

## **Sustainable and Efficient Production of Biopolymers from Industrial Waste Streams**

Koller M<sup>1,2,\*</sup>, Salerno A<sup>1</sup>, Muhr A<sup>1</sup>, Essl R<sup>1</sup>, Reiterer A<sup>1</sup>, Braunegg G<sup>2</sup>

<sup>1</sup> *Graz University of Technology; Institute of Biotechnology & Biochemical Engineering, Petersgasse 12/I, 8010 Graz, Austria*

<sup>2</sup> *ARENA Arbeitsgemeinschaft für Ressourcenschonende & Nachhaltige Technologien, Inffeldgasse 23, 8010 Graz, Austria*

\**martin.koller@tugraz.at*

### **Abstract**

The price of Polyhydroxyalkanoates (PHAs) for a defined application must be similar to competing “classical” plastics. Today, PHA production starts from costly carbon sources like sugars. Using carbon-rich waste streams which don't interfere with nutrition can make PHAs economically competitive. *Inter alia*, waste from slaughtering and biodiesel industry or whey from dairies is available.

Surplus whey causes growing environmental concern. Its main carbon substance, lactose, can act as substrate in bio-mediated processes like PHA production.

Surplus lipids from slaughterhouses also can be upgraded to bio-resources. They can be converted to biodiesel (FAE) consisting of saturated and unsaturated FAE. Saturated FAE compromises FAE quality as fuel, but can be used for PHA production. As 2<sup>nd</sup> surplus product from FAE production, glycerol is available as carbon source.

Different scientific fields are involved in the optimization of PHA production from the discussed waste streams. Studies are supported by LCA & feasibility studies covering the use and the marketing of the products.

### **Keywords:**

White Biotechnology; Biopolymers; Polyhydroxyalkanoates; Whey; Biodiesel; Lipids; Glycerol; Industrial waste

## General Introduction

The ecological necessity for industry to switch to raw materials independent of fossil resources is generally undisputed (Braunegg et al., 2004). Already in 1992 at the Earth Summit of the United Nations “*Rio Declaration on Environment and Development*”, most nations declared their political intention to support the development of biocompatible materials based on renewable resources. This is mainly important for goods like polymers, exerting considerable ecological pressures. Figure 1 illustrates the rising amounts of plastics produced annually on earth, underlining that nowadays mankind literally lives in the “plastic age”.

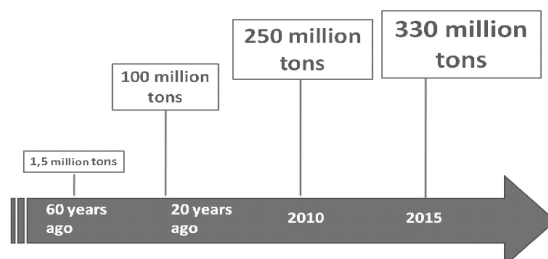


Figure 1: Rising amounts of plastic produced globally

The biopolymer market is emerging extremely fast; in 2010, the market value reached a magnitude of US-\$  $10^{10}$  with a clearly upwards trend. Only from 2008 to 2015, the quantity of global bioplastic production is estimated to increase from 180 to 1710 kt (Plastic Additives and Compounding 2008). Various polymeric materials exist for which the stylish attribute “*green plastic*” is claimed by the manufactures. Often, *de facto* properties of such materials do not match the strict definitions as prescribed for classifying them as “*biobased*”, “*biodegradable*”, “*compostable*” or “*biocompatible*”, thus making them “*green*”. These attributes only apply to plastics fulfilling strict requirements defined by standardized norms. Polyhydroxyalkanoates (PHAs) constitute a family of polyesters fulfilling these requirements (Koller et al. 2012). The spectrum of potential applications ranges from simple packaging materials featuring advantageous properties like a high oxygen barrier and UV-resistance, to high-quality materials to be used in special niches, e.g. in the medical and pharmaceutical field. Up to date, economic reasons are the major obstacles for replacing common plastics by PHAs on a relevant scale.

## Particularities and Significance of PHAs

PHAs constitute a family of biodegradable intracellular polyesters synthesized by a wide range of prokaryotic genera starting from renewable feedstocks (Koller et al. 2010). Among all bio-based plastics, PHAs are unique by being entirely produced and degraded by living cells. Figure 2 provides a scanning transmission electron microscopy (STEM) picture of *Cupriavidus necator* cells. PHA inclusions are well visible as bright, refractive granules.

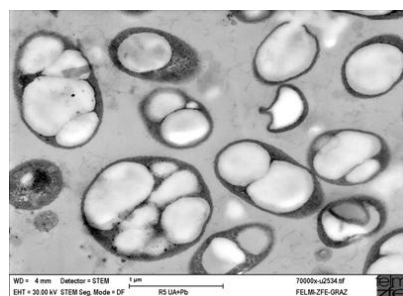


Figure 1: STEM picture of *Cupriavidus necator* cells harbouring PHA biopolymers. (Picture: Dr. Elisabeth Ingolić, FELMI/ZFE Graz)

PHAs fulfill important biological tasks, mainly as energy and carbon reserve materials for the microbial cells (Braunegg et al., 1998). Under conditions of lacking extracellular carbon sources, they can be

remobilized and utilized as carbon and energy substrates. PHAs are key compounds for the regulation of intracellular energy flow, e.g. for cell motility, and contribute to distribution and routing of carbon reserves to the metabolic pathways. In addition, a variety of important functions of PHAs in various ecosystems was elucidated, such as protection against environmental stress conditions like osmotic shock, UV irradiation, desiccation, or thermal and oxidative stress (Koller et al. 2011). More recently, also the role of stored PHA for maintenance of the intracellular redox state was reported (Ayub et al., 2009). In general, PHA accumulation is provoked by a sufficient availability of carbon source and restricted supply with macro-components like nitrogen, phosphate or dissolved oxygen or micro-components like magnesium, sulphate, and certain (heavy) metals (Kim and Lenz, 2001; Helm et al., 2008, Koller et al. 2010a).

PHAs mainly consist of 3-hydroxyalkanoates (3HAs) as monomeric building blocks. The general structure of PHA and its narrow biopolymeric relatives is provided in Fig. 3.

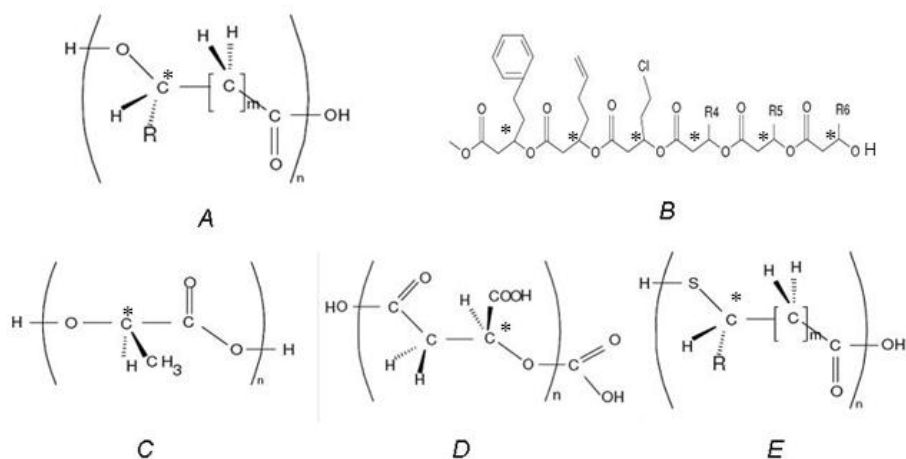


Figure 3: Chemical structures of biobased polyesters. A: General structure of PHA; B: example of a functional PHA harbouring 5-Phenyl-pentane-, 6-Hexene- and 5-Chloro-pentane groups (R4 to R6 indicate additional functional PHA building blocks); C: Poly-L-lactic acid; D: Poly-β-malic acid; E: General structure of Polythioesters (PTE). Asterisks indicate the chiral centers (Koller et al. 2010).

