHA production exhibited ethanol in concentration 0.5% (v/v) and 5 mM hydrogen peroxide, both applied at the beginning of stationary phase. Then after, PHA yields were improved about 20—30%. Use of waste whey substrate is one of the possible ways to enable broader use of bioplastics.

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3.1.30

Vegetable wastes as suitable biomass feedstock for biorefineries

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According to a widely accepted definition biorefinery is 'the sustainable processing of biomass into a spectrum of value-added products (chemicals, materials, food and feed) and energy (biofuels, power and heat).' The main sources of biomass feedstock consist of sugar and starch crops, vegetable oils, grasses, lignocellulosic materials and different organic wastes such as municipal solid wastes and residues from the food production chain.

In recent years vegetable biomass wastes produced by food processing industry revealed their intrinsic value and potentialities as biorefinery feedstocks. Indeed many studies carried out worldwide showed that such biomass represents an interesting source of value-added products including either bioactive molecules or additives for biopolymer production.

As a matter of fact a significant fraction of vegetable components such as antioxidants, fibers and biopolymers are lost after processing being discarded in the residual matter. In this context several researches has been devoted to investigate vegetable biomasses as a potential source of energy and bioactive molecules. In this study some examples are reported concerning the reuse and recovery of biomolecules from tomato processing industry wastes besides some preliminary results on extraction of lemon 'pastazzo', the main residue remaining after lemon processing for liquors production.

Tomato wastes are constituted by about 50% fibers—polysaccharides, 18% proteins, 10% fats besides carotenoids and other substances with antioxidant activity. Attention is focused on some tomato-derived polysaccharides that possess significant anti-inflammatory properties, in addition to potential applicability in design of biodegradable plastic films. Moreover the ability of tomato wastes is reported in promoting and sustaining microbial growth of extremophilic microorganisms, namely thermophiles and halophiles.

Citrus by-products comprise as main components dietary fibers (including polysaccharides and lignin) and different bioactive compounds (i.e. flavonoids and vitamin C) with antioxidant properties. In this report we show the outcome of different extraction treatments performed to investigate the chemical nature of both polyphenol fraction and polysaccharide components of lemon processing wastes.

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Supplemet VI

Production of Polyhydroxyalkanoates from Cheese Whey Employing *Bacillus megaterium* CCM 2037

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Abstract Poly(3-hydroxybutyrate) (PHB) is the polyester of the family of polyhydroxyalkanoates, which are accumulated in a wide variety of bacterial strains. PHB appears to be biodegradable alternative to traditional petrochemical polymers such as polypropylene and polyethylene. In this work we tested conversion of cheap waste cheese whey into PHB employing bacterial strain *Bacillus megaterium*. Optimization of medium composition improved PHB yields about 50 times (biomass and PHB yields 2.82 and 1.05 $g.l^{-1}$, respectively) as compared to whey itself. Furthermore, PHB yields were even improved about 40 % introducing 1 % ethanol into the media at the beginning of the stationary phase of the growth (biomass 2.87 $g.l^{-1}$, PHB 1.48 $g.l^{-1}$). According to results of the experiments carried out in Erlenmeyer flasks, *B. megaterium* CCM 2037 can be considered to be one of the few candidates for direct PHB production from waste cheese whey. Nevertheless, experiments in laboratory and semi-productive fermentors are needed to perform high cell density cultivation.

Keywords *Bacillus megaterium, polyhydroxyalkanoates, poly(3-hydroxybutyrate), cheese whey, exogenous stress*

Introduction

Polyhydroxyalkanoates (PHA) are biopolymers produced and accumulated in the form of intracellular granules by a number of bacterial strains. Of the big family of PHA, a homopolymer of 3-hydroxybutyrate, poly(3-hydroxybutyrate) (PHB), is the most widespread in nature and the best characterised one. PHB aroused much interest in industry and research thanks to its biocompatible, biodegradable, thermoplastic and piezoelectric properties. Therefore, PHB is considered to act as an alternative to common plastics derived from petrol (Kadouri et al. 2005).

High production cost is one of the main factors preventing broader use of PHB. Analysis and economic evaluation of the bacterial PHB production suggested, that the cost of substrate (mainly carbon source) contributed the most significantly (up to 50%) to the overall production cost (Choi and Lee 1997). Thus, PHB could be produced more economically using cheap waste substrates.

Cheese whey is the major by-product from the manufacture of cheese and casein, representing 80-90 % of the volume of transformed milk. A total cheese production in European Union corresponds to approximately 40 462 000 tons of whey per year. A major part is used for production of lactose and feeding, but an annual amount of 13 462 000 tons of whey per year containing about 619 250 tons of lactose constitutes a surplus product (Koller et al. 2008). That is why cheese whey is promising substrate for cheap production of PHB in large amounts.

Although biotechnological production of PHB from different sugars via condensation of acetyl-CoA units stemming from hexose catabolism is well described (Kessler and Wilholt 1999), only limited number of bacterial strains directly converts lactose into PHB. Few reports are available on PHB production from lactose and whey by recombinant *Escherichia coli* (Wong and Lee 1998, Ahn et al. 2000). *Methylobacterium* sp. ZP24 (Yellore and Desai 1998, Nath et al.

2007) and thermophillic bacterium *Thermus thermophilus* HB8 (Pantazaki et al. 2009) are also able to utilize whey lactose for PHA biosynthesis. Recently, Pandian et al. reported PHA production from mixture of rice bran, non-specified diary waste and sea water employing gram positive bacterium isolated from brackish water. Based on morphological, physiological properties and nucleotide sequence of its 16S rRNA, it was suggested that the isolate was closely related to *Bacillus megaterium* (Pandian et al. 2010). Also *Pseudomonas hydrogenovora* was used for PHA production using cheese whey, but this bacterial strain was not able to utilize lactose directly, therefore, lactose in whey was hydrolyzed prior cultivation (Koller et al. 2008).

We have previously reported, that *Bacillus megaterium* CCM 2037 is able to utilize lactose and accumulate poly(3-hydroxybutyrate) (Obruca et al. 2008). In this study we focused on optimization of whey medium in order to enhance PHB and biomass yields. Moreover, application of exogenous stress was studied as potential strategy enhancing PHB biosynthesis in cells.

Materials and methods

Micro-organism, media and growth conditions

Bacillus megaterium CCM 2037 was obtained from Czech Collection of Microorganisms (Brno, Czech Republic).

CCM Bacillus Medium, consisting of peptone 5 $g.l^{-1}$, yeast extract 3 $g.l^{-1}$, $MnSO_4$ 0.01 $g.l^{-1}$ and agar 20 $g.l^{-1}$, was used for maintaining the culture. Mineral Medium (MM) was used for inoculum preparation. MM contained lactose 8 $g.l^{-1}$, $(NH_4)_2SO_4$ 5 $g.l^{-1}$, Na_2HPO_4 2.5 $g.l^{-1}$, KH_2PO_4 2.5 $g.l^{-1}$, $MgSO_4$ 0.2 $g.l^{-1}$ and $MnSO_4$ 0.01 $g.l^{-1}$. Initial pH of medium was adjusted to 7.0. Inoculum was developed in 250 mL Erlenmeyer flasks containing 100 mL of media. MM

medium was inoculated with bacterial culture and cultivated at agitation speed of 150 rpm and 30° C for 24 hours. Subsequently, 5 ml of the culture was inoculated into 250 ml Erlenmeyer flask containing 100 ml of whey medium.

Whey was obtained from cheese manufactory Pribina Pribyslav (Pribyslav, Czech Republic). The whole whey was treated in order to remove excessive proteins. Whey was acidified to pH 4.0 with 1.0 M H_2SO_4 and heated (100° C for 20 minutes) cooled and centrifuged at 8000 rpm for 5 minutes. The treated whey was used in the experiments after adjusting the pH to 7.0 with 1.0 M NaOH and MM medium components (except lactose) and 0.1 $g.l^{-1}$ yeast extract were added in concentration according to MM unless otherwise indicated.

Analytical methods

Cell growth was monitored by measuring the absorbance of culture broth at 630 nm on Helios α (Unicam, UK) after suitable dilution with distilled water. Cell biomass was calculated using calibration curve for $A_{630\ nm}$ and dry cell mass. For dry cell mass determination, cells obtained after centrifugation (8 000 rpm, 10 min.) were dried at 105° C till constant weight was obtained.

The supernatant obtained by centrifugation of the culture broth at 8 000 rpm for 10 minutes was used for residual lactose analysis by Somogyi-Nelson method (Deng and Tabatabai 1994).

PHB content in dried cells was determined by gas chromatography according Brandl et al. (Finnigan Trace GC Ultra, USA, column DB-WAX 30 m by 0,25 mm) with mass spectrometry detection (Finnigan Trace DSQ, USA) (Brandl et al. 1988).

Analysis of treated whey

Concentration of dry matter was estimated after drying (105° C) of 10 ml of whey till the constant weight was obtained. Ash content was analysed as weight of solids after incubation of 2 ml of whey at 800° C for 2 hours. The phosphorus content was measured by molybdenum blue colorimetric method ($A_{610\ nm}$) using whey ash. The concentration of soluble protein was performed using biuret method with bovine serum albumin as a standard. Sugars content of whey was estimated by HPLC method (pump LCP 4020, thermostat LCO 101, degasser DG-1210, refractometric detector RIDK 102; Ecom, Czech Republic) with ZOBRAX NH_2 column (150 cm× 4,6 mm, 5 μm; Chromservis, Czech Republic), chromatographic conditions were: 25° C; acetonitril: water 75:25; mobile phase flow 1.0 ml/min.

Media optimization using Placket-Burman experimental design

The dilution of whey (carbon source) and concentrations of nitrogen source ($(NH4)_2SO_4$), mineral salts and yeast extract were tested using Placket-Burman experimental design. Each parameter was tested at two levels, high (+1) and low (-1) (Tab. 1). A design of 12 experiments was formulated for 5 factors using Minitab software. The experiments were done in 250 ml Erlenmeyer flasks containing 100 ml of whey media at 150 rpm, 30° C for 50 hours in duplicate. Response was measured in terms of A: Biomass production, B: PHB accumulation in cells (% w/w) and C: PHB yields.

Results and discussions

Analysis of whey composition

Used cheese whey contained about 6.8 % of dry matter. Main part of dry matter is composed of sugars and salts, moreover, some soluble proteins are still present in whey even after treatment. In spite of the high concentration of salts (expressed as ash), which could result in high osmotic pressure influencing bacterial growth negatively, tested waste cheese whey seems to be promising complex substrate for PHB production. Sugars (lactose and glucose, galactose was not detected) could be utilized as the carbon sources, soluble proteins could serve as a complex carbon and nitrogen source and low level of potential phosphorus sources was observed as well. In addition, it could be expected, that whey contains some minor components such as free amino acids and vitamins (not analyzed), which are likely to support bacterial growth.

Supplementation of whey by salts

In order to test whether whey itself is sufficient substrate, culture of *Bacillus megaterium* was inoculated into whey with and without salts added according to MM (Fig. 1). Biomass and PHB yields were analyzed after 48 hours.

In the whey medium without added salts, bacterial growth as well as PHB production was rather low. Oppositely, addition of salts promoted the growth of bacterial culture and improved PHB production as well (almost 10 times). The explanation could be that despite excess of salts present in whey, whey itself lacked in nitrogen, phosphorus, magnesium and manganese. Therefore, these components must be added in the form of mineral salts.

Optimization of whey supplementation

For multivariable processes such as biotechnological systems, in which numerous potentially influential factors are involved, it is not always obvious to determine which the most important ones are. Hence, it is necessary to submit the process to an initial screening design prior to optimization. The methodology of Plackett–Burman could be a tool for this initial screening, because it makes possible to determine the influence of various factors with only a small number of trials (Khanna and Srivastava 2005).

We used Placket-Burman methodology to optimize composition of whey medium for PHB production. Five factors were selected for optimization, each factor was tested at two levels, high and low (Tab. 1). PHB and biomass yields were analyzed after 50 hours. Experiment was designated by Minitab software.

According to the results of Placket-Burman study, concentration of $(NH_4)_2SO_4$, PO_4^{3-} and $MgSO_4$ are statistically important neither for biomass nor for PHB production ($P > 0.05$). Nevertheless, in previous experiments addition of salts strongly enhanced biomass and PHB production, therefore, in following experiments salts were added at the same concentration which was used as lower (-1) in Placket-Burman study. The addition of yeast extract, which was tested as potential source of vitamins, amino acids etc., had negative statistically important impact on PHB production. It is probably due to the fact that yeast extract could serve as nitrogen source and PHB biosynthesis is much more pronounced under nitrogen limiting condition (Kessler and Wilholt 1999). Therefore, in further experiment only low concentration (0.1 $g.l^{-1}$) of yeast extract was added into whey medium. Oppositely, whey dilution seems to be crucial for biomass as well as for PHB production ($P < 0.05$). High concentration of salts and lactose in undiluted whey medium probably caused high osmotic pressure which consequently inhibited bacterial growth

and PHB biosynthesis (t values < 0). High concentration of lactose could/can also induce growth inhibition by substrate, hence, whey dilution was optimized in order to achieve maximal biomass and PHB yields (Fig. 2).

The highest biomass and PHB yields were obtained, when whey was diluted to lactose concentration of 20 $g.l^{-1}$. Thereafter, biomass and PHB yields were 2.51 $g.l^{-1}$ and 0.79 $g.l^{-1}$, respectively.

The growth and PHB production courses of *B. megaterium* in optimized whey medium were estimated as well. The growth was accompanied with lactose utilization during the whole cultivation, while the biomass formation reached its maximum after 28 hours of cultivation. After then stationary phase occurred, followed by decrease of biomass concentration after 50 hours of cultivation. The highest PHB yields were observed at the 50^{th} hour (see Fig.3).

PHB production under stress conditions

We have recently reported, that stress response of *Cupriavidus necator* H16 to ethanol and hydrogen peroxide is accompanied with enhanced PHB accumulation. However, stress should be applied at the beginning of the stationary phase and its concentration must be optimized. Suggested strategy could be used biotechnologically as simple, cheap and effective tool enhancing total PHB yields (Obruca et al. 2010a, Obruca et al. 2010b). In order to study, whether this strategy could be used also in *Bacillus megaterium* cultivated on cheap whey medium, we decided to apply different concentrations of ethanol and hydrogen peroxide at the 25^{th} hour of cultivation (Tab. 5).

Both hydrogen peroxide and ethanol increased PHB biosynthesis in *B. megaterium* cells, but the most effective strategy was the application of 1 % ethanol, which enhanced PHB yields about 41 % as compared to control culture (Tab. 5). Oppositely, application of hydrogen peroxide

enhanced PHB yields very slightly. The reason why ethanol enhanced PHB yields is that ethanol is metabolized via oxidation to acetyl-CoA. During these reactions reduced coenzymes NAD(P)H stimulating flux of acetyl-CoA to PHB biosynthetic pathway are formed and free CoA, which inhibits PHB biosynthesis, is built into acetyl-CoA. Moreover, acetyl-CoA as the final product of ethanol metabolization, is the entering substrate of PHB biosynthetic pathway (Obruca et al. 2010b).

Total PHB yields we obtained in this work are relatively low as compared to those obtained in fermentor in fed-batch mode (Nath et al. 2007, Pandian et al 2010). This fact is caused predominantly by relatively low growth of bacterial culture in Erlenmeyer flasks. On the other side, optimization of medium composition as well as controlled introduction of stress factor significantly enhanced PHB content in cells. This is beneficial in terms of total PHB yields, but, furthermore, it is also likely to reduce cost of PHA recovery because PHB content in cells strongly affects the efficiency and the cost of down-stream processing (Lee and Choi 1999). Further experiments have to be focused on cultivation in fermentor in order to reach high cell density and improve PHB yields in this way. Nevertheless, we have proved that application of controlled stress conditions by ethanol is promising strategy improving process of PHB production from cheese whey employing *B. megaterium*.

Conclusions

In this work we tested *Bacilus megaterium* as bacterial strain able to utilize waste cheese whey and produce PHB. Because supplementation of cheese whey medium with salts is necessary to reach higher PHB yields, optimization of whey media composition was performed. In our experiments, PHB production was pronounced about 50 times by optimization of cheese medium as compared to the cheese whey itself. Furthermore, even higher PHB yields can be

obtained when bacterial culture is exposed to 1 % ethanol as exogenous stress factor applied at the beginning of stationary phase. This novel strategy enhanced PHB production about 41 %. Our results indicate potential of *Bacillus megaterium* for industrial PHB production from cheap whey substrate, nevertheless, further experiments carried out in laboratory and semi-productive bioreactors are needed to obtain high cell density and even improve production parameters.

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Figures:

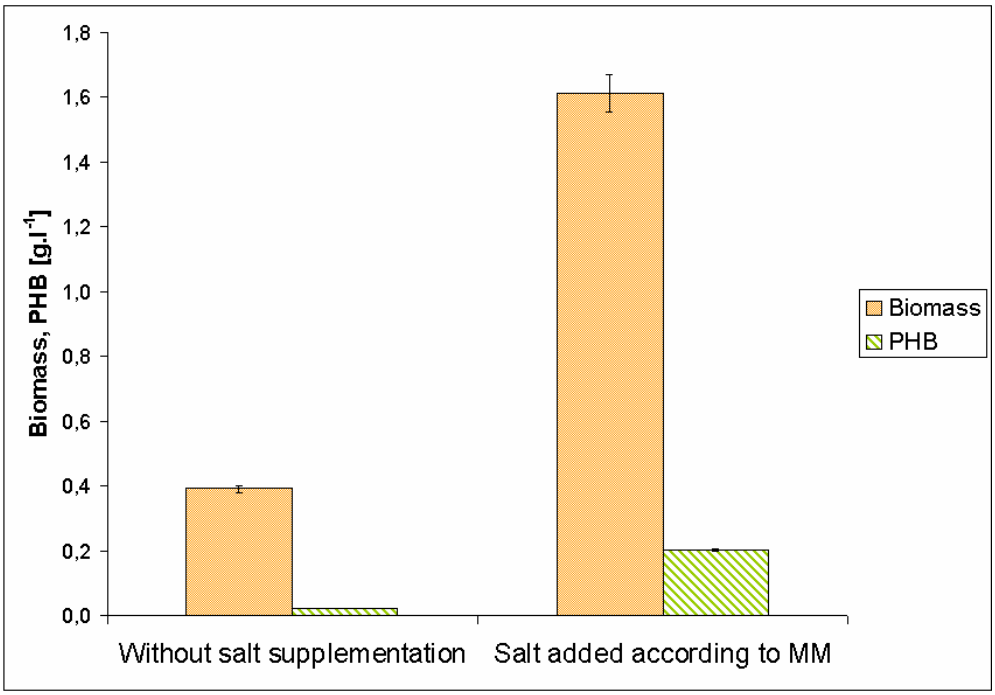

Fig. 1 Biomass and PHB yields by *Bacillus megaterium* on whey with and without salts addition.

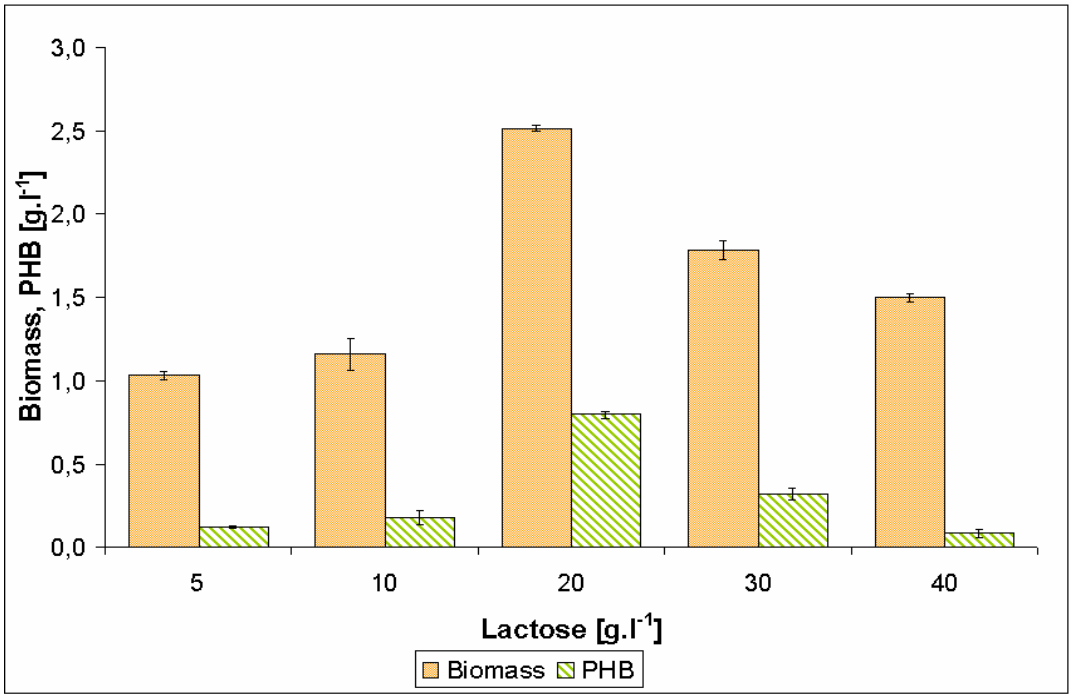

Fig. 2 The effect of whey dilution (expressed as lactose concentration) on PHB and biomass production.

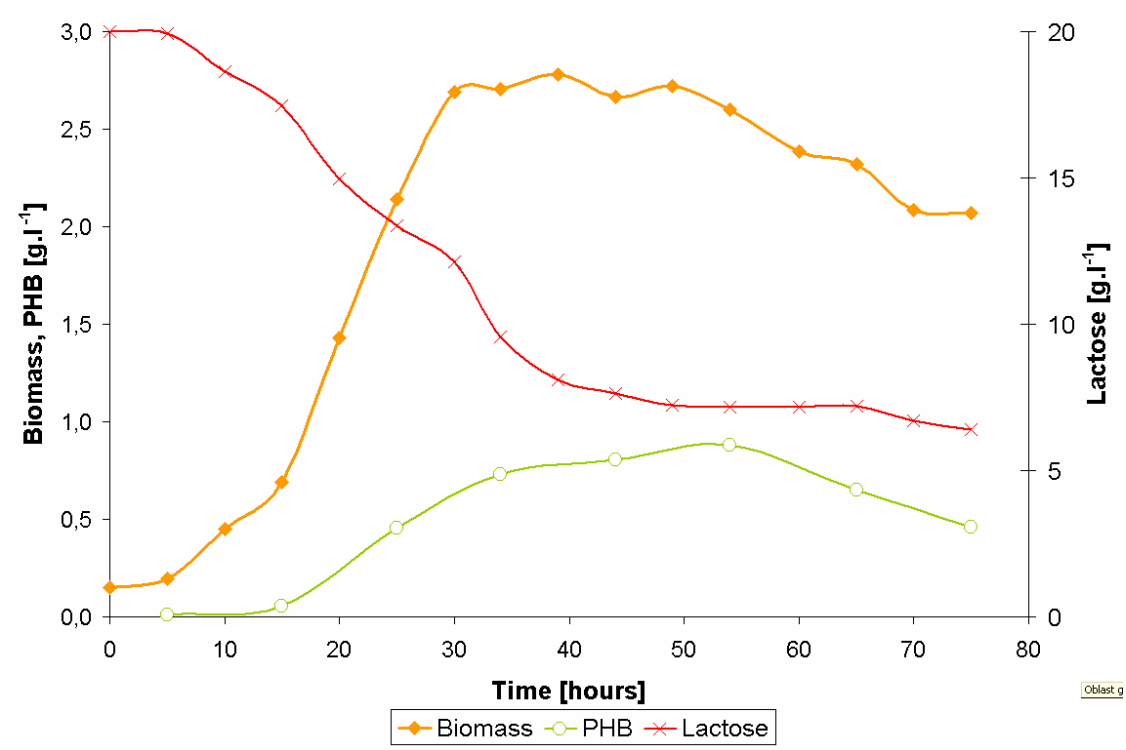

Fig. 3 The growth characteristics of *Bacillus megaterium* in optimized whey medium.

Tables:

Tab. 1 Range of factors studied in the Placket-Burman design

Factor	Name	Level	
		1	-1
A	Whey	Not-diluted (Lactose 40 $g.l^{-1}$)	Diluted (Lactose 40 $g.l^{-1}$)
B	$(NH4)_2SO_4$	5 $g.l^{-1}$	1 $g.l^{-1}$
C	Na_2HPO_4; KH_2PO_4 (1:1)	5 $g.l^{-1}$	1 $g.l^{-1}$
D	$MgSO_4$	0.2 $g.l^{-1}$	0.04 $g.l^{-1}$
E	Yeast autolysate	1 $g.l^{-1}$	0.1 $g.l^{-1}$

Tab. 2 Composition of used cheese whey

Substance	Concentration
Water	93 %
Dry matter	68 $g.l^{-1}$
Ash	27.10 ± 0.30 $g.l^{-1}$
Lactose	39.60 ± 0.45 $g.l^{-1}$
Glucose	0.35 ± 0.02 $g.l^{-1}$
PO_4^{3-}	63.0 ± 1.2 $mg.l^{-1}$
Soluble proteins	2.0 ± 0.1 $g.l^{-1}$

Results are in form average ± standard deviation, each analysis was performed in triplicate

Tab. 3 Experimental design and responses of Placket-Burman study

	Whey	$(NH_4)_2SO_4$ $[g.l^{-1}]$	PO_4^{2-} $[g.l^{-1}]$	$MgSO_4$ $[g.l^{-1}]$	Yeast Extract $[g.l^{-1}]$	Biomass $[g.l^{-1}]$	PHB [%]	PHB $[g.l^{-1}]$
1	Not-Diluted	1.0	5.0	0.04	0.10	0.90	2.06	0.02
2	Not-Diluted	5.0	1.0	0.20	0.10	0.76	3.20	0.02
3	Diluted	5.0	5.0	0.04	1.00	2.56	16.26	0.42
4	Not-Diluted	1.0	5.0	0.20	0.10	0.92	1.90	0.02
5	Not-Diluted	5.0	1.0	0.20	1.00	0.77	5.08	0.04
6	Not-Diluted	5.0	5.0	0.04	1.00	0.75	1.95	0.01
7	Diluted	5.0	5.0	0.20	0.10	2.40	27.36	0.66
8	Diluted	1.0	5.0	0.20	1.00	2.02	12.72	0.26
9	Diluted	1.0	1.0	0.20	1.00	1.95	13.51	0.26
10	Not-Diluted	1.0	1.0	0.04	1.00	1.01	2.17	0.02
11	Diluted	5.0	1.0	0.04	0.10	2.47	35.48	0.87
12	Diluted	1.0	1.0	0.04	0.10	2.99	35.49	1.06

Tab. 4 The results of data analysis (t-values and P values) for the effect of medium component on growth and PHB production

	Biomass [g.l^{-1}]		**PHB [%]**		**PHB [g.l^{-1}]**	
	t value	P value	t value	P value	t value	P value
Whey concetration	-11.46	0.000	-7.53	0.000	-6.40	0.001
$(NH_4)_2SO_4$	-0.10	0.923	1.30	0.241	0.73	0.493
PO_4^{2-}	-0.48	0.647	-1.98	0.095	-1.70	0.139
$MgSO_4$	-2.31	0.051	-1.79	0.123	-2.17	0.073
Yeast extract	-1.69	0.142	-3.26	0.017	-3.09	0.021

t value is statistically significant only if P value < 0.05

Tab. 5 PHB and biomass yields after stress factor application at the 25th hour of cultivation

	Biomass [g.l^{-1}]	**PHB [g.l^{-1}]**	**PHB [%]**
Control	2.82 ± 0.11	1.05 ± 0.05	37.23
EtOH 0.5%	2.79 ± 0.08	1.43 ± 0.07	51.23
EtOH 1 %	2.87 ± 0.08	1.48 ± 0.08	51.57
EtOH 1.5 %	2.68 ± 0.03	1.22 ± 0.12	45.52
H2O2 1 mM	2.62 ± 0.12	1.18 ± 0.16	45.04
H2O2 3 mM	2.75 ± 0.06	1.09 ± 0.03	39.61
H2O2 5 mM	2.77 ± 0.09	1.15 ± 0.05	41.53

Results are in form average ± standard deviation, each cultivation was performed in triplicate and analyzed in triplicate as well.

Supplement VI

Production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from waste rapeseed oil using propanol as a precursor of 3-hydroxyvalerate

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Keywords *Polyhydroxyalkanoates, Waste edible oils, Cupriavidus necator, Stress conditions, Propanol*

Abstract

In this study, waste rapeseed oil was observed to be promising carbon substrate for polyhydroxyalkanoates (PHA) production employing *Cupriavidus necator* H16. In addition, the application of propanol significantly enhanced both PHA and biomass formation, and also resulted in incorporation of 3-hydroxyvalerate units into PHA structure, which improves mechanical properties of material. Thus, propanol can be used as an effective precursor of 3-hydroxyvalarete for production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer. In fed-batch mode we gained high biomass and PHA yields - 138 and 105 g l^{-1}, respectively, yield coefficient and volumetric productivity yield reached were 0.83 g-PHA per g-oil and 1.46 g l^{-1} h^{-1}, respectively. During cultivation, propanol concentration was maintained at 1 % which resulted in 8 % content of 3-hydroxyvalerate in PHA.

Introduction

Polyhydroxyalkanoates (PHA) are polyesters of hydroxyacids, which are accumulated in bacterial cells in form of intracellular granules. Bacteria use PHA as carbon, energy and reducing power storage materials. Due to their mechanical properties, PHA have attracted much attention as biodegradable alternative to traditional petrochemical plastics (Kessler and Wilholt 1999). The member of PHA family, copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(HB-*co*-HV)] is one of the best characterized PHA copolymers because of its high commercial potential. Incorporation of 3-hydroxyvalerate (3-HV) units into PHA structure reduces the hardness, crystallinity and melting point of material as compared to hopolymer of 3-hydroxybutyrate (3-HB), poly(3-hydroxybutyrate) (PHB). Therefore, copolymer P(HB-*co*-HV) possesses better mechanical properties and processability (Du et al. 2001).

In spite of many satisfactory properties of PHA, their commercial applications have been limited by their high price. One approach to reduce the cost of PHA production is to use inexpensive carbon source for their production such as plant edible oils. In contrast to 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 (Kahar et al. 2003).

Waste edible oils exhausted from food industry and food service industry are recovered as industrial wastes. They can be converted into feed for animals, fatty acids, soap or bio-diesel, nevertheless, waste oils are largely destroyed in incinerators or lost in the environment, which causes problems with waste management or water pollution (Taniguchi et al. 2003). On the other side, problematic waste oils could be used as cheap substrates for microbial production of highly valuable environmentally-friendly materials such as PHA.

This report describes production of PHB and P(HB-*co*-HV) from waste rapeseed oil employing *Cupriavidus necator* H16. In our previous work we reported that exposition of *C. necator* to controlled stress conditions caused by ethanol or hydrogen peroxide enhances PHA accumulation in cells (Obruca et al. 2010a, Obruca et al. 2010b). Therefore, we decided to apply different alcohols as stress factors enhancing PHA yields. Finally, we performed fed-batch cultivation of *C. necator* in laboratory fermentor to test strategy of PHA production from waste oils under controlled stress conditions.

Material and Methods

Microorganism

Cupriavidus necator H16 (CCM 3726) purchased from Czech Collection of Microorganisms, Brno, Czech Republic, was used in all experiments.