Depended on various factors like the type of microbial production strain, the nutrient supply regime, and the process parameters during biosynthesis, PHA polymer chains contain a magnitude of  $10^2$  to  $10^5$  3HA monomers. 3HAs normally are enantiomerically pure, *R*-configured chiral compounds. Among all known PHAs, Poly([*R*]-3-hydroxybutyrate) (PHB) is the best characterized one. PHB is a homopolyester consisting merely of 3-hydroxybutyrate (3HB) building blocks; it features rather high crystallinity and restricted processability. The low difference between the decomposition temperature (around 270°C) and the high melting point (around 180°C) provides a too small window of processability for many processing techniques, e.g. in melt extrusion or production of polymeric films. This can be overcome by interrupting the crystalline PHB matrix by additional building blocks like 3-hydroxyvalerate (3HV) or the achiral building block 4-hydroxybutyrate (4HB). This results in copolyesters with enhanced material properties and a broader range of applications. The exact material properties are strongly dependent on the monomeric composition of the co-polyesters; this composition can be triggered during the PHA biosynthesis by feeding of precursor substrates to obtain the desired building blocks. For example, 3HV building blocks are produced by many PHA accumulating strains by supplying 3HV related precursors, namely odd-numbered fatty acids, such as propanoic or pentanoic acid (Braunegg et al. 1998).

By methods of “White biotechnology”, PHAs can be produced starting from renewable resources. For this purpose, pure sugars, lipids, methanol, agroindustrial wastes like hydrolysis products of various (ligno)cellulosics, starch, sucrose, glycerol from biodiesel production, or whey lactose from dairy industry, are available at sufficient quantities (Lee 1996; Braunegg et al. 2007; Koller et al. 2010b; Patel et al. 2005; Sudesh et al. 2011; Titz et al. 2012).

After production of PHA-rich cells, efficient methods of downstream processing are needed, encompassing cell harvest, PHA isolation and refining. PHAs can be recovered from surrounding cell

mass by extraction, digestion of the cell wall, or by mechanical cell disruption. After recovery from cells, PHAs can be processed and used as sustainable biodegradable substitutes for a variety of “classical” petrochemical plastics such as poly(ethylene) (PE), poly(propylene) (PP), poly(ethyleneterephthalate) (PET) and many others (Reddy et al. 2003; Khanna and Srivastava 2005; Ren et al. 2005; Chen 2009). According to well-grounded calculations accomplished by Akiyama et al. (2003), Harding et al. (2007), Pietrini et al. (2007), and Titz et al. (2012), the production of PHAs is more beneficial considering full cradle-to-gate life cycle analysis (LCA) if compared to the production of the petrochemical competitors.

### Global PHA Production

It is challenging to collect reliable data for the actual quantities of PHA that are currently produced on (semi)industrial scale. This is due to an obvious gap between the often highly enthusiastic public announcements done by the manufacturers, and the *de facto* realized PHA production. The subsequent lines provide prime examples for successful PHA production on larger scale and encompass typical industrial throwbacks.

Production on pilot scale is accomplished in Brazil by the company PHB Industrial / Copersucar (PHBISA). Here, PHB and its PHBV co-polyesters are produced starting from sugar cane sucrose. This process is economically feasible due to the integration into existing production lines for sugar and bio-ethanol. Based on the combustion of bagasse, the major by-product of cane sugar production, sufficient energy is generated “in house” to run the production of sugar, bio-ethanol and PHA energetically autarkic. Also chemicals required for PHA recovery are directly available at zero costs; here, distillation by-products of the bioethanol production act as extraction solvents. The contemporary PHA production by PHBISA, commercialized under the trade mark BIOCYLE™ is in the magnitude of about 100 annual t with a theoretical capacity for 5.000 t per year. The realization of an industrial production plant with an annual capacity of 10.000 t is announced since more than 10 years, but is not realized yet due to the economic crisis during the last years (Nonato et al. 2001; Koller et al. 2010).

The company Polyferm Canada commercializes medium chain length (*mcl*) PHAs with a variety of rather unusual building blocks, such as 3-hydroxyhexanoate (3HHx), 3-hydroxyheptanoate (3HHp), 3-hydroxyoctanoate (3HO), 3-hydroxynonanoate (3HN), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) and even unsaturated building blocks (3-hydroxynonenoate or 3-hydroxyundecenoate) under the trademark VersaMer™. Also here, the production occurs on pilot scale using wild type bacteria as cell factories, and vegetable oils and sugars as carbon feedstocks. A variety of PHAs with a broad spectrum of different building blocks are produced by Polyferm on a kg scale (personal communication Bruce Ramsay, PolyFerm Canada, April 2012).

In China, Enmat™ PHBHV co-polymers are commercialized by the company Tianan as the world's leading producer for PHBHV starting from corn carbohydrate. This copolyester is, on the one hand, used for disposable articles such as shopping bags, cups, or golf pins; or, on the other hand, is blended with the material Ecoflex produced by BASF SE. These blends are commercialized as durable goods, e.g. bathroom accessories. The entire production quantities of Enmat™ PHA are estimated with 10 kt per year; this value is expected to increase to about annually 50 kt until 2020.

Also in China, co-polyester of 3HB and 4HB (PHB4HB) are manufactured by Tianjin & DSM (trade mark GreenBio™). The reported production quantity for GreenBio™ amounts to some hundred t per year at a theoretical capacity of 10.000 annual t. The fields of application for GreenBio™ PHA encompass the food service, agriculture mulch film, packaging, toys, and housewares. In addition, the material is used for production of sutures and artificial organs like esophagus or blood vessels.

In 2007, Metabolix and Archer Daniels Midland (ADM) formed a commercial alliance named Telles. This joint venture aimed to produce and commercialize PHA in Clinton, Iowa, under the trade mark Mirel™. The industrial production was planned for two types of PHAs: injection grade and paper coating grade. Envisaged applications were shopping bags, compost bags, packaging, agriculture/horticulture, aquatic applications, and production of various durable consumer goods. Also in this case, the large scale production was announced. Only recently, caused by the withdrawal of ADM from the joint venture in February 2012, the production of Telles PHA abruptly stopped (press release Telles, January 2012). This is a real and unexpected body blow for all optimism concerning the ultimate market

penetration of PHA bioplastics. Nevertheless, Metabolix continues research activities on PHA production in genetically modified crops.