Culture media

Nutrient broth (NB) medium was used for inoculums development. NB consisted of 10 g pepton, 10 g beef extract, 5 g NaCl in 1 l of distilled water. Mineral salt (MS) medium was used for all production experiments. MS medium contained 3.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 11.1 g $Na_2HPO_4.12\ H_2O$, 0.2 g $MgSO_4$, 1 ml of microelement solution and 1 l of distilled water. The microelement solution was composed of 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 0.156 g $CuSO_4.5\ H_2O$, 0.119 g $CoCl_2$, 0.118 g $NiCl_2$, 0.062 g $CrCl_2$ in 1 l of 0.1 M HCl. Plant edible oils (20 g in 1 l of medium) were used as carbon sources for cultivations. Oils, salt solution and microelement solution were sterilised separately at 121°C for 25 minutes and then aseptically reconstituted at room temperature prior to inoculation. The pH was adjusted to 7.0 using 2 M NaOH /HCl. Pure oils were purchased in supermarkets, waste frying rapeseed oils were obtained from Universtity canteen of Faculty of Chemistry, Brno University of Technology and from Fried chips Manufactory in Straznice. Waste frying sunflower oils were provided by Restaurant in Valasské Mezirici and randomly chosen household.

Cultivation in flasks

NB medium was used for inoculum development. The microorganism was cultivated at agitation speed of 200 rpm, 30°C for 24 hours. For production cultivation, 100 ml of MS medium with particular oil (20 g l^{-1}) was taken into a 300 ml Erlenmeyer flask and inoculated with 5 ml of inoculum. The flasks were kept under constant shaking (200 rpm, 30°C).

Fed-batch cultivation in 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 grown on the NB medium. 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.

Analytical procedures

To measure biomass concentration (expressed as cell dry weight) and PHA content in cells, withdrawn samples (10 ml) were centrifuged (8000 rpm, 5 min) and harvested cells were washed with 5 % (vol/vol) Triton X and distilled water, respectively. Afterwards, biomass and PHA concentrations were analyzed as reported previously (Obruca et al. 2010b). Residual biomass was defined as biomass minus PHA concentration.

During the fed-batch cultivation, concentration of oil in medium was estimated according to Kahar et al. (2004), concentration of $(NH_4)_2SO_4$ was analyzed spectrophotometrically ($A_{436\,nm}$) using Nessler reagent. Propanol concentration was measured as amount of NADH (A_{340nm}) formed after 5 min treatment of sample (100 μl of cultivation medium diluted to 1 ml with 100 mM phosphate buffer pH 7.0) with 30 units of alcohol dehydrogenase (EC 1.1.1.1, Sigma A-7011).

RESULTS AND DISCUSSION

PHB production from various oils

To investigate the ability of *Cupriavidus necator* H16 to utilize plant oils and produce PHA, we performed cultivation of this bacterial strain on selected oils of various origins (Tab. 1). Bacterial culture grew well on all tested plant oils while produced homopolymer PHB. Surprisingly, the highest yields were observed on waste oils from various sources which had been used for frying. During the frying process the oil undergo few changes – fatty acid composition is slightly changed, the concentration of free fatty acids is enhanced and also some components of fried food such as proteins, lipids or carbohydrates migrate to oil (Matthäus 2007). It is likely that

these changes were beneficial for bacterial growth and PHB accumulation. Thus, waste oils seem to be very promising feedstock for PHA production, although only few authors reported PHA production on these cheap substrates. Taniguchi et al. (2003) reported PHA production from waste sesame oil employing *C. necator*, achieved yields were 4.6 g l^{-1} of PHB. Chan et al. (2006) studied 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. Also Vidal-Mas et al. (2001) cultivated *Pseudomonas aeruginosa* on waste frying oil and reached PHA yields 3 g l^{-1}. Nevertheless, in this work we obtained PHB yields which were significantly higher than those reported in literature. The highest PHA yields were produced on waste rapeseed oil coming from university canteen and, therefore, this oil was used as carbon substrate in further cultivations.

Introduction of stress conditions by alcohols

In our previous work we observed that exposition of *C. necator* to ethanol significantly enhanced PHB accumulation in cells Briefly, ethanol was metabolized via oxidation while reduced coenzymes NAD(P)H were formed and final product of ethanol metabolization was acetyl-CoA , the key substrate of PHB biosynthesis. Reduced coenzymes inhibited Krebs cycle which supported flux of acetyl-CoA into PHB biosynthetic pathway, reduced coenzyme NADPH is also necessary substrate for the second enzyme of PHB biosythetic pathway - acetoacetyl-CoA reductase. Moreover, as the consequence of Krebs cycle inhibition, less free CoA, which inhibits the first step of PHB biosynthesis catalyzed by β-ketothiolase, was formed (Obruca et al. 2010b).

Because we expected that also application of other alcohols may have caused the same response of culture resulting in enhanced PHA yields, we applied methanol (MetOH), ethanol (EtOH) and propanol (PrOH) into waste oil cultivation media at various times of cultivation (Tab. 2).

In terms of biomass as well as PHA yields, the most efficient was addition of alcohols at the 24th hour of cultivation. In this case application of alcohols did not only enhanced PHA accumulation in cells in comparison with control culture, but, surprisingly, it also increased bacterial growth while the more non-polar alcohol, the more pronounced growth was observed. The explanation of such an effect could be that alcohols supported solubilization of triacylglerols, which made them more susceptible to 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.

Especially EtOH and PrOH significantly enhanced PHA production. In addition, metabolization PrOH resulted in incorporation of 3-hydroxyvalerate into PHA structure, which significantly improves mechanical properties of produced material. For instance, incorporation of 10 % of 3-hydroxyvalerate increases elongation to break from 3 % (homopolymer PHB) to 20 % (copolymer P(HB-co-HV)) (Kessler and Wilholt 1999). According to our knowledge, the use of PrOH as a precursor of 3-hydroxyvalerate in P(HB-co-HV) has not been reported so far. In comparison with other commonly used precursors of 3-hydroxyvalerate, such as propionate or valerate, PrOH has also other advantages – it significantly enhances PHA yields and supports the growth of bacterial culture on oils as carbon substrate and, moreover, it is much cheaper. In Tab 3, there is comparison of PrOH with other commonly used precursors of 3-HV.

We assume that the way of propanol metabolization in *C. neactor* is the same as that of ethanol. Thus, 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 – by inhibition of the Krebs cycle followed by increased flux of acetyl-CoA into PHB biosynthetic pathway and simultaneous activation of acetoacetyl-CoA reductase. 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* (Slater et al. 1998). Following NADPH dependent stereospecific reduction of 3-ketovaleryl-CoA could be catalyzed by acetoacetyl-CoA. The final step of copolymer synthesis is catalyzed by PHB synthase which is, thanks to its low substrate specificity, able to built 3-hydroxyvalerate units into PHA structure (Fig. 1).

We tested various concentration of PrOH in order to find the most efficient one. The highest support of growth and PHA production was observed for 1 % (vol/vol) PrOH, on the contrary the highest content of 3-HV in PHA was obtained when 0.75 % (vol/vol) PrOH was applied (Tab. 4). Furthermore, controlled application of stress factor not only enhanced total PHB yields, but also increased PHB content in cells. This strategy would reduce costs of PHB recovery, because PHB content in cells strongly affects the efficiency and the cost of down-stream processing (Lee and Choi 1999).

PHA production in fed-batch mode

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 (Fig. 2).

After 72 hours of cultivation we gained high cell density (cell dry weight was 138.46 g l^{-1} at the 72^{th} hour of cultivation), moreover, cell contained 76 % of PHA with 8 % content of 3-HV. 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. According to our knowledge, the yields obtained in this work belong among the highest reported in literature for PHA production from oils. Kahar et al. (2004) performed fed-batch cultivation of *C. necator* on soybean oil, yield coefficient and volumetric productivity yield were 0.76 g-PHA per g-oil and 1.0 g l^{-1} h^{-1}, respectively. Thus, we gained better yields and, moreover, it should be taken into account that we used cheap waste oil instead of pure oil.

Conclusion

In this paper we show that utilization of waste edible oils could be a promising strategy facilitating economically feasible process of PHA production. Waste oils are lower in prize as compared to other pure substrates and, moreover, problematic waste would be turned into high value product. In fed-batch mode of cultivation we gained high yield coefficient and volumetric productivity yield 0.83 g-PHA per g-oil and 1.46 g l^{-1} h^{-1}, respectively. Furthermore, we suggest propanol as a novel precursor of 3-hydroxyvalerate for P(HB-*co*-HV) production. Besides the fact that propanol is built into PHA structure, which improves mechanical properties of material, it also enhances PHA yields and support bacterial growth on oils as substrates.

Ackonowledgements This work was supported by projects MSM 0021630501 and CZ.1.05/2.1.00/01.0012 of the Czech Ministry of Education.

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Tables

Table 1 Growth and PHB production of *C. necator* H16 on various oils as carbon sources[a]

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

[a] Cultivation conditions: MS medium, oil 20 g l^{-1}, Erlenmayer flasks, 30° C, 200 rpm, samples were withdrawn at the 72nd hour of cultivation.

Results in form: mean ± standard deviation, each cultivation was performed in triplicate and analyzed in triplicate as well.

Table 2 Biomass and PHA production after alcohols application[a].

	Time of application	Biomass [g l^{-1}]	PHA [g l^{-1}]	PHA [% w/w]	3-HB[b] [%]	3-HV[b] [%]
MetOH 1%	**24**	12.09 ± 0.77	8.32 ± 0.19	68.85	100	0
	48	10.48 ± 0.44	5.18 ± 0.46	49.45	100	0
	60	10.30 ± 0.45	6.67 ± 0.14	64.74	100	0
EtOH 1%	**24**	13.03 ± 0.48	10.39 ± 0.82	79.70	100	0
	48	12.25 ± 0.73	8.21 ± 0.23	67.03	100	0
	60	10.13 ± 1.28	5.44 ± 0.02	53.74	100	0
PrOH 1%	**24**	14.68 ± 0.33	11.71 ± 0.67	79.74	91	9
	48	12.74 ± 0.93	7.78 ± 0.74	61.10	97	3
	60	11.34 ± 0.16	7.12 ± 0.37	62.76	97	3
Control		11.17 ± 0.60	5.92 ± 0.39	53.17	100	0

[a] Cultivation conditions: MS medium, oil 20 g l^{-1}, Erlenmayer flasks, 30° C, 200 rpm, samples were withdrawn at 84th hour of cultivation. Alcohol concentration in medium was set to 1 % (vol/vol)

[b] Molar content (%) of particular monomer in polymer

Results in form: mean ± standard deviation, each cultivation was performed in triplicate and analyzed in triplicate as well.

Table 3 Production of P(HB-co-HV) on waste rapeseed oil using selected precursors of 3-HV[a].

Precursor	Biomass [g l^{-1}]	PHA [g l^{-1}]	PHA [% w/w]	3-HB[b] [%]	3-HV[b] [%]
PrOH 1 %	14.68 ± 0.33	11.71 ± 0.67	79.74	91	9
Propionate 5 g l^{-1}	14.08 ± 0.34	7.30 ± 0.08	51.81	87	13
Valerate 5 g l^{-1}	12.13 ± 0.47	6.45 ± 0.19	53.18	82	18
Control	11.17 ± 0.60	5.92 ± 0.39	53.17	100	0

[a] Cultivation conditions: MS medium, oil 20 g l^{-1}, Erlenmayer flasks, 30° C, 200 rpm, samples were withdrawn at 84th hour of cultivation. Precursors were applied at the 24th hour of cultivation. PrOH concentration in medium was set to 1 % (vol/vol) propionate and valerate concentration.

[b] Molar content (%) of particular monomer in polymer.

Results in form: mean ± standard deviation; each cultivation was performed in triplicate and analyzed in triplicate as well.

Table 4 Optimization of PrOH concentration[a] .

PrOH [% vol/vol]	Biomass [g.l^{-1}]	PHA [g.l^{-1}]	PHA [% w/w]	3-HB [%]	3-HV [%]
0.50	12.25 ± 0.65	7.75 ± 0.05	63.25	94	6
0.75	12.93 ± 0.35	9.09 ± 0.96	70.29	86	14
1.00	13.71 ± 0.18	10.84 ± 0.30	79.07	89	11
1.25	12.95 ± 0.14	8.74 ± 0.47	67.45	90	10
1.50	12.13 ± 0.21	8.33 ± 0.72	68.67	91	9
Control	11.16 ± 0.68	6.44 ± 0.59	57.69	100	0

[a] Cultivation conditions: MS medium, oil 20 g l^{-1}, Erlenmayer flasks, 30° C, 200 rpm, samples were withdrawn at 84th hour of cultivation. PrOH was applied at the 24th hour of cultivation.

[b] Molar content (%) of particular monomer in polymer.

Results in form: mean ± standard deviation, each cultivation was performed in triplicate and analyzed in triplicate as well.

Figures:

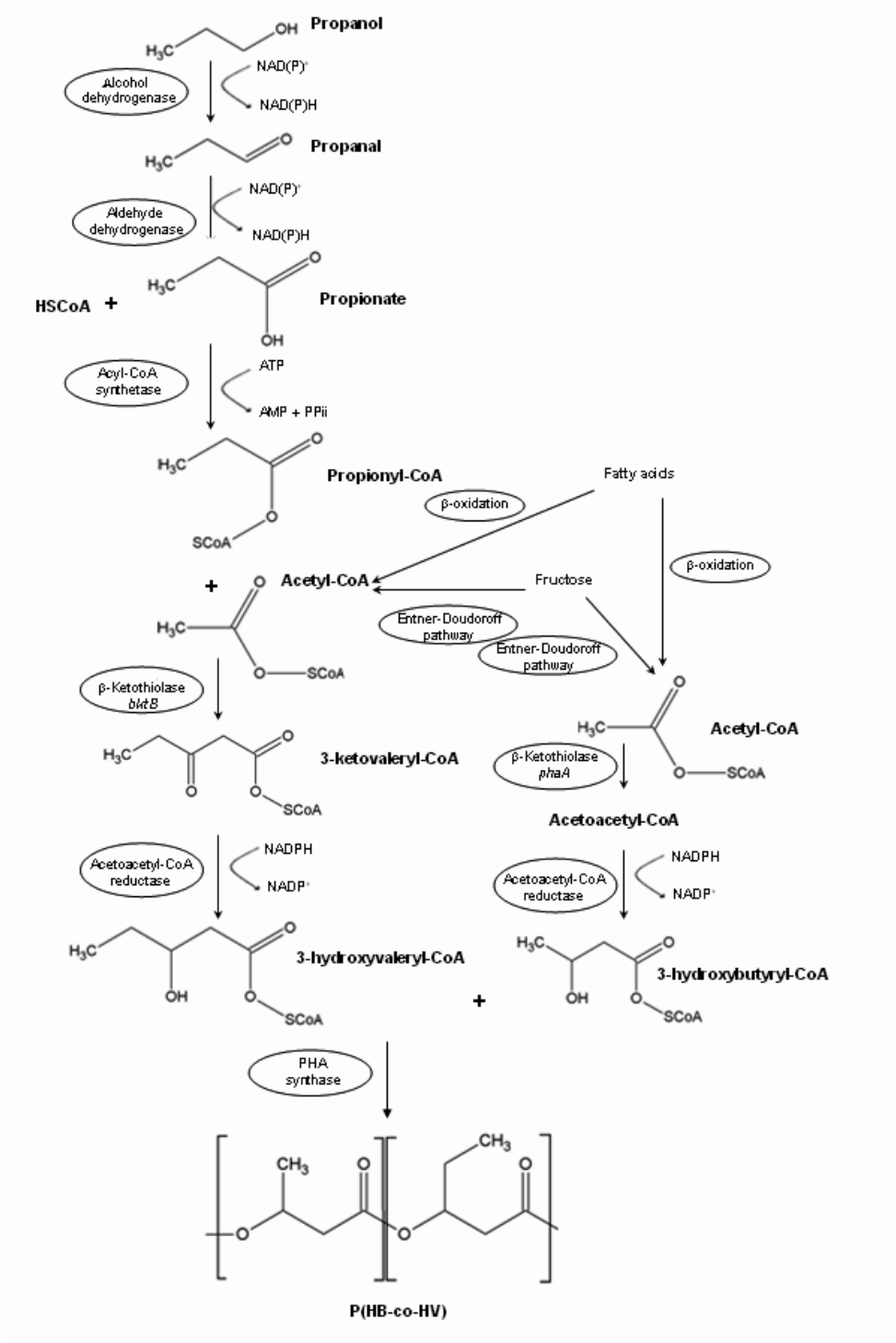

Fig. 1 Proposed mechanism of metabolization of propanol by *C. necator* H16 and its incorporation into P(HB-co-HV) copolymer structure.