Tepha is the worldwide most important manufacturer of PHA for application in the medical and surgical field. TephaFLEX™ is a homopolymer of 4-hydroxybutyric acid (4HB) (wwe.tepha.com, press release, April 17th, 2008). Poly-4-hydroxybutyrate (P4HB) is the only known example of a natural PHA consisting of solely achiral building blocks. The strength of P4HB fibers prepared by melt extrusion are comparable with that of traditional suturing materials; in addition, P4HB is typically more flexible, an often desired property for application as suture material. Properties like glass transition temperature and melting temperature of P4HB are similar to the values known for polycaprolactone (PCL), another well-studied biocompatible plastic material. P4HB has a tensile strength similar to ultrahigh molecular mass PE and it displays an extremely high elongation to break of about 1000%, meaning that it can be stretched 10 times its length without breaking. All these parameters indicate that P4HB is a promising material for production of bio-resorbable sutures, especially for cardiovascular surgeons (Martin and Williams, 2003).

### **Potential Fields of Applications of PHAs**

Concerning the potential applications, PHAs display very versatile materials that raise the attention of different industrial branches.

#### *Packaging and commodity items*

As the best-known and least pretentious application, these biopolymers are of interest for packaging purposes, especially in such areas where compostable packaging is wanted like for easily spoiling food: Here, especially the high oxygen barrier of PHA films is very beneficial. In addition, bottles for shampoos made of PHAs were commercially available in the past based on Biopol™ products, encompassing PHB and PHBV. PHAs can be used for paper coating, production of daily commodity items like razors, diapers, hygiene products, or cups and dishes. In agriculture, mulch films can be produced from PHAs. For these applications, PHAs can be processed by techniques of injection molding or film blowing by using the same equipment as known from the well-established processing of petrochemical plastics. As an example, an ongoing Austrian research project develops biodegradable carriers for electrical spools made of PHAs. Tests carried out on laboratory scale already demonstrated the suitability of PHA for this special application (Koller et al. 2011b).

#### *Medical applications*

In the medical field, PHAs were investigated as bone implant materials, for tissue engineering, as implants, surgical pins, screws, meshes and sutures, and as carrier matrices for controlled drug release. Also the production of highly sophisticated surgical articles such as artificial blood vessels and vein valves, spinal fusion cages, bone marrow scaffolds, and meniscus regeneration devices is reported (Chen and Wu 2005; Zinn et al. 2001; Valappil et al. 2006).

At the moment, implant for children femoral fracture healing are developed by the project Consortium of BRIC (“BioResorbable Implants for Children”) coordinated by the Medical University Graz (www.medunigraz.at/bric). Additional partners are distributed all over Austria: Graz University of Technology, University of Natural Resources and Life Sciences Vienna, This research is realized within a Laura Bassi Center of Excellence, financed by the Austrian Research Promotion Agency FFG and the industrial partners AT&S Austria Technologie und Systemtechnik AG, and Hereus Medical. Strong support in material development is also provided by ETH Zürich, CH. Beside the development of PHA-based implants, BRIC also focuses on novel biodegradable metal alloys for the purpose of femoral fracture healing. In principle, the applied implants are degraded and absorbed by the children’s organism within an optimized time period, thus avoiding a physically and psychologically wearing secondary surgery for removal of the implant (Koller et al. 2010b).

It is important to distinguish between such medical applications where high hydrolytic stability of the material is desired, and applications demanding fast degradation. Especially the variable composition of PHA allows the manufacture of materials with tailor-made mechanical properties and a fine-tuned degradation rate under *in-vivo* conditions. In fact, one can expect a quick increase of the number of

medical applications for PHAs and its composites; a first step in this direction was done by the approval of P4HB as implant material.

#### *Application of the monomeric building blocks*

Hydrolysis of PHA to the monomers generally results in a rich source of chiral synthons that can be used as starting materials for synthesis of fine chemicals and marketable products such as pheromones, aromatics, vitamins or antibiotics, or can even be used as pharmaceutically active compounds (Ren et al. 2005), e.g. 3HB and its oligomers reveal therapeutic effects. They promote cell proliferation and prevent necrotic cell death. As one of the well-known ketone bodies, 3HB is a naturally occurring metabolite in human blood plasma. It was successfully tested as carbon- and energy source in order to prevent major loss of proteins, e.g. for obese patients experiencing provoked therapeutic starvation (Pawan and Semple 1983; Mizobata et al. 1996; Williams and Martin, 2002). Some of these chiral acids also display biological activity against pathogenic bacteria or viruses (Ruth et al. 2007).

#### *Smart materials*

Further, PHAs harboring special building blocks can be applied as so-called “functional materials” for different niche applications. Here, they can act as heat sensitive adhesives, latex materials, or smart gels (reviewed by Chen et al. 2010).

#### *Controlled release of active agents*

Carrier materials and degradable matrices can be produced from PHAs and are desired in many fields, such as agriculture, food technology, or pharmacy. Compounds that can be released from these matrices at controlled rates include drugs, hormones, pesticides, antibiotics, or flavors (Steinbüchel and Fuchtenbusch 1998).

#### *Novel biofuels*

A completely new field of application for PHAs is their conversion towards alkyl esters by means of transesterification. Here, the conversion leads to 3-hydroxyalkanoate methyl esters (3HAME) (Zhang et al., 2009). Chemically, 3HAME have a composition similar to biodiesel stemming from the alkaline transesterification of vegetable oils or tallow. In fact, PHA-based alkyl esters of 3HAs (3HAMEs) were successfully tested as engine fuels; similar combustion heats were determined for 3HAME if compared with gasoline. The conversion of PHA to “biofuels” seems to be reasonable for such biopolymer fractions that show modest material properties, e.g. PHA blends stemming from mixed cultures grown in sewage water. PHA produced by such microbial consortia often contains building blocks with a rather long carbon side chain (*mcl*-PHAs). Examples for such *mcl*-building blocks are 3HHx, 3HO, 3HD, and 3HDD. The longer the carbon chain, the higher the expected combustion heat for the deriving 3HAMEs should be.

### **Economic Challenges in the Production of PHAs and Attempts to overcome them**

Despite all efforts globally devoted to biopolymer research, PHAs are still not really competitive to petrochemical plastics mainly considering production costs and, to a certain extent, also regarding the material properties (Choi and Lee 1999, Sudesh and Iwata 2008, Koller et al. 2010b). A major share of up to half of the entire production costs is related to the carbon substrates. It is decisive that the explosive nutritional situation for mankind in many global regions strictly interdicts the utilization of various renewable feedstocks for production of chemicals, plastics or fuels from an ethical point of view. This goes especially for the application of carbohydrates, high-value proteinaceous materials or edible oils. But, diverse wastes exist which represent severe disposal problems for the concerned industrial branches, and, at the same time, do not interfere with the nutrition chain. Their utilization is a viable strategy to overcome this ethical conflict; it can be considered as the most promising approach in making PHAs economically competitive. Such materials are mainly produced in agriculture and such industrial branches that are closely related to agriculture (Braunegg et al. 1998, Solaiman et al. 2006a, Khardenavis et al. 2007, Khanna and Srivastava 2005a).