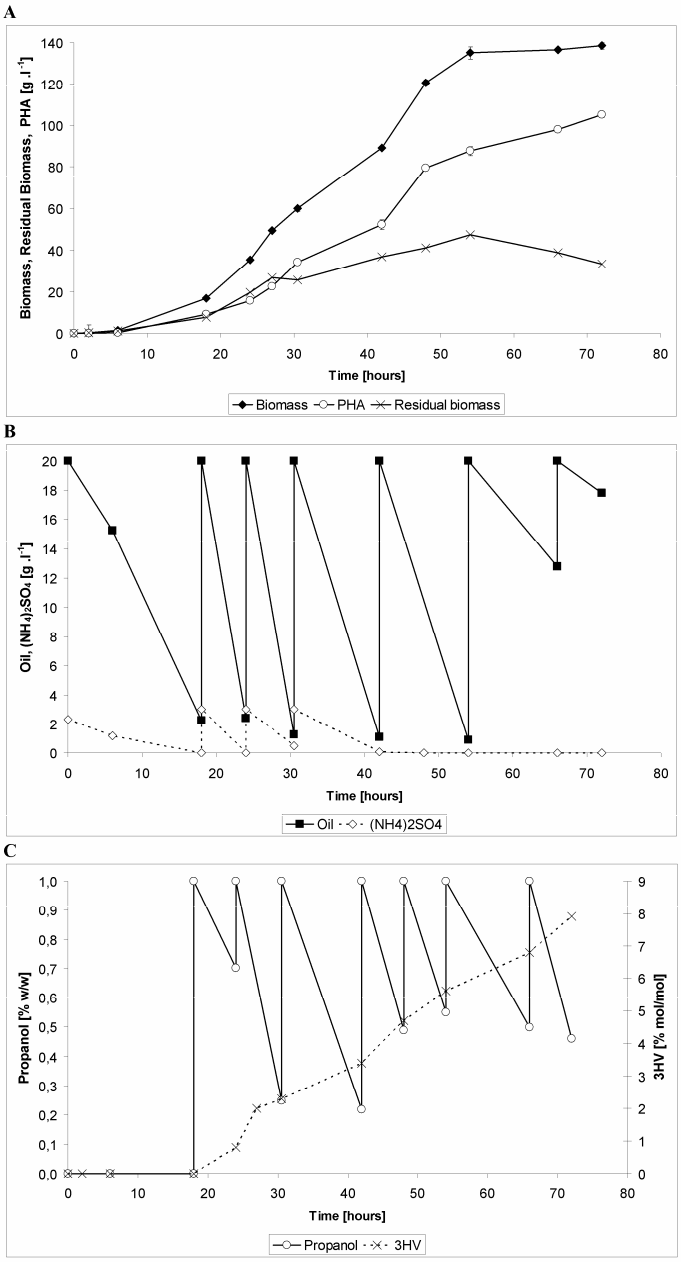

Fig. 2 PHA production form waste rapeseed oil in fed-batch mode. PrOH was applied at the 18th hour of cultivation and after that its concentration was maintained at 1 % (vol/vol), nitrogen source ($(NH_4)_2SO_4$) level was maintained at 3 g l^{-1} until the 30th hour of cultivation when nitrogen feeding was stopped to induce nitrogen limitation, concentration of carbon source – waste rapeseed oil was kept at 20 g l^{-1}. **A** pictures biomass, PHA and residual biomass concentrations. **B** shows ammonium sulfate and oil concentration. **C** describes PrOH concentration and 3-HV content in PHA.

Supplement VII

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L06 BIODEGRADATION OF MODIFIED POLYURETHANE FOAMS

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Introduction

Polyurethanes (PUR) are unique family of polymeric materials that is able to pose wide range of properties to suit many requirements. Some of the applications of this versatile polymer include foams, elastomers, paints, fiber, adhesives and sealants[1].

Biodegradation is natural process of material decomposition which is based on the fact that synthetic polymeric materials could serve as carbon and energy sources for many microorganisms including bacteria, moulds and yeasts. Despite its xenobiotic origin PUR was found to be susceptible to biodegradation by naturally occurring microorganisms[2,3].

Biodegradability of material can be influenced by many factors. Very important are structure properties such as molecular orientation, crystallinity, cross-linking and chemical groups presented in the molecular chain. For example, increase of molecular weight or crystallinity usually leads to increasing material durability. Thus, variations in the degradation patterns of different PUR are attributed to many properties of PUR such as topology and chemical composition[3,4].

Other important factors which influence process of biodegradation in nature are presence of susceptible microbial population and environmental conditions. PUR biodegradation activity was observed for many microorganisms including bacteria (e.g. *Corynebacterium sp.*, *Pseudomonas aureginosa*, *Comamonas acidovorans*, *Pseudomonas chlororaphis*, *Pseudomonas flourscens*, *Acinetobacter calcoaceticus* and *Arthrobacter globiformis*) and fungi (*Culvularia senegalensis*, *Fusarium solani*, *Aerobasidium pollulans*, *Cladosporium sp.*, *Cheatomium globosum* and *Aspergillus terreus*)[3].

In this work, biodegradability of modified PUR foams was tested using single bacterial strain (*Arthrobacter globiformis*), mixed bacterial culture (*Thermophillus* sp.) and selected moulds strains (*Fusarium solani*, *Alternaria alternata*). In a complex comparative study standard biodegradability tests in laboratory conditions, in model composting conditions and also in different natural environments were used.

Experimental

PUR foams were modified by following biopolymer components: 10 % of acetyl- (AC), hydroxyethyl- (HEC) cellulose, 10; 20; 30 % of carboxymethyl cellulose (CMC), 10% acetylated starch (AS), 10 % glutein (PB). Modifying agents partially replaced polyether polyol. Reference sample without modification agent was tested too.

Flask biodegradation tests were carried out in 50 ml of defined media under permanent shaking (120 rpm). One sample of PUR was represented by 0.25 g of PUR. Growth characteristics and chemical oxygen demand were determined in regular intervals. At the end of cultivation (about 300 hours) PUR mass decreases were measured gravimetrically and PUR surface changes were analysed microscopically. PUR foams were tested in complete medium as well as the only carbon and/or nitrogen source. Single strain *Arthrobacter globiformis* CCM 193 was cultivated at 25 °C in Nutrient broth NB1 and in Minimal inorganic medium containing glucose as carbon source and $(NH_4)_2SO_4$ as nitrogen source. Mixed aerobic thermophillic culture *Thermophillus sp.* was originally obtained from sludge of wastewater treatment in Bystřice pod Hostýnem. Cultivation was carried out at 60 °C on Minimal inorganic medium with minerals and vitamins. The mould strain *Fusarium solani* F-552 was cultivated on Minimal inorganic medium according *A. globiformis*. *Alternaria alternata* was identified during the study with *A. globiformis* as a common air contaminant. *A. alternata* was cultivated on Minimal inorganic medium too.

Model composting experiments were carried out under controlled conditions in presence of culture *Arthorbacter globiformis*. Model experiment under composting conditions was performed according to ref.[1]. PUR mass changes and surface patterns were analysed at the end of the experiment.

Test of biodegradability in natural condition. PUR foams were exposed to three different natural envitoment for 50 days. After the end of exposition, mass decreases of foams were analysed and a total bacterial microflora (quantified as colony forming units) in each environment was determined.

Results

All flask biodegradation tests proved that crucial for the course of PUR degradation are type and concentration of modification agent (see Fig. 1. and Fig. 2.).

Generally, PUR mass decreases during flask biodegradation tests were practically identical in presence and/or in

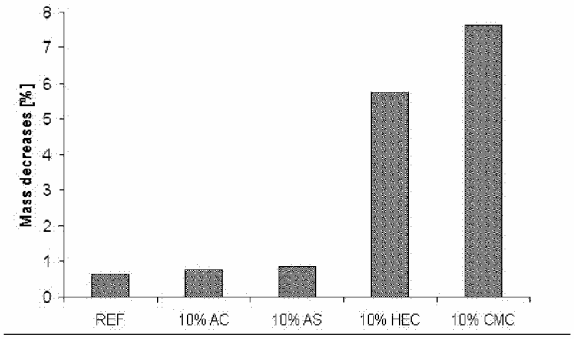

Fig. 1. **Mass decreases of PUR samples modified by different agents (flask biodegradation test)**

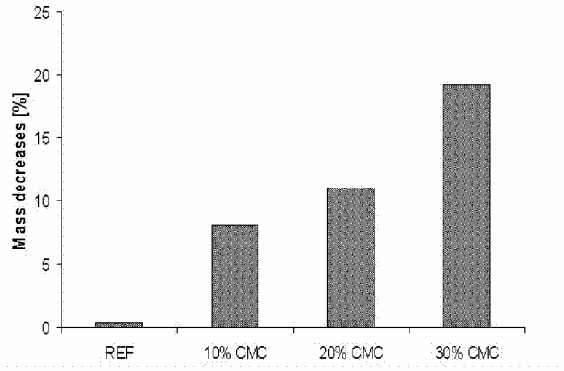

Fig. 2. **Mass decreases of PUR samples modified by different concentration of the same modification agent (flask biodegradation test)**

absence of bacterial culture. On the other side, the presence of PUR foam influenced the growth of culture. These two effects could be explained as two-step biodegradation process. First, abiotic destruction of foams occurred, which was followed by the second step – utilization of degradation products by the bacterial culture.

The growth of culture in presence of individual PUR samples was dependent on type of modifying agent. The highest growth of both cultures was observed in PUR modified by CMC.

Experiments with PUR foams used as the only carbon or nitrogen source for thermophillic culture showed that all modified PUR foams could serve as the only nitrogen source. Surprisingly, PUR modified by CMC and AS were better nitrogen sources than inorganic salt. On the other side, only PUR foam modified by AS supported the growth of culture when no other carbon source was present.

In all experiments with fungi, a typical distribution of biomass between PUR surface and medium was observed. *Alternaria alternata* utilized all PUR as the only carbon source. Foams modified by CMC and by AS strongly supported absorption of biomass on PUR surface. Under nitrogen limitation practically the whole biomass was adsorbed on foam surface.

Fusarium solani was able to use modified PUR as the only carbon as well as nitrogen source. This strain also strongly preferred growth on surface of PUR foams, mainly under both carbon and nitrogen limitation.

In model composting conditions, the highest loss of biomass was observed in PUR modified by 10 % of CMC.

All tested natural environments disposed PUR biodegradation activity. In environments different total bacterial activity was found. Individual environments exhibited also different degree of exposition of foams to natural conditions (weather, water, etc.) which could be considered to be important abiotic factors.

It seems that in early stages of long-term natural degradation microbial and abiotic factors act simultaneously, but abiotic processes are more efficient. The highest degradability degree was shown repeatly in PUR modified by CMC and AS.

Conclusions

Tested PUR foams could be biodegraded by microorganisms used in this study. Degree of degradation is strongly dependent on type and concetration of used modifying agents. The highest tendency to biodegradability by most of tested microorganisms was observed in PUR modified by CMC and AS. Mainly in early stages of degradation abiotic and microbial factors act simultaneously. It seems that microorganisms are able to utilize degradation fragments formed during abiotic decomposition.

The effect of mould sorption on PUR surface could be important for biotechnological application. Surface sorption degree depended on type of used modifying agent, mould strain and also on cultivation conditions.

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Supplement VIII

Bacterial biodegradation of modified polyurethane foams: comparison of single and mixed culture

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Abstract

Waste polyether polyol based polyurethane (PUR) foams present a serious problem for environment. In this work polyether polyol based PUR foams were modified by biopolymers (hydroxyethyl-, acetyl- and carboxymethyl cellulose and acetylated starch) which partially replaced polyether polyol. Biodegradability of newly prepared foams was tested using two different bacterial system – single culture of *Arthrobacter globiformis* and mixed aerobic thermophillic culture originally used for waste water treatment. It was observed that modification of foams supports bacterial growth and improves bioavailability of PUR foams to bacterial culture. However, biodegradation mechanism involves two steps. Abiotic degradation of foams is followed by utilization of degradation products by bacterial culture. Mixed culture seems to be more effective in utilization of abiotic degradation products as compared to single culture. This is probably due to relationships of individual strains in mixed culture which facilitated utilization of PUR foams fragments. Despite the fact that under conditions of short term incubations mass losses of PUR foams were relatively low, the mixed culture was able to use foam modified by acetylated starch and hydroxyethyl-cellulose as the sole carbon source and practically all tested foams as the sole nitrogen source. This is necessary presumption of successful biodegradation of resistant polyether polyol based PUR foams in environment by naturally presented mixed cultures. Therefore, modification of PUR foams by biopolymers seems to be promising strategy with potential to reduce solid PUR waste by environment friendly attitude.

Keywords:
Polyurethane foam; biodegradation; *Arthrobacter globiformis*; mixed thermophillic culture

1. Introduction

Polyurethanes (PUR) are interesting family of polymeric materials, derived from the condensation of polyisocyanates and polyols, which are able to possess wide range of properties to suit many requirements. Some of the applications of this versatile polymer include foams, elastomers, paints, fiber, adhesives and sealants (Young and Sung, 1998).

PUR materials in form of foams are widely used throughout the world for diverse applications, mainly in automotive and furniture industry. Therefore, enormous quantities of used PUR foams are disposed of in landfills and incinerators, which cause resource depletion and litter problems (Zheng et al., 2005). Biodegradation of waste PUR materials may provide innovative solution to these problems.

Biodegradability of polymer material is influenced by many factors. Very important factors are the material structure properties such as molecular orientation, crystallinity, cross-linking and chemical groups presented in the molecular chain. For example, an increase of molecular weight or crystallinity usually leads to increasement of material durability. Thus, variations in the degradation patterns of different PUR are attributed to the many properties of PUR such as topology and chemical composition (Howard, 2002).

Generally, the main factor influencing the biodegradability of PUR material is type of used polyol. PUR materials synthesized using polyester polyol are relatively easily degradable by microorganisms, PUR based on polyether polyols are more resistant to microbial attack (Nakajima-Kambe et al., 1999). Darby and Kaplan (1968) tested the biodegradability of various types of polyether PUR. They reported, that polyether PUR were scarcely susceptible to biodegradation. Jansen et al. (1991) reported that some kinds of polyether-based PUR were degraded by *Staphylococcus epidermis* KH11, but the degradation progressed very slowly. Generally, the relative resistance of polyether PUR to microbial degradation is considered to be due to its mechanism of degradation, which involves exo-type depolymerization, whereas the degradation of polyester PUR involves endo-type depolymerization (Kawai et al., 1985).

In spite of their poor biodegradability, most of PUR materials (including PUR foams) used at present are based on polyether polyols. Therefore, it is really needed to increase biodegradability of polyether-based polyurethanes. In our work we tested biodegradability of polyether-based PUR foams that were modified using selected biomaterials in order to increase their bioavailability and biodegradability. Moreover, the aim of this work was also to compare two bacterial systems. Firstly, single culture of *Arthrobacter globiformis* that was previously reported to be able to degradate PURs (Halim et al., 1996). Secondly, mixed culture of thermophillic bacteria containing mostly bacteria of the genes *Bacillus* and *Thermus,* which is originally used for waste water treatment and which disposes with high range of enzymatic activities.

2. Experimental

2.1 Tested polyurethane materials

The PUR foam materials tested in this work were synthesized using polyether polyol *Slovaprop G-48s polyol* (purchased from Gumotex), diisocyatnate *Toluene-2,4(2,6)-diisocyanate (80/20)* (TDI) (Gumotex) and catalyst *Dibutyltin dilaurate* (Ciba). Water was used as foaming agent. For modification of PUR foams, polyether polyol was partially (10%) replaced by following biopolymers: acetyl-cellulose (AC), hydroxyethyl-cellulose (HEC), carboxymethyl cellulose (CMC) and acetylated starch (AS). Non-modified reference sample (REF) was synthesized and tested as well.

The foams were prepared by a three-step reaction process. At first, the polyol polyether was mixed with the calculated amount of biopolymer in order to dissolve biopolymer in polyol. In the second step the pre-mixed catalyst (including water) was added and stirred. At the end, the TDI was added, stirred up to cream time and quickly poured into a mold. The foams were let to cure for about 48 hours in the fume hood in open air.