In addition to the substrate expenses, costs have to be saved by optimizing the downstream processing for PHA recovery after cell harvest. As intracellular products, PHAs have to be separated from the

surrounding non-PHA cell mass (NPCM), mainly consisting of proteins, lipids, nucleic acids and polysaccharides. Here, high input of often hazardous solvents and enormous energy demand still constitute the state-of-the art in PHA recovery, antagonizing the claims of these bio-plastics to be ecologically benign materials (Koller et al. 2010a, Kunasudari and Sudesh 2011).

Apart from the selected raw materials and the downstream processing, the increasing of productivity by designing of the optimal engineering set-up is indispensable for the final break-through of PHAs on the market. Batch and fed-batch discontinuous fermentation mode are up to date the most common techniques for microbial PHA production (Kim et al 1994, Ahn et al. 2000, Nonato et al. 2001). In contrast, continuous biotechnological production mode is a well-known tool for achieving high productivities, lower production costs and constant product quality. Due to these facts, a growing number of research activities were accomplished during the last couple of years, investigating and assessing the potential of continuous PHA production processes (Zinn et al. 2003, Sun et al. 2007, Atlić et al. 2011). Recently, the continuous production of PHB in a five-stage cascade of bioreactors was investigated. Here, the biopolymer was produced on glucose by the eubacterial strain *Cupriavidus necator*. As a main results, the authors report high productivities of 1,85 g/L h for PHB and a constant and superior product quality (Atlić et al. 2011).

Nowadays, the research in the field of PHAs focuses on several key topics. The application of growth additives that shorten the time for production of catalytically active biomass is a pre-requisite to enhance the entire volumetric productivity of the process. Such cheap growth additives can be found in agriculture, e.g. side streams from the cultivation of green grass land, and were already tested successfully on laboratory scale (Koller et al. 2005b, Koschuh et al. 2012). Concerning the raw materials, simple “unrelated” carbon sources that are available at low prices, or even constitute waste streams, should act as sole feedstocks for production of high-value PHA co-polyesters using specialized microbial production strains. This provides the possibility to save costs for precursor compounds normally needed for co-polyester production.

Efforts done in the field of genetic engineering mainly intend the increase of volumetric PHA productivity and higher molecular masses of the biopolymers. This can be accomplished via the knock-out of enzymes responsible for intracellular PHA degradation. Also metabolic bottle necks that can hamper a fast and complete substrate conversion can be overcome by genetic modifications, as well as the enhancement of the microbial oxygen uptake by inserting genes encoding catalase or peroxidase (Ouyang et al. 2007).

In the field of downstream processing, environmentally safe and efficient solvents are investigated for enhanced recovery of PHA from the cells. This goes in parallel with the examination of novel biological lysis methods and enhanced strategies for mechanical cell disruption. In any case, enhanced downstream processing has to manage with lower energy consumption if compared to contemporary methods. For efficient polymer recovery, the increase of the intracellular polymer content as well as the increase of the PHA granule size is beneficial; these factors are determined during the PHA bio-production (Chen 2010). Also the remaining NPCM has to be converted in a sustainable, value-adding way. Currently, research in this direction is devoted to the anaerobic digestion of NPCM in biogas plants, or to the chemical or enzymatic hydrolysis of NPCM to a rich carbon- and nitrogen source for subsequent microbial cultivations. As an alternative, NPCM can be applied in agriculture as “green fertilizer”. Also downstream processing can be facilitated by genetic modification; an excretion of high quantities of nuclease enzymes after cell disruption results in decreased amounts of nucleic acids in the medium, leading to lower viscosities that facilitate the separation of PHA granules from the surrounding liquid phase by centrifugation or flocculation.

The technological drawbacks of the bio-production itself can be handled by the application of robust microbial production strains that remain genetically stable for a long time period under continuous cultivation conditions, and, at the same time, can resist the risk of microbial contamination by microbial competitors that can endanger whole fermentation batches, generating a high economic loss (Koller et al. 2011c). Here, extremophilic species like the highly salt requiring archaeon *Haloferax mediterranei* might be a viable solution in order to minimize the normally indispensable, highly energy demanding sterility requirements for PHA production set-ups (Koller et al. 2007a,b). In future, continuous PHA production should not only aim at the increase of volumetric productivity, but also open the door for tailor-made material properties by fine-tuning the polyester composition. This can be accomplished by the formation of block-copolymers, where the sequential arrangement of softer and harder polymer parts

can result in well-adjusted novel polymeric materials. Here, a multistage bioreactor cascade for PHA production as presented by Atlić and colleagues (2011) might be the adequate process engineering equipment.

During the last two to three decades, the preparation of composites and blends has become one of the key research fields in biopolymer science. For enhancement of the material properties, PHAs can be processed together with a variety of compatible matters, resulting in the creation of novel PHA-based blends and composites. For this purpose, the utilization of polymeric materials like poly(vinyl alcohol) (PVA), poly(lactic acid) (PLA), poly( $\epsilon$ -caprolactone) (PCL) etc., including synthetic analogues of PHA (e.g. atactic PHB), inorganic fillers (clays, sepiolites, Montmorillonite, or calcium carbonate), and organic fillers of agricultural origin was already tested (Chiellini et al. 2004, Pietrini et al. 2007). Concerning fillers from agriculture, the application of surplus materials like lignocelluloses like sugar cane bagasse, wheat flour, fruit peels, crop fruit fibres, saw dust and wheat straw is reported in literature (Chiellini et al. 2004). In general, nanocomposites and natural fibers composites can be distinguished. Nanocomposites have the potential to improve special polymer properties, such as gas permeability and thermal and mechanical characteristics. For creation of nanocomposites, rather small amounts of filler, commonly an organophilic modified clay, are needed for efficient enhancement of the properties. Natural fibers composites often display excellent mechanical properties, and, as desired for many applications, they lower the density of the final product. Due to the fact that in most cases fibers constituting agricultural residues are used as fillers, the biodegradability of the final product is enhanced. Because such fibers do not feature a considerable price, this normally goes in parallel with a reduction of the entire production cost of the marketable product (Pietrini et al. 2007).

### **The WHEYPOL Project: From Surplus Whey to PHA Biopolyesters**

Cheese whey is a surplus product in dairy industry. From the feedstock milk, casein is precipitated enzymatically or by acidification. This so called “transformation” results in the generation of solid curd cheese (predominately consisting of caseins), and liquid full fat whey. After removing the major part of lipids from full fat whey by skimming, skimmed whey remains. Sweet skimmed whey is subjected to a concentration step, removing 80% of its water content. This whey concentrate is separated via ultra-filtration in whey permeate (carbohydrate fraction) and whey retentate (protein fraction with considerable lactose residues). Whereas whey permeate that contains about 80% of the lactose originally included in milk can be used as carbon source for fermentative production of e.g. PHA, special proteins of the retentate like lactoferrin and lactoferricin are of significance for pharmaceutical application. The predominant proteins in whey retentate, namely  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin, are potential candidates for food- and feed supplements. Biotechnologically, they can be applied as nitrogen source for enhanced cultivation of microbial PHA production strains. More recently, plastic films coated by whey proteins are developed; also here, classical polymers are replaced by recyclable, bio-based materials featuring low oxygen and moisture permeability, making them especially interesting for food packaging (<http://www.wheylayer.eu/project.html>).