2.2 Microbial culture

2.2.1 Single strain: Arthrobacter globiformis CCM 193

Bacterial strain *Arthrobacter globiformis* CCM 193 was purchased from Czech Collection of Microorganisms. Culture was maintained on Nutrient Broth agar. For biodegradation experiments following mineral medium was used: 8 $g.l^{-1}$ glucose (carbon source), 5 $g.l^{-1}$ $(NH_4)_2SO_4$ (nitrogen source); 5 $g.l^{-1}$ NaCl; 1 $g.l^{-1}$ KH_2PO_4; 0.5 $g.l^{-1}$ $MgSO_4$. 7 H_2O, pH was adjusted to 7.

2.2.2 Mixed aerobic thermophillic culture

Mixed aerobic thermophillic culture was originally obtained from sludge of wastewater treatment in Bystrice pod Hostynem (Czech Republic). The cultivations were carried out in synthetic minimal medium: 8 $g.l^{-1}$ glucose (carbon source); 5 $g.l^{-1}$ $(NH_4)_2SO_4$ (nitrogen source); 2.44 $g.l^{-1}$ Na_2HPO_4; 1.52 $g.l^{-1}$ KH_2PO_4; 0.2 $g.l^{-1}$ $MgSO_4$. 7 H_2O; 0.05 $g.l^{-1}$ $CaCl_2$. 2 H_2O, 10 $ml.l^{-1}$ solution of trace elements (0.2 $g.l^{-1}$ $FeSO_4$. 7 H_2O; 0.01 $g.l^{-1}$ $ZnSO_4$; 0.003 $g.l^{-1}$ $MnCl_2$. 4 H_2O; 0.03 $g.l^{-1}$ H_3BO_3; 0.02 $g.l^{-1}$ $CoCl_2$. 6 H_2O; 0.001 $g.l^{-1}$ $CuCl_2$. 2 H_2O; 0.002 $g.l^{-1}$ $NiCl_2$. 6 H_2O; 0.003 $g.l^{-1}$ Na_2MoO_4. 2 H_2O); 2.5 $g.l^{-1}$ vitamin solution (0.01 $mg.l^{-1}$ p-Aminobenzoate; 0.02 $mg.l^{-1}$ Biotin; 0.2 $mg.l^{-1}$ Nicotine acid; 0.1 $mg.l^{-1}$ Thiamin; 0.05 $mg.l^{-1}$ Ca-Pantothenate; 0.5 $mg.l^{-1}$ Pyridoxamin). Temperature of cultivation was 60°C, pH was adjusted at 7 and flasks were shaken (120 rpm) during cultivations.

2.3 Biodegradation experiments

2.3.1 Biodegradation experiments in complete medium

A series of batch cultures was set up to screen PUR foams biodegradability by single as well as mixed bacterial culture. In a batch assays, 5 plugs (1 cm x 1 cm x 1 cm, cca 0.05 g each) were placed in 100 ml Erlenmeyer flask containing 50 ml of medium used for cultivation of *Arthrobacter globiformis* or mixed thermophillic bacterial culture. Flasks were kept at desired temperature (30° C *Arthrobacter globoformis*, 60° C *Thermophillus sp.*) under permanent shaking (120 rpm). For each of foams, four identical samples were used. Two of them were

inoculated by bacterial culture and two flasks were used as controls. Bacterial growth and chemical oxygen demand (COD) were monitored during the test. At the end of cultivation (about 300 hours) PUR weight losses were measured gravimetrically (as described bellow).

2.3.2 Test of PUR foams bioavailability as only carbon or nitrogen source

The PUR bioavailability assays were performed in the defined mineral medium where PUR served as sources of carbon or nitrogen for growth of bacterial cultures. Growth tests were performed under different conditions which are summarized below:

1. PUR as the sole source of carbon (alternative nitrogen source, ammonium sulphate, was added, glucose omitted).
2. PUR as the sole source of nitrogen (alternative carbon source, glucose, was added, ammonium sulphate omitted).
3. PUR as well as added sources of carbon (glucose) and nitrogen (ammonium sulphate) as nutrients for growth.
4. Only added nitrogen (ammonium sulphate) and carbon (glucose) sources were available (PUR was not present; positive control).
5. Nitrogen sources were not present, but alternative carbon (glucose) source was added (negative control for nitrogen limitation).
6. Carbon sources were not present, but alternative nitrogen (ammonium sulphate) source was added (negative control for carbon limitation).

The experiment was designed according to Urgun-Demirtas et. al. (2007).

2.3.3 Analysis of bacterial growth and chemical oxygen demand

Cell growth was monitored by measuring the absorbance of culture broth at 630 nm. The cells harvested by centrifugation (8 000 rpm, 10 min.) were dried (105°C) till constant weight was obtained. Cell biomass was determined using standard plot of relationship between $A_{630\ nm}$ and dry cell mass. Chemical oxygen demand (COD) was analyzed spectrophotometrically according to Thomas et al. (1997).

2.3.4 Analysis of PUR weight losses

PUR degradation was monitored by measuring the weight of PUR plugs before and after cultivation. The PUR plugs were taken out and washed with distilled water and ethanol to remove debris on the surface. Then, plugs were dried to constant weight overnight at 40° C and weighed. The weight loss percentage was calculated as

$$\%\text{Weight loss} = (m_o - m_t)/ m_o \,.\, 100\%$$

where m_0 is the initial weight of the plug, m_t is the final weight of the plug after experimentation.

2.3.5 Analysis of abiotic degradation products in medium

Proposed abiotic degradation products of biopolymers, reducing sugars and polysaccharides, were analyzed spectrophotometrically after 300 hours incubation of foams in

distilled waster without culture. Reducing sugars were determined using Somogyi-Nelson method and calibration was done using glucose (Deng & Tabatabai 1994). Polysaccharides were estimated according to Dubois et al. (1956) and calibrated using starch.

3. Results and discussion

3.1 Biodegradation in complete medium

The presence of PUR samples influenced the growth of both bacterial cultures as compared to control – culture without PUR sample. This effect was especially evident for mixed culture (see Fig 1. and Fig. 2). Generally, the influence of PUR on the growth of culture depended on the type of used modifying agent. As was assumed, modification of PUR foams by biopolymers partially improves bioavailability of PUR foams to bacterial culture which resulted in supported growth of culture in comparison with non-modified reference sample. Mainly PUR foam modified by CMC supported the growth of the mixed as well as the single culture. On the other hand, foam modified by HEC inhibited the growth of the cultures as compared to either reference sample or control culture. This effect may be caused by release of some toxic degradation products into cultivation media.

Mass losses of PUR foam plugs were relatively low probably due to the fact that the 300 hours incubation was most likely too short to obtain more significant mass losses. The obtained mass losses ranged from 0.62 to 7.68 %. However, these mass losses are similar to those of Urgun-Demirtas et al. (2007) who tested biodegradability of PUR foams under anaerobic conditions and Gautam at al. (2007) who used bacterial strain *Pseudomonas chlororaphis* for biodegradation of PUR foams. Both studies were carried out under comparable condition with this study and lasted similar time period. Moreover, mass losses of foams modified by CMC and HEC are significantly higher than observed in any studies mentioned above.

Mass losses of PUR plugs were independent of the fact whether a bacterial culture was presented or not (see Tab. 1). Therefore it can be assumed that the first step of PUR foam degradation probably involves mainly abiotic degradation of foams. The measure of mass losses strongly dependent on the type of used modification agent, which could influence polymer stability. The highest mass losses were observed in PUR foam modified by CMC that also strongly supported the growth of bacterial culture. Thus, it can be assumed that the first abiotic step of PUR foam degradation is subsequently followed by utilization of abiotic degradation products by bacterial culture. This mechanism of PUR biodegradation was previously proposed by Albertson et al. (1987).

In order to explain why modification of PUR foams by biopolymers influences the growth of bacterial culture, expected abiotic degradation products of used biopolymers (polysaccharides and reducing sugars) were analyzed after incubation of PUR foams in distilled water without bacterial culture (see Tab. 2). The results show that significant part (more than 50%) of degradation products of PUR foams (mainly those which were modified by CMC and HEC) is constituted by polysaccharides, which can be utilized by bacterial strains and supports their growth.

The fact, that the differences in the growth are more evident in mixed culture, indicates that mixed culture seems to be more efficacious in utilization of PUR abiotic degradation products. Moreover, mixed culture strongly eliminated values of COD in comparison with single culture (see Tab. 3). COD value expresses the amount of organic compounds in water. It can be assumed that the significant part of COD value consisted of abiotic degradation products of PUR foams. Higher potential of utilization of abiotic degradation product observed in mixed culture is probably caused by relationships of strains present in mixed culture which facilitated more efficient utilization of PUR foams fragments.

3.2 Test of PUR foams bioavailability as only carbon or nitrogen source

In order to test whether the PUR foams can be utilized by bacterial culture as the sole carbon or nitrogen source, test of PUR foams bioavailability was performed. *Arthrobacter globiformis* was able to use PUR foams neither as carbon nor as nitrogen source. Oppositely, mixed culture utilized some of tested PUR foams as the sole carbon as well as the sole nitrogen source (See Fig. 3 and Fig. 4). PUR foams modified by CMC and HEC supported the growth of bacterial culture as compared to negative control and therefore they can be expected to serve as the sole carbon source for mixed culture. Similarly, foams modified by CMC, AC as well as REF sample are utilizable nitrogen source for mixed culture. The fact, that mixed culture was able to use PUR foams as the sole nitrogen and carbon source whereas single culture was not, also proves significantly higher degradation potential of mixed culture.

4. Conclusions

In conclusion, we observed that modification of PUR foams by biopolymers supports bacterial growth and improves bioavailability of PUR for bacterial culture. According to our results, biodegradation mechanism of polyether PUR foams involves two following steps. Abiotic degradation is followed by utilization of degradation products by both bacterial cultures. Mixed culture seems to be significantly more effective in utilization of abiotic degradation products than single culture. This is probably caused by the relationships of individual strains in mixed culture which resulted in broader range of enzyme activities in medium that facilitated utilization of PUR foams fragments. Although under conditions of short term incubations mass losses of PUR foams were relatively low, the mixed culture was able to use foam modified by acetylated starch and hydroxyethyl-cellulose as the sole carbon sources and practically all foams as the sole nitrogen source. This is necessary presumption of successful biodegradation of resistant polyether polyol based PUR foams in environment by naturally presented mixed cultures. From this point of view, the modification of PUR foams by partial replacement of polyether polyol by biopolymers seems to be promising strategy for environmental reduction of amount of solid waste of PUR origin by biodegradation.

5. Acknowledgements

This work has been supported by project MSM 0021630501 of Czech Ministry of Education

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Figures

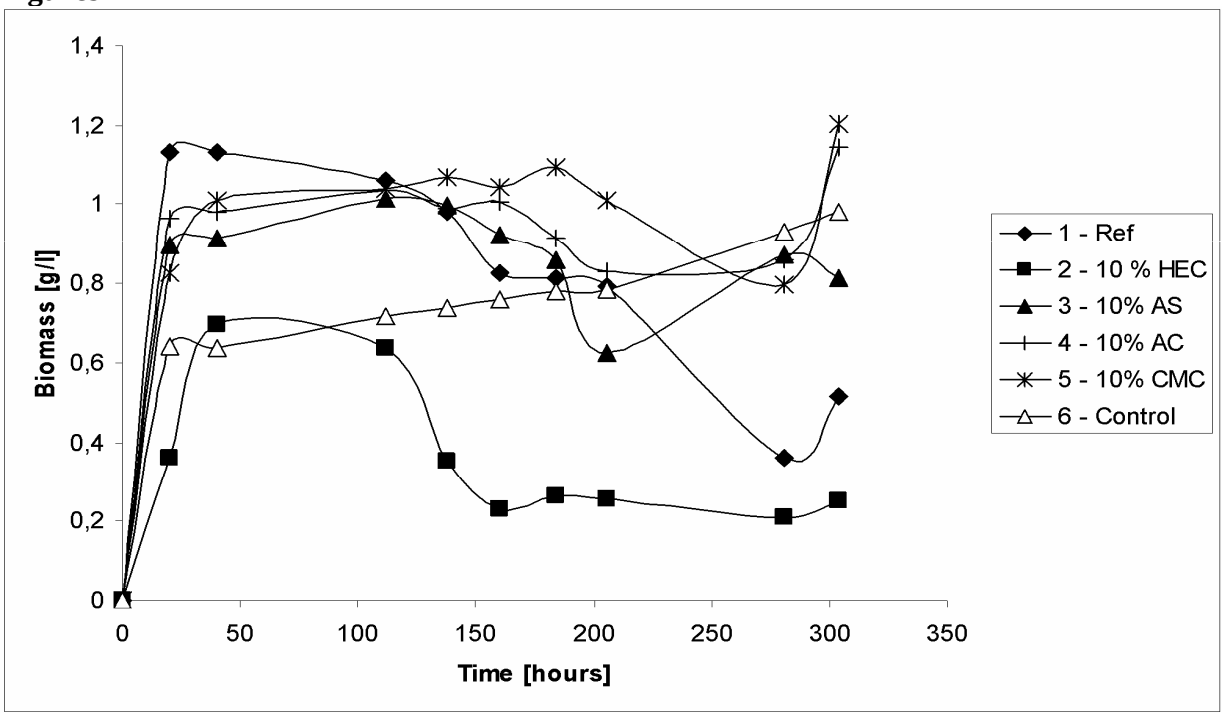

Fig 1: The growth of mixed culture in presence of PUR foams

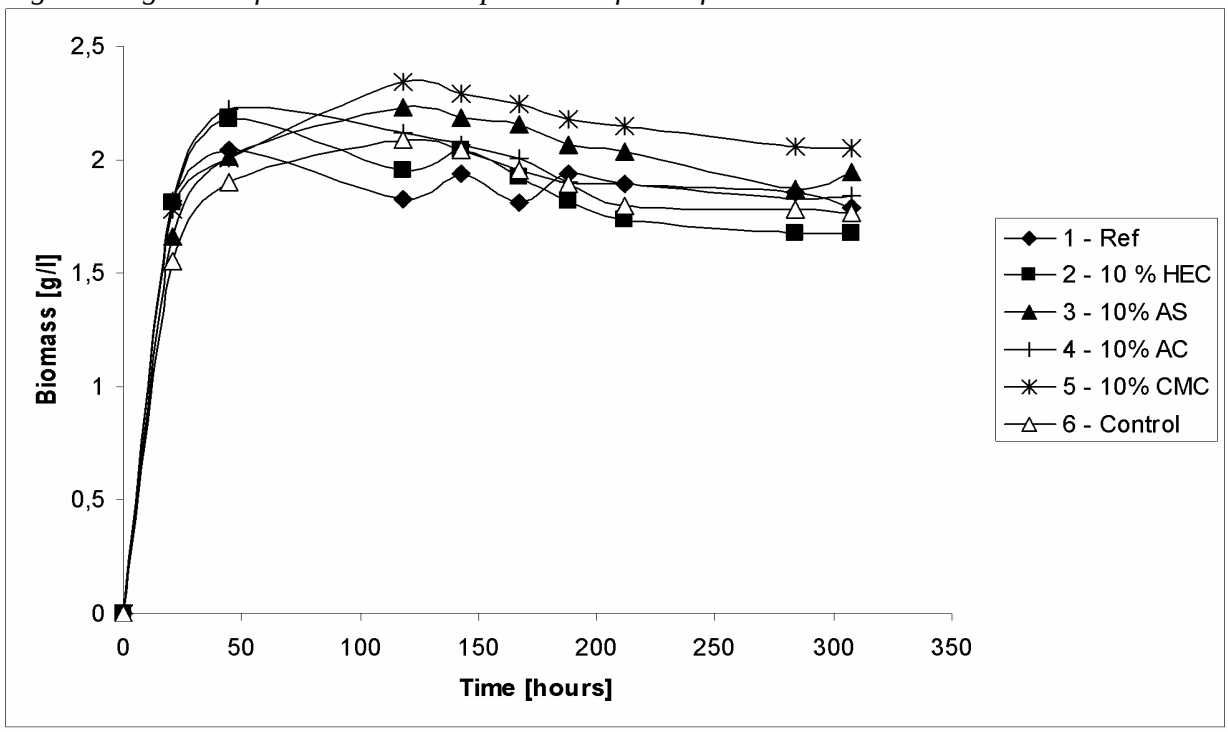

Fig 2: The growth of single culture Arthrobacter globiformis in presence of PUR foams