Whey constitutes a surplus material from dairy and cheese industries. Reported amounts of whey that are produced globally vary from  $1.15 \cdot 10^8$  t (Peters 2006) to  $1.40 \cdot 10^8$  t (Audic 2003) per year. OECD and FAO even estimate  $1.60 \cdot 10^8$  t with annual increase of 1-2% (values for 2008; Guimarães et al. 2010). Mainly in North America and Europe, huge quantities of whey are available; in 2008, the estimated accruing values are reported with  $4 \cdot 10^7$  t for the USA, and  $5 \cdot 10^7$  t for the EU. Reliable data for Canada report  $2,2 \cdot 10^5$  t annually (year 1997, Ghaly and El-Taweel 1997).

Bovine whey is not only a cheap raw material, but also causes severe disposal problems for the dairy and cheese industry due to its high biochemical oxygen demand (BOD, 40,000 –60,000 ppm) and chemical oxygen demand (COD, 50,000–80,000 ppm), making the disposal of surplus whey rather expensive (Kim et al. 1995, Viñas et al. 1994). During cheese production, whey accrues in almost equal volumes to the processed milk. This means that the processing of one million litres of milk causes the challenging task of disposing of nearby one million litres of whey. This exemplary quantity contains up to 50 t of whey’s main carbon ingredient, namely lactose. The resulting problem becomes especially obvious considering the fact that today surplus whey is very often disposed of just by being poured into rivers and the sea. For example, in the Northern Italian Po-region, where a variety of well-known dairies is located, about 1 million litres of whey has to be disposed daily.



Hence, a detailed research was missing, resulting in an integral process for PHA production from surplus whey by an organism, preferentially a wild type strain that is superior to other lactose-utilizing PHA producers. The process has to include the following features as listed below (Koller 2005):

- Optimized upstream technology for the feedstock whey
- Optimized conditions for growth and PHA formation from whey by the selected strain
- Knowledge of triggering the polymer composition (optimized precursor feeding strategies) as a tool for triggering the final polymer properties
- Optimized downstream strategy that is very much dependent on the strain
- Detailed characterization of the products
- Accompanying economic and ecologic appraisal of the process under development
- Evaluation of possible applications of the products

It is clear that the discussed points can only be achieved, if there are intensive interactions between experts in strongly differing scientific fields: microbiology (search for the “ideal” microbial production strain), biotechnology (fermentation technology as the core part), chemical engineering (tailor made downstream processing, design of industrial plant), polymer science (on-going characterization of the products), and life cycle assessment (LCA).

Within the FP5 Growth Program of the European Union, the project WHEYPOL realized these ideas between 2001 and 2004. The demanded interactions between different research areas were put into practice by the formation of a network consisting of seven scientific groups and three industrial partners each specialized in a certain field needed for the process development.

Starting from whey permeate that constitutes a surplus material at the Italian dairy company Latterie Vincentine, microbial strains were investigated for growth and PHA formation based on this feedstock. This was partly done at the University of Padua (Italy), and mainly at Graz University of Technology (TUG). Here, the know-how for fermentation was developed as the core part of the WHEYPOL process. Also at TUG, downstream processing was established for safe and efficient product isolation. Characterization of the produced polyesters was done by different research groups at several European universities and research centres (Pisa, Ljubljana, Bratislava, and Zabrze). The engagement of different groups specialized in polymer characterization enabled the investigation of the produced materials using a broad spectrum of special state-of-the-art analytical equipment, and further to perform different tests for blending, mixing and processing of the materials. Industrial partners were needed on the one hand to plan the scale-up for realization of the developed process in semi-industrial scale, and to evaluate the cost effectiveness of the process. This was done by experts from Biodiesel International (Austria). The company Idroplax (Italy), on the other hand, was responsible for large scale processing of the products by film blowing or injection moulding.

In a study that was accomplished within the WHEYPOL project, Koller and colleagues (2007a) compare the potential of three prokaryotic wild type strains for utilization of whey as carbon feedstock for PHA production. In this work, the archaeon *Haloferax mediterranei* and the eubacterial strains *Pseudomonas hydrogenovora* and *Hydrogenophaga pseudoflava* were investigated in bioreactors at laboratory scale. Among these organisms, *H. mediterranei* turned out to be the most promising candidate for eventual industrial scale PHA production starting from whey. This is due to the strain's high robustness and stability; the risk of microbial contamination during cultivation is restricted to an absolute minimum, thus a lot of energy can be saved by the lower sterility demands. Additionally the strain produces a P(3HB-co-8%-3HV) co-polyester directly from the 3HV-unrelated carbon source whereby the normally high costs for propanoic acid or pentanoic acid as precursors can be saved. The strain grew well on hydrolyzed whey permeate with a maximum specific growth rate  $\mu_{max}$  of 0.11 1/h. PHA was accumulated at a maximum specific production rate of 0.08 g/gh. The conversion yield for whey sugars to PHA was calculated with 0.33 g/g. After further optimizing of the production conditions, the productivity for this strain on hydrolysed whey permeate was increased to 0,09 g/ Lh (specific rate 0,15 g/gh); 16,8 g/L biomass containing 73% PHA were obtained (Koller et al. 2007b, Koller et al. 2012). By co-feeding of precursors for 3HV and 4HB production (pentanoic acid and  $\gamma$ -

butyrolactone, respectively) together with hydrolysed whey permeate as main carbon source, a high value P-(3HB-co-21.8%-3HV-co-5.1%-4HB) terpolyester was produced by *H. mediterranei*. Also in this case, the polymer was recovered from the cells and underwent a detailed characterization of thermal properties and molecular mass distribution. The promising results for polymer characterization indicate that the material might be of special interest for application in the medical field (Koller et al. 2007b). The partial conversion of whey sugars to 3-hydroxyvalerate (3HV) units and the excellent polymer characteristics (low melting temperature, high molecular masses within narrow distribution) together with a viable cheap and simple downstream processing (Munoz et al. 1994) make the strain especially interesting. The estimated production price amounted to € 2.82 per kg PHA. The recycling of the highly saline side streams has to be tested and optimized. Additionally, high salinity requires special material demands for the bioreactor equipment and sondes (Hezayen et al. 2000).

*P. hydrogenovora* features the disadvantage of low final polymer contents, low productivities and product yields due to redirection of the carbon flux towards unwanted by-products such as organic acid. Using this organism, the final PHB homopolyester content amounted to 12 wt.-% ( $q_p$ : 2.9 mg/gh). By co-feeding of pentanoate, the strain accumulated 12 wt.-% of poly-3(HB-co-21%-HV) ( $q_p$ : 2.0 mg/gh) (Koller et al. 2007a, Koller et al. 2008).

*H. pseudoflava* produces biopolyesters of rather good quality (high molecular masses and low polydispersities) directly from whey lactose at acceptable specific production rates and yields, but is not competitive with *H. mediterranei* in terms of strain stability and robustness. In details, using this strain, 40 wt.-% of poly-3(HB-co-5%-HV) in cells with addition of pentanoic acid ( $q_p$ : 12.5 mg/gh) were obtained. Without pentanoic acid, the strain accumulated 30 wt.-% of the homopolyester PHB in cells ( $q_p$ : 16.0 mg/gh). (Koller et al. 2007a).

Figure 5 provides the fermentation pattern for PHB biosynthesis in a laboratory bioreactor by *H. pseudoflava* on hydrolyzed whey permeate.