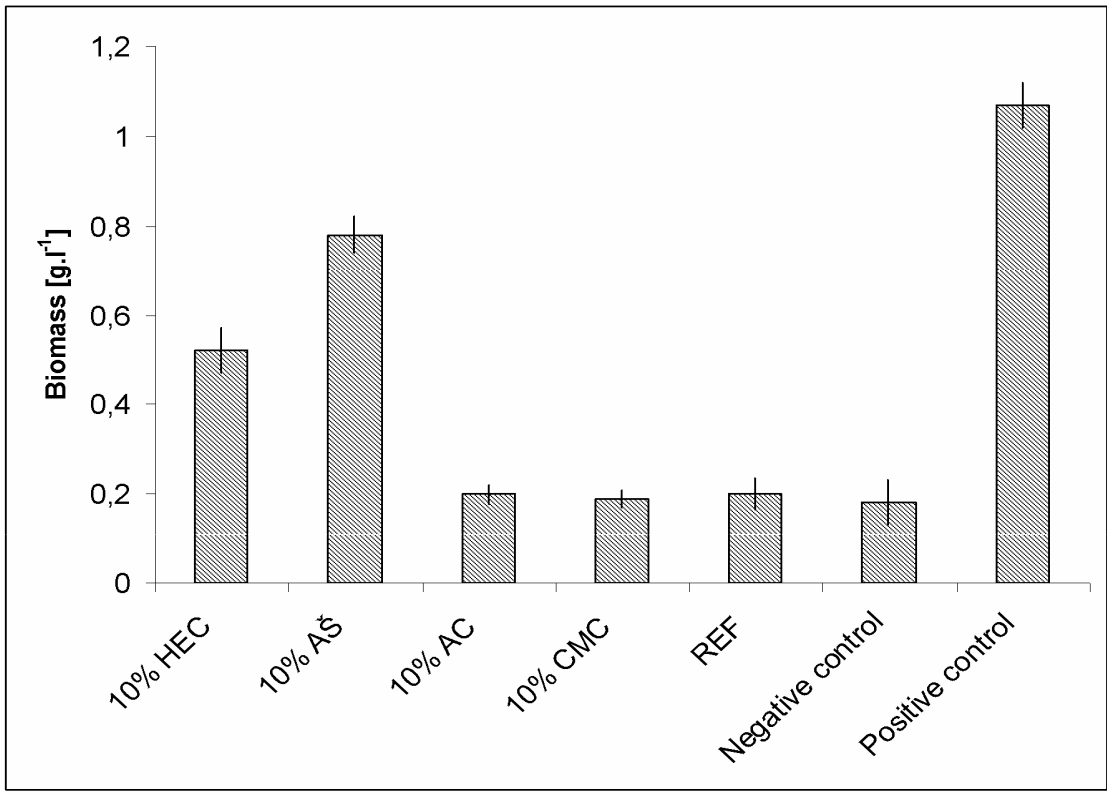

Fig. 3: The test of bioavailability of PUR foams as the only carbon source (negative control – glucose as well as PUR foam omitted, positive control – glucose added, PUR foam omitted)

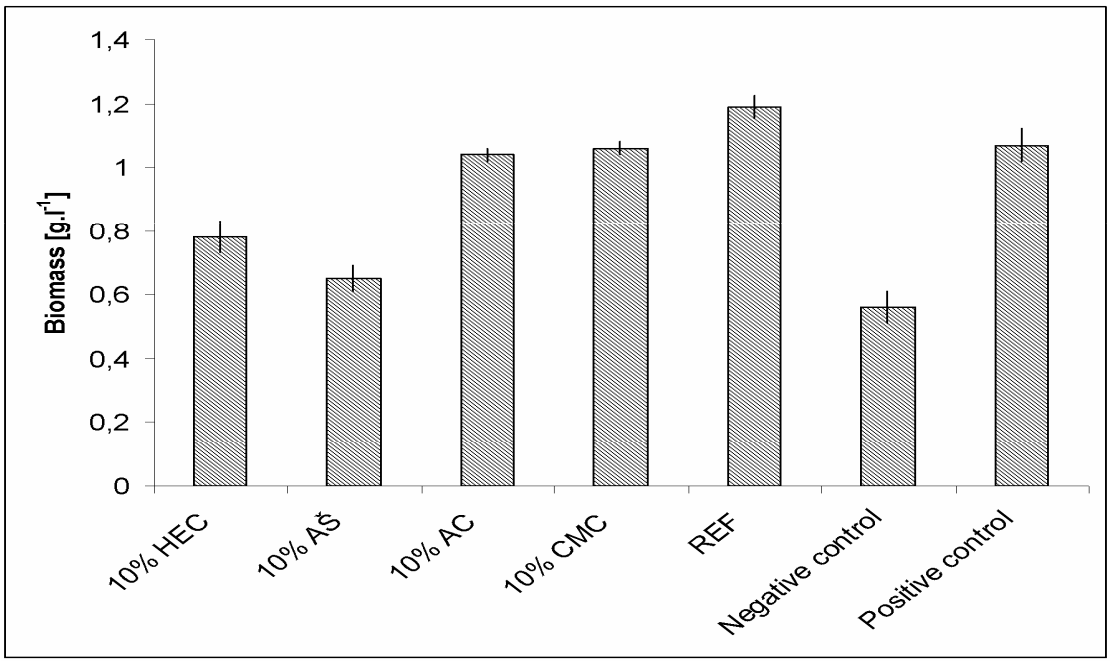

Fig. 4: The test of bioavailability of PUR foams as the only nitrogen source (negative control – ammonium sulphate as well as PUR foam omitted, positive control – ammonium suplhate added, PUR foam omitted

Table 1: PUR mass losses [%] during flask biodegradation tests

	Without culture	*Thermophillus sp.*	*Arthrobacter globiformis*
REF	0.91	0.93	1.15
10% AC	1.12	0.76	0.62
10% CMC	7.68	7.61	7.25
10% HEC	5.58	5.74	5.19
10% AS	1.29	0.84	1.08

Tab. 2: Analysis of abiotic degradation products of biopolymers in medium after incubation of PUR samples in water without bacterial culture

	Reducing sugars (mg per 50 ml of media)	Polysaccharides (mg per 50 ml of media)	Total mass losses of PUR foams (mg)
REF	*	*	2.8
10% AC	*	0.451 ± 0.08	6.1
10% CMC	0.25 ± 0.05	13.971 ± 0.43	21.2
10% HEC	1.34 ± 0.12	11.795 ± 0.23	20.1
10% AS	*	0.245 ± 0.09	3.4

Data are shown in form: mean ± standard deviation, * - not detected

Table 3: Elimination of COD [%] during flask biodegradation tests

	Thermophillus sp.	*Arthrobacter globiformis*
REF	38.14	35.82
10% AC	50.07	39.96
10% CMC	52.76	36.64
10% HEC	55.44	36.44
10% AS	41.58	38.51
Control	50.52	30.85

Supplement IX

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P09 COMPARISON OF BIODEGRADABILITY OF MODIFIED POLYURETHANE FOAMS AND POLYURETHANE ELASTOMERIC FILMS

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Introduction

Polyurethanes (PUR) are a widespread group of polymeric materials involved in many aspects of modern life. They are widely used in medical, automotive or industrial fields. PUR are very variable group of materials because they could be prepared in many forms ranging from flexible or rigid foams, solid elastomers, coating, adhesives and sealants[1].

Polymeric materials could serve as carbon (but also nitrogen and other atoms) and energy source for heterotrophic microorganism including bacteria, moulds and yeasts. This natural process of material decomposition is called biodegradation[2].

After the years of PUR production manufactures found PUR susceptible to biodegradation. Microbial degradation of PUR as well as other polymeric materials is dependent on many properties of the polymer such as molecular orientation, crystallinity, cross-linking and chemical groups present in the molecular chain. Very small variations in the chemical structure may result in large differences in the term of biodegradability[3].

This work was focused on the comparison of biodegradability of two different forms of the same PUR material - modified PUR foams and modified PUR elastomeric films. Several types of biopolymers were used as modyfying agents that should enhance biodegradability of both PUR forms. For laboratory biodegradtion tests mixed thermophillic culture *Thermophillus sp.* was used.

Experimental

PUR foams and elastomeric films were modified by 10 % of acetyl-cellulose (CA), 10 % carboxymethyl cellulose (CMC) and 10 % acetylated starch (AS). Modifying agents partially replaced polyether polyol. Both PUR foams and PUR elastomeric films were prepared from the identical raw materials. The only difference was that in film preparation blowing agent (water) was not used.

Biodegradation Tests

Biodegradation tests were performed with mixed thermophillic culture *Thermophillus sp.* Cultivations were carried out at 60 °C under permanent shaking in 100 ml of defined media. One sample of PUR was represented by 0.25 g of PUR. Growth characteristics, chemical oxygen demand and glucose concentration were determined in regular intervals. At the end of cultivation (about 100 hours) PUR mass decreases were measured gravimetrically and PUR surface changes were analysed microscopically.

Results

The presence of PUR material influenced growth of bacterial culture in comparison with culture without PUR. Generally, PUR foams supported the growth of bacterial culture, on the contrary PUR elastomeric films weakly inhibited biomass production. The inhibition effect was exhibited mainly during early stages of growth when long lasting lag-phase was observed. This effect was probably caused by formation and/or by release of some toxic component(s) which inhibited the start of the growth. Because toxic effect was not observed in PUR foams, it is possible that toxic component(s) was by some way removed during the foaming process.

Modifying agent type was other important factor which influenced the growth of culture. From this point of view, CMC seems to be the best modifying agent because it strongly supported the growth of culture grown both in presence of PUR foams as well as PUR films.

Mass decreases of foams and films were very similar, however, in some experiments higher degradation degree was observed in PUR films. It is surprising because PUR films are expected to be more rigid than PUR foams.

It seems that modification agent type is probably more important factor than the form of PUR material. The highest losses of material were observed in PUR foams and films modified by CMC (see Fig. 1.). High mass decreases of CMC PUR materials could be partially caused by water solubility of carboxymethyl cellulose itself.

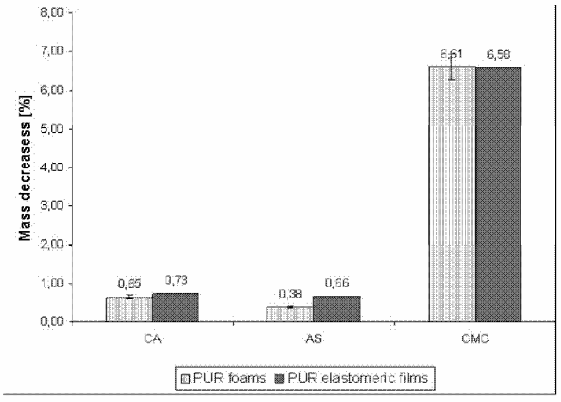

Fig. 1. **Mass decreases of PUR foams and films**

Conclusions

In conclusion, modified PUR foams as well as elastomeric films could be degraded by thermophillic bacteria. Differences between foams and films are surprisingly small. It seems that for biodegradation degree more important factor is the modification agent type than PUR material form. Carboxymethyl cellulose appears to be the best modifying agent

from biodegradation point of view. In relatively short-time lasting tests (about 100 hour) PUR materials modified by carboxymethyl cellulose exhibited almost 7 % of weight loss. CMC PUR materials also strongly supported the growth of bacterial culture.

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Supplement X

Biodegradation of polyether-polyol based polyurethane elastomeric films: influence of partial replacement of polyether-polyol by biopolymers of renewable origin

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Abstract:
In this work we investigated the process of degradation of polyether-polyol based polyurethane elastomeric films (PUR) in presence of mixed thermophillic culture as a model of natural bacterial consortium. The presence of PUR material 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 material. Lag-phase was significantly shortened and biodegradability of PUR materials was enhanced by partial replacement (10%) of polyether-polyol by biopolymers (carboxymethyl-, hydroxyethyl-, and acetyl-cellulose and actylated starch). The process of material degradation consisted of two steps. First, materials were mechanically disrupted and, secondly, bacterial culture was able to utilize abiotic degradation products, which resulted in supported bacterial growth. Direct utilization of PUR by bacterial culture was observed as well, but bacterial culture contributed only slightly to the total mass losses. The only exception was PUR material modified by acetyl-cellulose. In this case, direct biodegradation represented the major mechanism of material decomposition. Moreover, PUR material modified by acetyl-cellulose did not tend to undergo abiotic degradation. In conclusion, the modification of PUR by proper biopolymers is promising strategy reducing potential negative effects of waste PUR materials on environment and enhancing their biodegradability.

Key words:
Polyether-polyurethanes, elastomeric films, biodegradation

1. Introduction

Polyurethanes (PUR) are materials which are presented in many aspects of modern life. They represent a class of polymers that have found a widespread use in the medical, automotive and industrial fields [1]. Generally, PUR is a term used for materials derived from the condensation of polyisocyanates and polyols resulting in intermolecular urethane bond. PUR can

adopt various forms depending on used raw materials. Therefore, PUR can be used as a foam, elastomer, paint, adhesive, elastomeric fiber or artificial leather [2].

The raw materials used in the synthesis of PUR are classified into polyisocyanates, polyols, catalyst and auxiliary materials. As polyols, polyether and polyester polyols are generally used, nevertheless, most PUR used in present are based on polyether polyols. Polyisocyanates used for PUR production include aromatic and aliphatic compounds. Among these, tolylene diisocyanate and diphenylmethane diisocyanate are the most common in use. Since an isocyanate group generates carbon dioxide while reacts with water, PUR foams are synthesized by addition of a small quantity of water during the synthetic process [2].

Generally, PUR materials are considered to be susceptible to microbiological degradation. However, the biodegradability of PUR is strongly dependent on its structure and predominantly on type of polyol used. Polyester based PUR possesses many ester bonds that are vulnerable to enzymatic hydrolysis. Therefore, polyester PUR biodegradation ability was observed among fungal (*Fusarium solani*, *Culvularia senegalensis*, *Aerobasidium pollulans*, *Cladosporium* sp.) [3] as well as bacterial strains (*Comamonas acidovorans*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*) [4, 5, 6]. On the contrary, polyether PUR seems to be more resistant against enzymatic hydrolysis. Darby and Kaplan observed very low biodegradation degree of polyether PUR as compared to polyester based PUR [7]. Jansen et al. reported biodegradation of polyether PUR by *Staphylococcus epidermis*, but the process of degradation progressed very slowly [8]. Urgun-Demirtas et al. tested biodegradability of polyether PUR foam under anaerobic conditions. The experimental results showed that PUR foam was resistant against the action of anaerobic microorganisms [9]. It is proposed, that resistance of polyether PUR is due to mechanisms of biodegradation, which involves exo-depolymerization [10], whereas that of polyester PUR involves endo-type of depolymerization [2].

Because of high quantities of PUR produced, whose main part is constituted of resistant polyether PUR, waste PUR materials represent serious environmental problem. For instance, the half-time of polyether PUR foam in environment was predicted as 400 years [11]. Enhancement of biodegradability of polyether PUR materials is strategy which could contribute to the solution of mentioned environmental issue. Therefore, we tested biodegradability of polyether polyol based PUR elastomers, which were modified by partial replacement of polyether polyol by biopolymers of renewable origin. Such a strategy might reduce resistance of PUR to microbial action and enhance biodegradability of polyether PUR materials. In our experiments we used mixed thermophillic aerobic culture as a model of natural microbial population. This bacterial culture was originally used for waste water treatment. It disposes with wide range of enzymatic activities which facilitate utilization of various substrates, for instance we reported the ability of this mixed culture to contribute to degradation of polyether PUR foams modified by various polysaccharides [12].