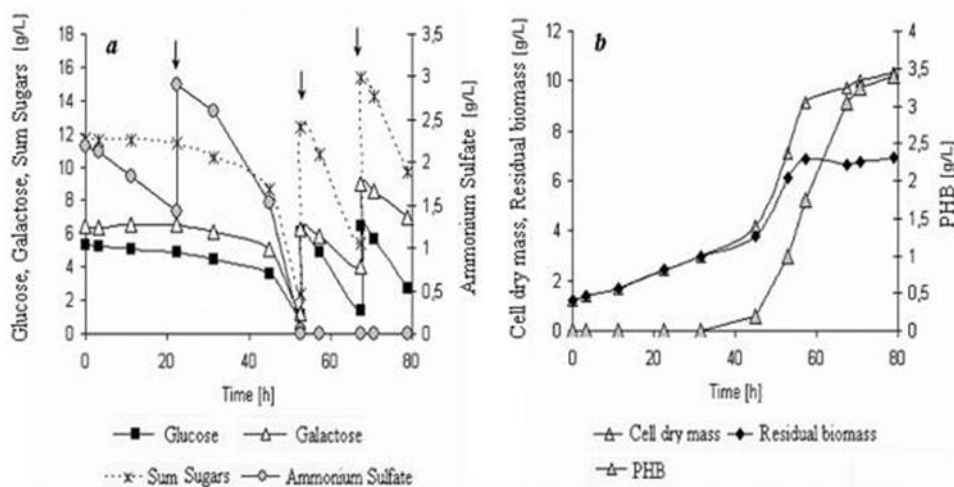


Figure 5: Fermentation pattern PHB production by *H. pseudoflava* on hydrolyzed whey permeate.

### The ANIMPOL-Project: Slaughtering Waste for PHA production

The amounts of animal lipids from the slaughtering process in Europe amounts to more than 500.000 t per year. The available saturated biodiesel (SFAE) fraction from this waste amounts to annually 50.000 t. This SFAE causes problems in fuel at cold temperatures due to an elevated cold filter plugging point that results in precipitation. From SFAE, the amount of PHA biopolyesters that can theoretically be produced amounts to 35.000 t annually (conversion yield of 0.7 g product per g substrate). The surplus glycerol phase (CGP) from the biodiesel production is estimated for Europe with annually 265.000 mt. If applied for production of microbial PHA accumulating biomass, one can expect about 0.4 to 0.5 gram biomass per gram of glycerol. This is visualized in Fig. 4.

At the 1st of January 2010, the project “*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*”, Acronym ANIMPOL, was launched, funded by the 7<sup>th</sup> Framework Program of the EU and coordinated by Graz University of Technology.

The project aims at the development of a sound industrial process for conversion of lipid-rich animal waste from meat processing industry towards biodiesel via innovative methods. Those biodiesel fractions negatively influencing the biodiesel properties as fuel (highly saturated fractions) are separated and subsequently used as feedstock for the biotechnological production of polyhydroxyalkanoates (PHA), a versatile group of biopolymers for production of bioplastics items. The remaining unsaturated biodiesel fraction features an excellent 2<sup>nd</sup> generation biofuel. The significance of the project is obvious considering the enormous amounts of available waste materials in Europe to be used for the process developed in ANIMPOL.

Fig. 4 illustrates the available amounts of raw material and the quantities of PHA polyesters that can be produced thereof.

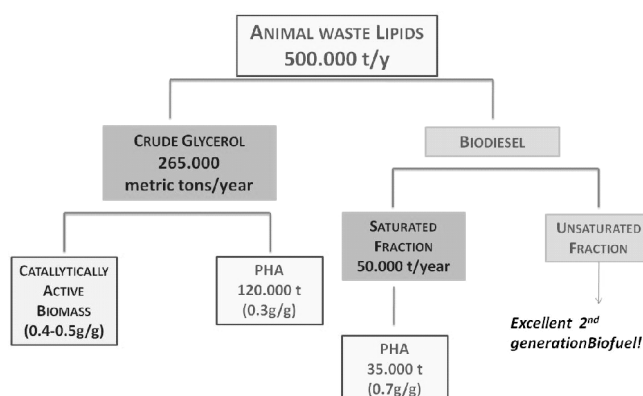


Figure 4: Amounts of available waste streams, and potential bio-plastic production thereof

The project brings together players of waste resources from slaughterhouses, rendering industry, and the biodiesel production with polymer processing industry. ANIMPOL creates transnational added value and further benefit for industrial development by the cooperation of partners from different European countries having complementary expertise. Different branches of European industry will be able to handle their tremendous waste streams, and polymer producing and processing industry will be able to switch to alternative, sustainable products. The project contributes to socioeconomic benefit by job creation directly in meat converting factories and biodiesel factories, branches with strong implementation in Austria and various other European countries. ANIMPOL will result in further research activities and distribution of the developed value-added bioplastic products.

The project consortium is distributed all over Europe and well balanced between academic and industrial partners. Beside the Styrian meat converter U. Reistenhofer GesmbH (SME) and the large Scottish biodiesel producer Argent Energy (UK) Limited Contact Point, additional industry is represented by the Italian producer of plastic packaging materials Termoplast and the German company Argus Umweltbiotechnologie, responsible for Downstream Processing. Concerning the academic partners, Graz University of Technology acts as project coordinator and expert for biotechnology as well as for process engineering and LCA and Cleaner Production Studies. Support is provided in microbiology and genetic engineering by the University of Padova and by the University of Zagreb (mathematical modeling of bioprocesses). University of Graz carries out the optimized conversion of animal lipids to biodiesel by novel transesterification strategies, while University of Pisa, the National Institute of Chemistry (Ljubljana) and the Polish Academy of Science fulfill special tasks in PHA characterization. Termoplast finally converts the produced biopolyesters and assesses the market potential of the novel materials.

An Advisory Board consisting of several industrial entities was installed in order to give advice to the project Consortium. It acts as an “Enduser Group” with focus on the quality demands of the final polymeric materials for industrial applications. Further, the Advisory Board provides the input to define the needed experiments for planning of a pilot scale plant. At the moment, the Advisory board consists of three industrial members (Novamont, Italy, polymer company specialized in biodegradable packaging; ChemTex Italia (Mossi & Ghisolfi group), Italy, experienced in chemicals, polymers and biofuels; KRKA, Slovenia, expertized in large-scale biotechnology) (Koller et al. 2010e).

Experimentally, the most promising results for PHA production from SFAE were achieved by the ANIMPOL consortium using a wild-type eubacterial strain (*C. necator*). Fast microbial growth, satisfying cell densities, and high volumetric productivities for PHA homo- and co-polyesters, together with promising material characteristics were obtained starting from SFAE. Fig. 6 illustrates the fermentation pattern of the production of poly-3(HB-co-4.5%-HV) on SFAE as main carbon source by *C. necator*.

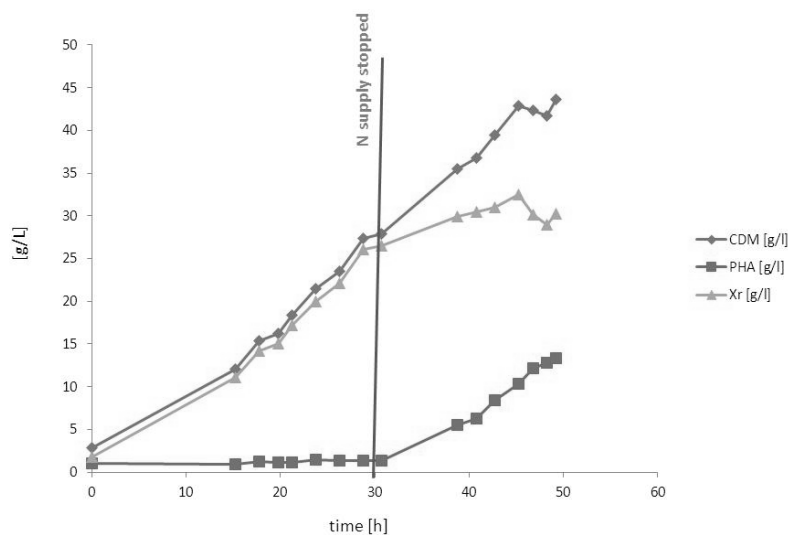


Figure 6: Fermentation pattern for poly-3(HB-co-4.5%-HV) production by *C. necator* on SFAE. Time curves for CDM, PHA, and  $X_r$  indicate cell dry mass, PHA biopolyester and catalytically active non-PHA biomass, respectively. The line at time 30 hours indicates the limitation of nitrogen supply, provoking PHA accumulation.

## Conclusion

The study presents strategies to upgrade industrial wastes like surplus whey and residues from the slaughtering and biodiesel industry to the substrates for biopolymer production. Applying such waste streams can be regarded as the most promising route to make the entire PHA biopolymer production process economically competitive; this is valid for bulk-plastics made of petrochemical competitors and special polymers as currently applied for niche products. The selection of the raw materials discussed in the article at hand is especially important to provide European industry an advantage on the one hand to handle tremendous wastes streams by value-added conversion, and, on the other hand, to break new ground in developing and commercializing bio-polymeric materials.

It is generally undisputed that the global demand in bio-plastics will quickly increase. In this context, it has to be considered that especially in many emerging and developing countries, huge efforts are devoted at the moment to research and industrial production of biopolymers. Applying feedstocks that are available in Europe as waste streams is a viable route to avoid that, in future, bio-plastics have to be imported from other global areas.

Beside the raw material costs and the fermentation process itself, downstream processing for polymer recovery from the surrounding cells is a decisive cost-determining factor in biopolymer production. Especially here, additional research progress is required in order to decrease the demands for energy and chemicals that still counteract the sustainability of PHA production.

Uniting the potential enhancements of each process step, one can definitely make substantial progress towards an environmentally benign and cost-efficient technology. In any case, the development of really efficient biopolymer production processes needs the narrow cooperation of experts from industry and different scientific fields; hence, a multidisciplinary approach is required. Decision makers from companies, chemical engineers, microbiologists, enzymologists, polymer scientists, genetic engineers, and experts for LCA and Cleaner Production studies have to concentrate their special know-how. This can close existing gaps between the meritorious successes on laboratory scale that in most cases affect

only singular aspects of biopolymer production, and the final market success of competitive bioplastics.

Independent from the selected microbial production strain, facilities for PHA production from whey and animal processing industry should be integrated into existing process lines of large dairies, or biodiesel companies, where the raw material whey directly accrues. This can be considered as a viable strategy to minimize production costs by taking profit of synergistic effects.

Regarding the high number of available data from literature, one can conclude that important progress has been achieved in terms of combining the environmental benefit of future-oriented bio-polyesters with economic viability of their production. This should finally facilitate the decision of responsible policy-makers from various waste-generating industrial branches and from polymer industry to break new ground in sustainable production.

## Acknowledgement

The authors gratefully acknowledge the support of the European Commission by granting the projects *WHEYPOL* (GRD2-2000-30385) and *ANIMPOL* (Contract No: 245084) as well as the support in the “*Laura Bassi Center of Expertise*” project *BRIC* by *FFG* and the industrial partners *AT&S Austria Technologie und Systemtechnik AG*, and *Hereus Medical*. Further, the authors are thankful for the STEM picture of *C. necator* provided by Dr. Elisabeth Ingolić, FELMI-ZFE Graz.