2. Material and methods

2.1 PUR materials

The PUR elastomers tested in this work were synthesized using polyether polyol Slovaprop G-48s polyol (PEP) (purchased from Gumotex), diisocyanate Toluene-2,4(2,6)-diisocyanate (80/20) (TDI) (Gumotex,) and catalyst Dibutyltin dilaurate (DBTL) (Ciba). For modification of PUR elastomers, polyether polyol was partially (10 %) replaced by one of following biopolymers with different degree of substitution (DS) of –OH groups: acetyl-cellulose (AC) (DS = 2.4), hydroxyethyl-cellulose (HEC) (DS = 0.6), carboxymethyl cellulose (CMC) (DS = 0.7), acetylated starch (AS) (DS = 0.1). Non-modified reference sample (REF) was synthesized and tested as well.

Water-free tolylene diisocyanate (TDI), water-free polyether polyol (PEP), tin catalysts (DBTL) and particularly degassed biopolymer were mixed together in one shot process, stirred under vacuum and poured into the 1 mm thick metal frame placed between two polypropylene sheets. The reacting mixture was left to cure for 2 days at laboratory temperature. Synthesis and mechanical properties of PUR elastomeric films were described in details by Vojtova et. al. [13].

In order to compare biodegradability of PUR foams and PUR elastomeric films synthesized using the same raw materials, we used non-modified PUR foam. The foam was prepared by a two-step reaction process. At first, the polyol polyether was mixed with the catalyst and with water and stirred. Secondly, the TDI was added, stirred up to cream time and quickly poured into a mold. The foam was let to cure for about 48 hours in the fume hood in open air.

2.2 Biodegradation experiments

Mixed aerobic thermophillic culture was originally obtained from sludge of wastewater treatment in Bystrice pod Hostynem (region North Moravia, Czech Republic). The cultivation was carried out in minimal synthetic medium containing: 8 $g.l^{-1}$ glucose (carbon source); 5 $g.l^{-1}$ $(NH_4)_2SO_4$ (nitrogen source); 2.44 $g.l^{-1}$ Na_2HPO_4; 1.52 $g.l^{-1}$ KH_2PO_4; 0.2 $g.l^{-1}$ $MgSO_4$. 7 H_2O; 0.05 $g.l^{-1}$ $CaCl_2$. 2 H_2O, 10 $ml.l^{-1}$ solution of trace elements (0.2 $g.l^{-1}$ FeSO4. 7 H_2O; 0.01 $g.l^{-1}$ $ZnSO_4$; 0.003 $g.l^{-1}$ $MnCl_2$. 4 H_2O; 0.03 $g.l^{-1}$ H_3BO_3; 0.02 $g.l^{-1}$ $CoCl_2$. 6 H_2O; 0.001 $g.l^{-1}$ $CuCl_2$. 2 H_2O; 0.002 $g.l^{-1}$ $NiCl_2$. 6 H_2O; 0.003 $g.l^{-1}$ Na_2MoO_4. 2 H_2O); 2.5 $g.l^{-1}$ vitamin solution (0.01 $mg.l^{-1}$ p-Aminobenzoate; 0.02 $mg.l^{-1}$ Biotin; 0.2 $mg.l^{-1}$ Nicotine acid; 0.1 $mg.l^{-1}$ Thiamin; 0.05 $mg.l^{-1}$ Ca-Pantothenate; 0.5 $mg.l^{-1}$ Pyridoxamin). Temperature of cultivation was 60°C, pH was adjusted to 7 and flasks were shaken (120 rpm) during cultivations.

A series of batch cultures were set up to screen PUR biodegradability. In a batch assays, plug of PUR elastomeric film (1 cm x 1 cm x 0.1 cm, cca 0.5 g) was placed in 100 ml Erlenmeyer

flask containing 50 ml of medium used for cultivation of mixed thermophillic bacterial 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. During the test, bacterial growth was monitored. At the end of cultivation (about 300 hours) PUR weight losses were measured gravimetrically and changes of material surfaces were analyzed microscopically (described bellow).

To assay the influence of individual components of raw composite materials on culture growth, individual chemicals or their combinations were applied into cultivation media in amount corresponding to 10 % of each chemical presented in 0.5 g sample of PUR elastomeric film. After that, medium was inoculated by bacterial culture

2.3 Analysis of bacterial growth

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 (CDW) content. For CDW determination, the cells obtained after centrifugation (8 000 rpm, 10 min.) were dried (105°C) until constant weight was obtained.

2.4 Analysis of PUR weight losses

PUR degradation was monitored by measuring the weight of PUR plugs before and after cultivation. The PUR plugs were taken out and washed with distilled water and ethanol to remove debris on the surface. Then, plugs were dried to constant weight overnight at 40° C and weighed. The weight loss percentage was calculated as

$$\%\text{Weight loss} = (m_0 - m_t)/ m_0 . 100\%,$$

where m_0 is the initial weight and m_t is the final weight of the plug after experimentation.

2.5 Microscopic analysis of PUR surface

Washed and dried pieces of PUR elastomeric films were examined by optical microscope L1100A (Alltion, Germany) to determine changes of PUR surface and consistence of biopolymer granules in PUR elastomeric films before and after degradation experiments.

2.6 Analysis of PUR abiotic degradation products

Reducing sugars and polysaccharides as proposed abiotic degradation products of biopolymers were analyzed spectrophotometrically after 300 hours incubation of foams in distilled water without culture. Reducing sugars were determined using Somogyi-Nelson method and calibration was done using glucose [14]. Polysaccharides were estimated according to Dubois et. al. and calibrated using starch [15]. Each sample was analyzed in triplicate.

3. Results and discussion

3.1 Comparison of PUR foam and PUR elastomeric film

Previously we reported that the presence of PUR foams in cultivation media had enhanced the growth of bacterial culture [12]. Nevertheless, non-foamed PUR materials possess physical and chemical properties that are distinct from that of foamed PUR materials. These differences in properties might influence the biodegradation process parameters such as rate of biodegradability or microbial growth [16]. Therefore, we decided to test the differences between PUR foams and PUR elastomeric films synthesized using the same raw materials. In order to investigate the effect of different form of PUR materials, we performed cultivation with non-modified PUR foam as well as non-modified PUR elastomeric film.

Significant differences between the growths of culture in presence of PUR foam and PUR elastomeric film were observed in the first series of experiments (see Fig. 1). PUR foam supported the growth of bacterial culture from the beginning of cultivation, so that the biomass

concentration was higher than in control culture (culture without any PUR material). On the contrary, the presence of PUR elastomeric film in cultivation media strongly inhibited the growth of bacterial culture for period longer than 150 hours. However, after this unusually long lag-phase, culture started to growth very intensively and biomass content was higher than in control as well as in PUR foam exposed culture.

This lag-phase is probably due to release of some toxic substance(s) from PUR elastomeric films. Because the same raw materials were used for synthesize of PUR foam and PUR elastomer, but foam did not exhibit any toxic effect on bacterial culture, it could be expected that the toxic substances were not presented in foam. They are likely to have been removed during the process of foaming. However, presence of such toxic substances in PUR material could have serious consequences not only for biodegradability of PUR elastomeric film, but also for environment, which could be easily contaminated from waste PUR material. On the other side, mixed bacterial culture was able to either eliminate the toxic substances or adapt to them, which resulted in delayed but intensive growth.

The mass losses of PUR materials were very low (see Tab. 1). This is in consistence with previous observation of resistance of polyether polyol PUR against microbial attack [7, 8, 9]. 300 hour lasting cultivation was probably too short to observe more significant mass losses. Nevertheless, despite the low mass losses, both PUR materials significantly enhanced the growth of culture as compared to control. Therefore, it can be expected that bacterial culture was able to utilize PUR as additional carbon and/or nitrogen source which supported bacterial growth, although such a process progresses very slowly and complete elimination of material would last really long time.

3.2 Identification of toxic substances responsible for long lag-phase

Because the unusually long time lasting lag-phase observed in PUR elastomeric films could be an important factor influencing the fate of PUR elastomers in the environment, we decided to investigate which of used raw materials or combination of them is responsible for such unusual growth characteristic of bacterial culture. Individual chemicals or their combination were applied into the media which were afterwards inoculated by bacterial culture.

Although individual chemicals inhibited the growth of bacterial culture, none of them presented solely in the medium caused prolongation of lag-phase (see Fig. 1A). However, extended lag-phase was clearly evident when combination of polyether polyol (PEP) and catalyst (DBTL) was added into cultivation medium and, of course, when all the raw materials were added together (see Fig. 1B).

Our experimental results indicate that prolonged lag-phase was caused by small residues of free PEP and DBTL which were not built into the structure of PUR material and released from material to water environment. It could be expected that these chemicals consequently reacted with each other or with some component(s) of media which resulted in toxic substance(s) preventing bacterial culture from rapid growth. Nevertheless, bacterial culture was able to eliminate these toxic substances or adapt to them which resulted in delayed growth of culture. According to our knowledge, this effect of prolonged lag-phase caused by these chemicals, which are commonly used for PUR production, has not been observed so far. Biodegradation of polyethers, such as polyethylene glycol, is process which progress relatively slowly, but the growth characteristics of involved bacterial strains are not strongly influenced by low concentrations of polyesters. Similarly, elimination of dibutyltin compounds by bacterial culture was already reported, but none extra long lag-phase was observed during the process [18].

PUR materials containing PEP and DBTL are produced in high amounts, thus, PUR waste can present environmental problem not only because of accumulation of solid waste but also because of release of potentially harmful components. Therefore, it is important to find a way how to overcome these problems for instance by partial replacement of potential sources of toxins either PEP or DBTL.

3.3 Influence of partial replacement of PEP by biopolymer

Partial replacement of PEP by biopolymer could be an interesting strategy resulting in enhanced biodegradability of PUR elastomeric films. Moreover, according to our results, PEP is responsible for prolongation of lag-phase, so that even partial replacement of PEP is likely to shorten or eliminate unusual lag-phase. We have reported previously, that partial replacement of PEP by biopolymer in PUR foams supported the growth of mixed thermophillic culture and, furthermore, bacterial culture was able to utilize some of the PUR foams as the sole carbon and nitrogen source [12]. In order to investigate the effect of partial replacement of PEP on biodegradability of PUR elastomeric films, we exposed PUR materials modified by different biopolymers to mixed bacterial culture.

In spite of the fact that exposition of bacterial culture to all tested PUR elastomeric films resulted in unusually long lag-phase, replacement of PEP by biopolymers seems to be an interesting strategy. Not only that all modifications of PUR materials by biopolymers resulted in shortened lag-phase as compared to the non-modified REF sample, but also bacterial growth was significantly enhanced in presence of modified PUR material (see Fig. 3). The shortest lag-phase was observed in PUR material modified by acetylated starch. Simple replacement of 10 % of PEP by acetylated starch shortened lag phase more than 3 times. Furthermore, PUR film modified by

acetylated cellulose strongly supported bacterial growth in comparison with either control or reference sample.

In addition, we observed that modification of PUR elastomeric films resulted in significantly enhanced mass losses of samples during cultivation both with and without presence of bacterial culture. PUR modified by HEC and CMC lost more than 7 % of its weight during the short time cultivation (304 hours) with culture, which is significantly more than in case of non-modified sample (REF – 0.28 %). Despite the fact that the main part of mass losses was probably caused by mechanical disruption of PUR samples in water environment without action of bacterial culture (Tab. 2, column Mass losses without culture), also bacterial culture contributed to degradation process. For instance presence of bacterial culture with AC sample enhanced its degradation more than 4 times.

Photomicrographs of un-degraded and degraded PUR elastomeric films are shown in Fig. 4. The analysis of surface of PUR samples revealed significant changes in biopolymer particles which were built in PUR matrix. It was observed, that particles of AC, HEC and CMC in samples, which were exposed to microbial culture, were damaged, moreover, in some cases it was possible to observe holes in PUR structure caused by complete granule degradation or solubilization. The same changes were observed also in PUR degraded without presence of bacterial culture, so the mechanism of biopolymer particle damaging is more or less of abiotic origin.

In order to analyze the measure of spontaneous release of biopolymer into cultivation media, we incubated the PUR elastomeric films in distilled water for 300 hours. Then the contents of the reducing sugars and polysaccharides in medium were analyzed and the mass losses of samples were estimated (see Tab. 3). According to our results, reducing sugars are released from PUR samples modified by HEC and CMC while polysaccharides were detected as

abiotic degradation product in all samples (except non-modified REF sample). Reducing sugars and polysaccharides released into water presented approximately 50 % of total mass losses. Only in case of AC, the mass loss was about 90%. Rest part of mass losses probably consisted of PUR matrix itself.

Our results suggest that the degradation of PUR elastomeric films involves two steps. The first step is abiotic degradation, during which PUR elastomeric films exposed to water environment spontaneously degrade and the main part of released degradation product is constituted of modifying agent. Therefore, the mass losses of particular samples are strongly dependent on the type of modifying agent used. Secondly, bacterial culture is able to utilize the degradation product which results in enhanced growth of bacterial culture. Furthermore, because the mass losses of PUR samples were always higher in presence of bacterial culture, it can be expected, that bacterial culture contributes to degradation of PUR elastomeric films and is able to utilize films directly. This is in consistence with Albertsson et al. who reported that the abiotic and biotic mechanism of degradation of polymeric materials can occur simultaneously or subsequently [19]. Nevertheless, direct utilization of PUR samples modified with CMC, HEC and AS is very slow process as compared to the abiotic degradation. On the contrary, in the case of sample modified by AC, 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 AC also supported the growth of culture the most intensively of 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, AC could be the modification agent of choice in order to enhance biodegradability of PUR elastomeric films.

According to our results, un-reacted PEP released from PUR films into cultivation media is co-responsible for the unusual growth characteristics of bacterial culture. The release of toxic

components from PUR waste could bring serious environmental consequences, therefore, particular attention should be paid to these effects. We observed that simple replacement of 10 % of PEP by biopolymer, which is by the way of renewable origin, results in significantly reduced lag-phase of bacterial growth. Thus, it can be expected that toxic effect of PUR material is reduced. So, the partial substitution of PEP by biopolymers not only enhances its biodegradability, but, moreover, also reduces its toxicity. Nevertheless, chemical substance responsible for unusual lag-phase should be identified in order to understand mechanism of its formation and impact on bacterial cells.

4. References

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Tab. 1 Mass losses of PUR materials after incubation with bacterial culture

PUR material	Weight losses [%]
PUR foam	0.81 ± 0.08
PUR elastomeric film	0.28 ± 0.03

Results are presented in form mean ± standard deviation.

Tab. 2 Mass losses of PUR elastomeric films after cultivation in media with or without bacterial culture (320 hours).

Biopolymer used for PUR modification	Mass losses in presence of culture [%]	Mass losses without culture [%]
HEC	7.27 ± 0.29	6.09 ± 0.24
CMC	7.05 ± 0.01	5.63 ± 0.05
AS	0.97 ± 0.08	0.51 ± 0.09
AC	1.66 ± 0.30	0.40 ± 0.21
REF	0.28 ± 0.03	0.20 ± 0.03

Results presented in form mean ± standard deviation.

Used modifying agents: HEC – hydroxyethyl-cellulose, AS – acetylated starch, AC -acetylated-cellulose, CMC-carboxymethyl cellulose, REF – non-modified PUR sample, Control – sample without PUR elastomeric film, modification – replacement of 10 % of PEP by particular biopolymer.

Tab. 3 Analysis of biopolymers released from PUR samples in non-inoculated medium after 300 hours lasting incubations.