## References

- Ahn, W.S., Park, S.J. & Lee, S.Y. (2000). *Applied Environmental Microbiology* 66(8): 3624–3627.
- Akiyama, M., Tsuge, T. & Doi, Y. (2003). *Polymer Degradation and Stability* 80: 183-194.
- Atlic, A.; Koller, M.; Scherzer, D.; Kutschera, C.; Grillo Fernandes, E.; Horvat, P.; Chiellini, E.; Brauneegg, G. *Applied microbiology and biotechnology* 91 (2011), 295 – 304
- Audic, J.L., Chaufer, B. & Daufin, G. (2003). *De Lait* 83(6): 417-438.
- N.D. Ayub, Tribelli P.M., Lopez N.I. (2009) *Extremophiles* 13:59–66
- Brauneegg, G., Koller, M., Hesse, P.J., Kutschera, C., Bona, R., Hermann, C., Horvat, P., Neto, J. & Dos Santos Pereira, L. (2007). Production of Plastics from Waste Derived from Agrofood Industry, in: Graziani, M. & Fornasiero, P. (eds.), *Renewable resources and renewable energy: a global challenge*, Taylor and Francis Group, Boca Raton, New York, London, pp. 119 – 135.
- G. Brauneegg, G. Lefebvre, K.F. Genser, J. *Biotechnol.* 65 (1998) 127-161
- Brauneegg, G.; Bona, R.; Koller, M. *Polymer plastics technology and engineering* 43 (2004) 6, 1779 – 1974
- Chen, G.Q. & Wu, Q. (2005). *Biomaterials* 26:6565-6578.
- Harding, K.G., Dennis, J.S., Blottnitz, H.V. & Harrison, S.T.L. (2007). *Journal of Biotechnology* 130: 57-66.
- Chen, C.Q. (2009) *Chemical Society Review* 38:2434–2446.
- Chen, G.Q. (2010) Plastics completely synthesized by bacteria: Polyhydroxyalkanoates, in: G.-Q. Chen (ed.), *Plastics from Bacteria*, Alexander Steinbüchel, Series (ed.) *Microbiology Monographs*, Springer-Verlag, Berlin, Heidelberg, pp. 17-37.
- Chiellini, E., Cinelli, P., Chiellini, F. & S.H. Imam (2004). *Macromolecular Biosciences* 4 (3): 218-231.
- Choi, J. & Lee, S.Y. (1999). *Applied Microbiological Biotechnology* 51:13-21.
- Ghaley, A.E. & El-Taweel, A.A. (1997). *Biomass and Bioenergy* 12(6), 461-472.
- Guimarães, P.M.R., Teixeira, J.A. & Domingues, L. (2010). *Biotechnology Advances* 28:375–384.
- Helm, J., Wendlandt, K.D., Jechorek, M. & Stottmeister, U. (2008). *Journal of applied microbiology* 105(4):1054-61.
- Hezayen, F.F., Rehm, B.H., Eberhardt, R. & Steinbüchel, A. (2000). *Applied Microbiology and Biotechnology* 54(3): 319-25.
- Khanna, S., Srivastava, A.K. (2005). *Process Biochemistry*. 40, 607–619
- Khardenavis, M.S. Kumar, S.N. Mudliar, T. Chakrabarti. *Bioresource Technol.* 98 (2007) 3579-3584.
- Kim, Y.B. & Lenz, R.W. (2001). *Advances in Biochemical Engineering/Biotechnology* 71:51-79.
- Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K. & Woo S.I. (1994). *Biotechnology and Bioengineering* 43:892-898.
- Kim, H.O., Wee, Y.J., Kim, J.N., Yun, J.S., Ryu, H.W. (1995). *Applied Biochemistry and Biotechnology* 129-132:694-704
- Koller, M. (2005) Innovative and sustainable approaches in the biotechnological production of Polyhydroxyalkanoates from surplus materials. Doctoral Thesis, Graz University of technology
- Koller, M.; Bona, R.; Brauneegg, G.; Hermann, C. et al. *Biomacromolecules* 6 (2005), 561 - 565
- Koller, M.; Bona, R.; Hermann, C.; Horvat, P. et al. *Biocatalysis and biotransformation* 23 (2005) 5, 329 – 337
- Koller, M.; Hesse, P. J.; Bona, R.; Kutschera, C. et al. *Macromolecular bioscience* 7 (2007a), 218 – 226
- Koller, M.; Hesse, P. J.; Bona, R.; Kutschera, C. et al. *Macromolecular symposia* 253 (2007b), 33 – 39
- Koller, M.; Bona, R.; Chiellini, E.; Grillo Fernandes, E. et al. *Bioresource technology* 99 (2008), 4854 – 4863
- Koller, M.; Salerno, A.; Miranda de Sousa Dias, M.; Reiterer, A. et al. *Food technology and biotechnology* 48 (2010a) 3, 255 – 269
- Koller, M.; Reiterer, A.; Malli, K.; Salerno, A. et al. *Nachwachsende Rohstoffe* 56 (2010b), 7.
- Koller, M.; Atlic, A.; Miranda de Sousa Dias, M.; Reiterer, A. et al. *Plastics from Bacteria: Natural Functions and Applications* . (2010c), 85 – 119
- Koller, M.; Hesse, P. J.; Kutschera, C.; Bona, R. et al. Sustainable Embedding of the Bioplastic Poly-(3-hydroxybutyrate) into Sugarcane Industry: Principles of a Future-Oriented Technology in Brazil. - in: *Polymers - Opportunities and Risks II*. (2010d), 81 – 96
- Koller, M.; Salerno, A.; Reiterer, A.; Brauneegg, G.: FP7-Projekt ANIMPOL. - in: *Nachwachsende Rohstoffe* 55 (2010e), 9.
- Koller, M.; Gasser, I.; Schmid, F.; Berg, G. *Engineering in life sciences* 11 (2011a) 3, 222 – 237
- Koller, M.; Reiterer, A.; Salerno, A.; Malli, H. et al. *Nachwachsende Rohstoffe* 60 (2011b), 22 – 22
- Koller, M.; Hesse, P. J.; Salerno, A.; Reiterer, A. et al. *Biomass & bioenergy* 35 (2011c), 748 – 753
- Koller, M.; Salerno, A.; Reiterer, A.; Malli, H. et al. Sugarcane as feedstock for biomediated polymer production. - in: *Sugarcane: Production, Cultivation and Uses*. (2012) In Press

## Koller et al., Production of Biopolymers from Industrial Waste Streams

- Koschuh, W.; Koller, M.; Essl, R.; Kromus, S.: Green Biorefinery - Chloroplast Rich Biomass (CRB) as alternative Bio Feedstock. – Poster presentation at: 25th VH Yeast Conference. Hamburg, Germany, 23.04.2012
- Lee, S.Y. (1996). *Biotechnology and Bioengineering* 49: 1-14.
- Martin, D.P., Williams, S.F. (2003) *Biochemical Engineering Journal* 16, 97–105
- Mizobata, Y., Hiraide, A., Katayama, M., Sugimoto, H. et al. (1996) *Surgery today* 26(3), 173-178
- Nonato, R.V., Mantelatto, P.E. & Rossell, C.E. (2001). *Integrated Production of Biodegradable Plastic, Sugar and Ethanol*, *Applied Microbiology and Biotechnology* 57: 1-5.
- Ouyang, S.P., Liu, Q., Fang, L. & Chen, G.Q. (2007). *Macromolecular Bioscience* 7(2): 227–233.
- Patel, M.K., Bastioli, C., Marini, L. & Wurdinger, G.E. (2005). *Biopolymers online* 21-24.
- Pawan G.I.S., Semple, S.J.G (1983) *The Lancet* 321(8314-8315), 15-17
- Peters D. (2006) *Biotechnology Journal* 1(7-8), 806-814
- Pietrini, M., Roes, L., Patel, M.K. & Chiellini, E. (2007). *Biomacromolecules* 8: 2210-2218.
- Reddy, C.S.K., Ghai, R., Rashmi, K.V.C. (2003). *Bioresource Technology* 87:137–146.
- Ren, Q., Grubelnik, A., Hoerler, M., Ruth K., Hartmann, R., Felber, H., Zinn M (2005). *Biomacromolecules* 6:2290–2298.
- Ruth, K., Grubelnik, A., Hartmann, R., Egli, T., Zinn, M., and Ren Q. (2007) *Biomacromolecules*, 8(1), 279–286
- Solaiman, Daniel K. Y.; Ashby, Richard D.; Foglia, Thomas A.; Marmer, William N. *Applied Microbiology and Biotechnology* (2006), 71(6), 783-789
- Steinbüchel A, Fuchtenbusch B (1998) *Trends Biotechnol* 16:419–427
- Sudesh, K. & Iwata, T. (2008). Sustainability of biobased and biodegradable plastics, *Clean* 36(5-6):433-442.
- Titz, M.; Kettl, K.-H.; Shahzad, K.; Koller, M.; Schnitzer, H.; Narodoslowsky, M. Process Optimization for Efficient Biomediated PHA Production from Animal-Based Waste Streams. *Clean technologies and environmental policy* (2012) Available online ahead of print (doi: <http://dx.doi.org/10.1007/s10098-012-0464-7>)
- Valappil, S.P., Misra, S.K., Boccaccini, A.R., Aldo, R., Roy, I. (2006) *Expert Review of Medical devices* 3(6), 853-868
- Viñas, M., Borzacconi, L. & Martínez, J. (1994). *Environmental technology* 15: 79–85.
- Williams, Martin Applications of PHAs in medicine and pharmacy (2002) In: .Doi Y, Steinbüchel A (eds) *Biopolymers*, vol 4. *Polyesters III—applications* 91-103
- Zhang, X., Luo, R., Wang, Z., Deng, Y. & Chen, G.Q. (2009). *Biomacromolecules* 10(4): 707-711.
- Zinn, M., Witholt, B. and Egli, T. (2001) *Advanced drug delivery review* 53, 5-21
- Zinn, M., Weilenmann, H.U., Hany, R., Schmid, M. & Egli, T. (2003). *Acta Biotechnologica* 23:309-316.