	Reducing sugars in 50 ml of medium [mg]	**Polysacharides in 50 ml of medium [mg]**	**Mass loss of PUR sample (starting mass about 500 mg) [mg]**
AC	*	0.27 ± 0.04	0.30 ± 0.07
CMC	0.37 ± 0.07	11.97 ± 0.08	24.90 ± 0.89
HEC	1.22 ± 0.10	9.97 ± 0.17	18.30 ± 1.21
AS	*	1.30 ± 0.09	2.70 ± 0.25
REF	*	*	1.80 ± 0.04

** - not detected, data are presented in form mean ± standard deviation.*

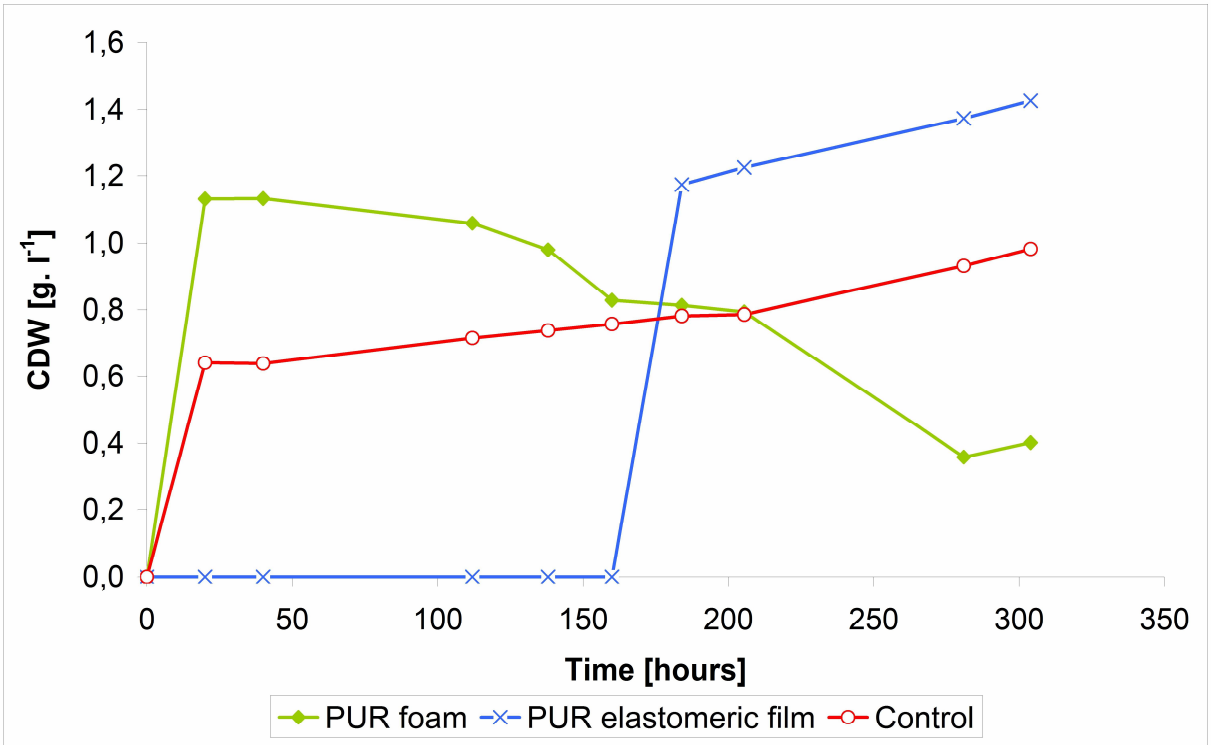

Fig 1. The growth of culture in presence of PUR foam or PUR elastomeric film.

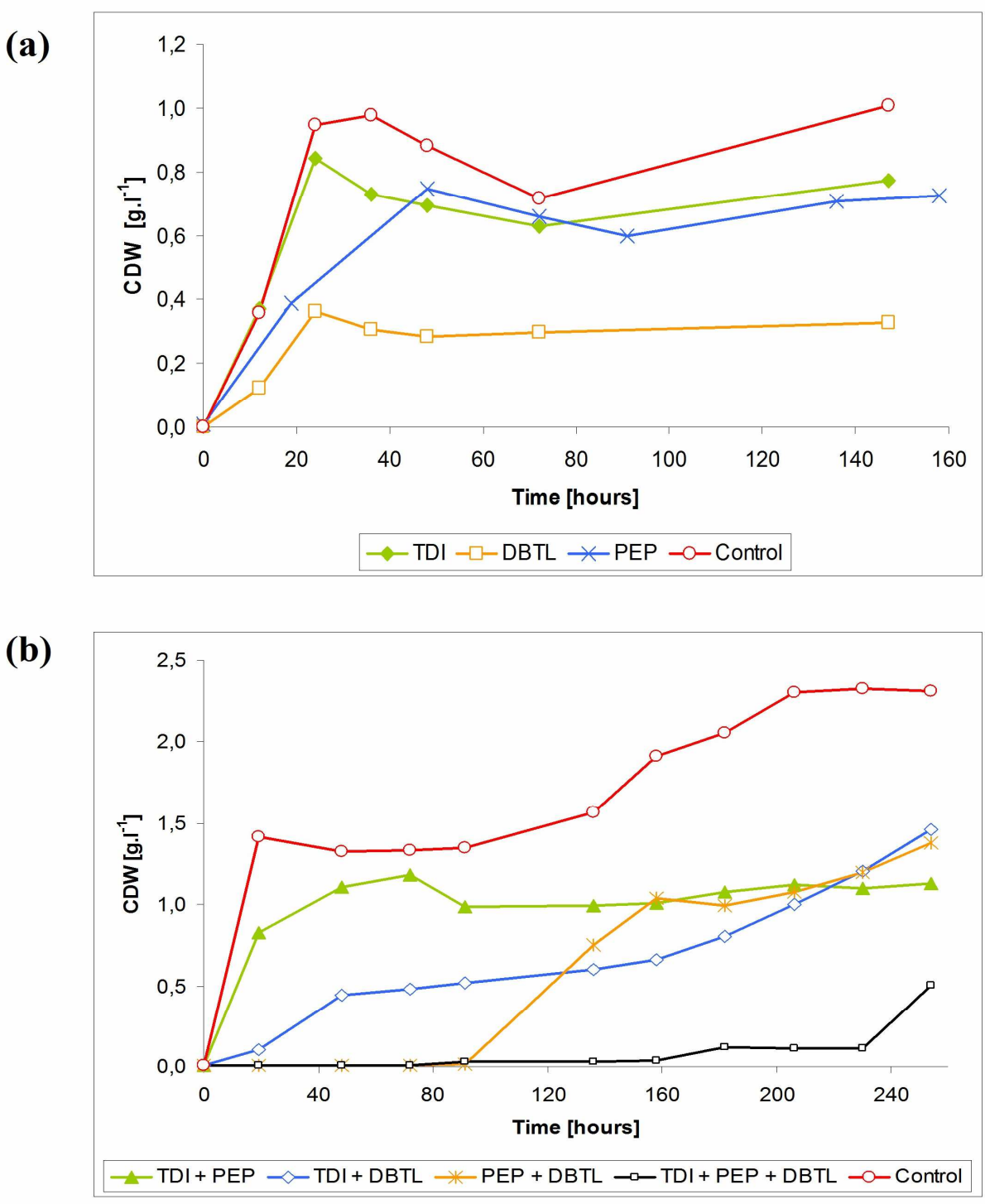

Fig 2. (A) – influence of individual raw materials on growth of bacterial culture, (B) – influence of combination of raw materials on growth of bacterial culture. PEP - polyether polyol, TDI - diisocyanate toluene-2,4(2,6)-diisocyanate, DBTL - catalyst dibutyltin dilaurate (DBTL).

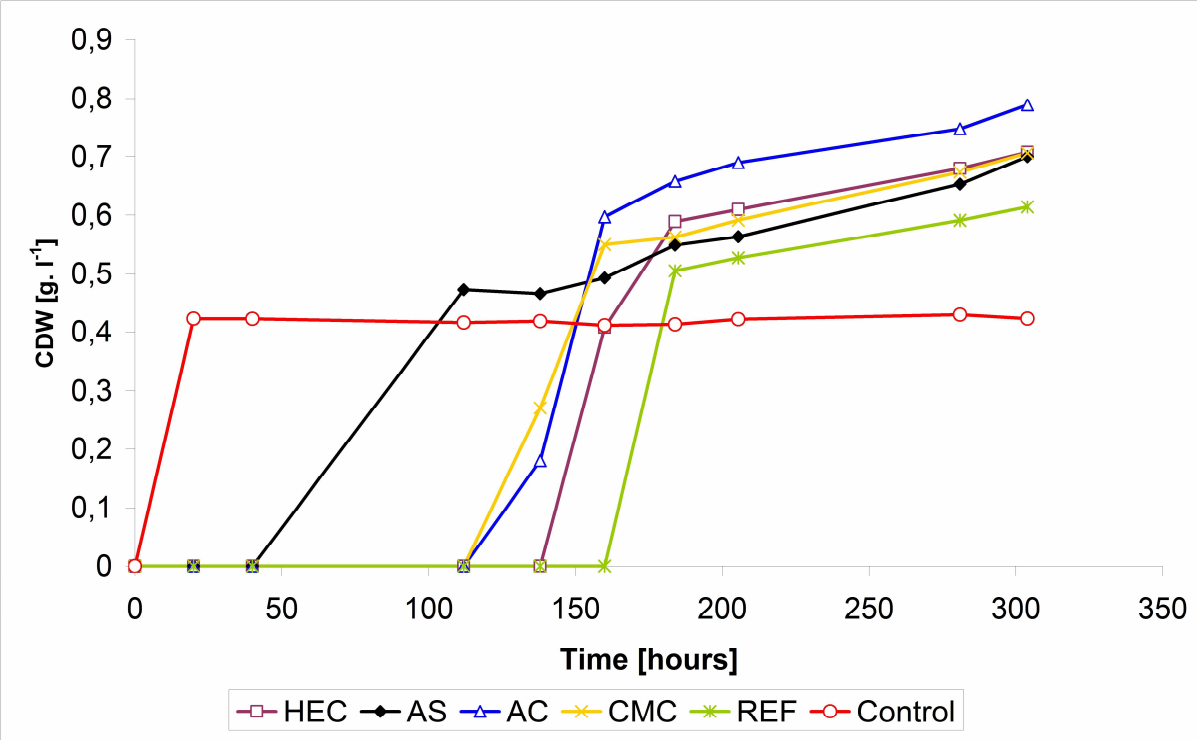

Fig. 3 Growth of bacterial culture in presence of modified PUR elastomeric films. Used modifying agents: HEC – hydroxtehyl-cellulose, AS – acetylated starch, AC -acetylated-cellulose, CMC-carboxymethyl cellulose, REF – non-modified PUR sample, Control – sample without PUR elastomeric film, modification – replacement of 10 % of PEP by particular biopolymer.

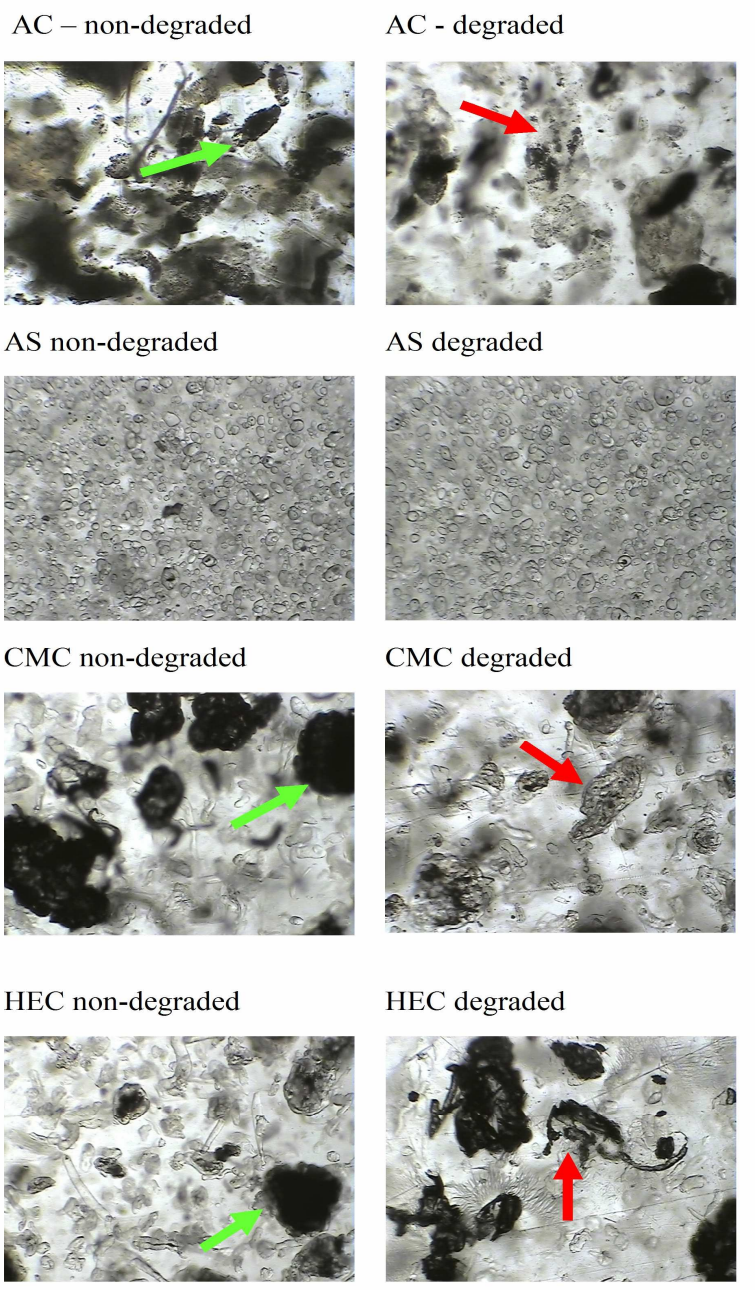

Fig 4. Surface microscopy of modified PUR elastomeric films – samples were taken before and after exposition to bacterial culture. Black narrows – granules of biopolymers in PUR matrix or damaged biopolymers granules and holes in PUR matrix. Used modifying agents: HEC – hydroxyethyl-cellulose, AS – acetylated starch, AC -acetylated-cellulose, CMC-carboxymethyl cellulose, REF – non-modified PUR sample, Control – sample without PUR elastomeric film, modification – replacement of 10% of PEP by particular biopolymer.

10. LIST OF ABBREVATIONS

ACP	Acyl carrier protein
aPHA	Amorphous PHA granules
Asp	Aspartate
ATP	Adenosine triphosphate
CoA	Coenzyme A
CCM	Czech Collection of Microorganisms
CDS	Coding sequence
DEG	Diethylene glycol
dPHA	Denaturated PHA granules
EDTA	Ethylenediaminetetraacetic acid
FID	Flame ionization detector
FBP	Fructose-1,6-bisphosphate
GC	Gas chromatography
Gly	Glycine
His	Histidine
HIV	Human immunodeficiency virus
HPLC/UV-VIS	High performance liquid chromatography with UV detection
ICI	Imperial Chemical Industries
LuxR	Regulatory protein of bioluminescence (*Vibrio harveyi*)
MCL	Medium Chain Length
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
nPHA	Native PHA granules
ntrB	Gene involved in nitrogen metabolism
ntrC	Gene involved in nitrogen metabolism
ORF	Open reading frame
pB1	Overlapping promoter (*Azotobacter vinelandii*)
pB2	Overlapping promoter (*Azotobacter vinelandii*)
PHA	Polyhydroxyalkanoates
PhaC	PHA synthase, or PHA synthase unit
PhaE	The second PHA synthase (class III) subunit
PhaF	Regulatory protein of PHA synthetic pathway
PhaP	Phasin protein
PhaR	The second PHA synthase (class IV) subunit
PhaQ	Regulatory protein of PHA synthetic pathway
PhaZ	PHA depolymerase
PHB	Polyhydroxybutyrate
$PhbR_{Ps}$	Transcriptional regulator (*Pseudomonas* sp. 61-3)
P(HB-*co*-HV)	Copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate
PMFS	Phenylmethylsuplhonylfluoride
ppGpp	Guanosin teraphosphate

PudA	PUR esterase (*Comamonas acidovorans*)
PUR	Polyurethane
RNA	Ribonucleic acid
RpoH	Sigma factor of RNA polymerase
RpoN	Sigma factor of RNA polymerase
RpoS	Sigma factor of RNA polymerase
SCL	Short Chain Length
SDS	Sodium dodecyl sulfate
SIM	Selected Ion Monitoring mode
SspD	Spore specific storage protein
Ser	Serine
*T*g	Glass transition temperature
*T*m	Melting point
TCA	Tricarboxylic acid cycle, Krebs cycle

11. COMPLETE LIST OF AUTHOR PUBLICATIONS

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