Executive summary:

ANIMPOL elaborated an environmentally sound biotechnological process for conversion of residues of the animal-processing industries towards marketable plastic items.

Until the end of the project, specific animal residues of porcine, bovine and avian origin without interference with human nutrition, were allocated and delivered for production of biodiesel by advanced catalytic methods. Catalytic transformations of this biodiesel by cracking and isomerization to yield more odd numbered carbon chains were a major task of the final project phase. Detailed information was pulled together relating to biodiesel production and rendering for the economic model for assessing feasibility. Other technical aspects regarding the combustion of distillate, effluent treatment, and others, were also considered. In addition, an appraisal of the saturated biodiesel market was carried out which considered changes that might impact on the economics of the ANIMPOL process if realized on an industrial scale.

To further enhance the fermentation performance and to facilitate PHA recovery, nuclease activity of stable transconjugants of Cupriavidus necator and Pseudomonas oleovorans harbouring the nuc gene was investigated. In several clones of both the mutant strains excellent nuclease activity was detected, indicating a significant nuclease expression. Further, the construction of strains with inactivated PHA-depolymerase by inserting a cassette containing a kanamycin resistance gene has been completed. The effect on reduction of PHA depolymerization was successfully tested under conditions of carbon starvation; the wild type C. necator reduced the intracellular PHA to 30% of the initial content, the transconjugant strain C. necator-phaZ1-Kmr maintained a content of 85%.

Using C. necator, sufficient experimental data were elaborated to enable the planning of a pilot-scale production facility. With this organism, it was possible to achieve PHA productivities of 1.34 g/L h, a PHA concentration of 30 g/L, and PHA contents in biomass exceeding 80% on animal-based biodiesel. Such values, until now, were only obtained using expensive carbon sources and/or recombinant microbial production strains. In the case of glycerol, the second waste stream from the biodiesel production, it was possible to achieve high PHA productivities of 2.79 g/L h, a PHA concentration of 30 g/L, and PHA contents in biomass of 65%. For developing latex-like mcl-PHA, work focused on the application of the strains Pseudomonas chlororaphis and Pseudomonas citronellolis. Here, promising productivities of the polyesters comparable with literature data for well-established mcl-PHA production processes for were obtained, and sufficient amounts of material were produced for detailed characterization of the material properties and the ecological performance. Since the project start, a total of about 100 PHA samples from selected microbial strains with different properties (thermoplasts, elastomers, latex) were delivered to the polymer scientists for characterization and processing.

A low structured mathematical model was compiled describing fed-batch cultivation of Ps. chlororaphis on biodiesel. For this purpose, adapted modeling principles, model equations and values of model parameters were established. This model was validated by simulation of additional fermentation data for different cultivation conditions along with models for fed-batch cultivation of C. necator on glycerol and fed-batch

cultivation of C. necator on biodiesel with odd-numbered 3-hydroxyvalerate precursors.

Process design and process development was finalized. The design has been optimized via a Pinch analysis, based on a model of the entire process with the tool CAPAD. A detailed economic analysis based on different scenarios regarding organic nitrogen sources and effect of possible market price fluctuations for by products (biodiesel and meat-and bone - meal) was accomplished. Using these scenarios, a sensitivity analysis feasibility of the process has been calculated. In addition, the ecological assessment of the entire process has been calculated.

The accomplished calculations resulted in an economic assessment of the elaborated ANIMPOL process .The cost appraisal indicated that ANIMPOL-BASED PHA CAN BE PRODUCED AT A PRICE OF LESS THAN 2 EUROS / kg which is below reported values for PHA production starting from pure substrates of nutritional value (glucose, edible oils), but also below the estimated PHA production price based on other agro-industrial waste streams like whey lactose (nearly 3 EUROS / kg). Most importantly, this value is already approaching the production price for petrochemical plastics like polypropylene (about 1 EURO / kg) to be replaced by biopolymers in the not too distant future. This economic assessment is taking into account the allocation of animal-based raw materials, the actual situation on the biofuel market, the optimized data for the bioprocess, a downstream processing without excessive use of chemicals, and enhanced energy-input scenarios. Especially for downstream processing, the new method, based on high-pressure homogenization, is an additional benefit of the ANIMPOL process, both in terms of economics and for environmental safety.

The ecological footprint of the ANIMPOL process is compared to competitors like fossil based PE polymer and PHA based on 'classical' feedstocks of nutritional value, indicating the ecological benefit of this new technology. The effect of use of renewable energy as well as comparison of plant geographic location and CO2 emission per ton of PHA production was studied in details, indicating that the ecological foot print of ANIMPOL-PHA shows a bandwith from 373,000 to 956,000 m²/t PHA, depending on the applied energy source. This is a clear benefit to the ecological foot print of 2,508.000 m²/t polypropylene as the main petrochemical competitor.

Regarding ANIMPOL- based marketable products, formulations were developed for production of PHA foils displaying enhanced properties such as decreases crystallinity and improved processability. In total, 14 formulations were developed for processing PHA with bio-compatible materials; among them, 5 were used for preparing prototype items via melt extrusion or compression molding. Cyto- and genotoxicity of the new plastic formulations were assessed, indicating no adverse effects on living cells.

The high public acceptance of the ANIMPOL process is manifested by the numerous contributions in the European broadcast (TV, radio), internet, and international print media. In addition lectures and poster presentations were accomplished at scientific and industrial events. The scientific impact of the ANIMPOL results is indicated by a total of more than 20 original articles in peer-reviewed scientific journals and many more in preparation. The polymer-scientific part of ANIMPOL already achieved one patent application together with several additionally envisaged patent applications accruing from the entire process.

The consortium was able to reach all envisaged Milestones, and is able to offer a holistic process for production of biopolyesters and follow-up products (composites, blends) based on waste streams from the animal processing industry. This ANIMPOL process now is ready to be implemented on (semi)industrial scale by interested industry willing to break new ground.

Project context and objectives:

Specific objectives of the project and how they were addressed during the project period

- Design of an integrated industrial process dedicated to the bio-mediated, cost-efficient production of value-added, biodegradable polyhydroxyalkanoate (PHA) biopolyesters, by starting from lipid waste (tallow) from slaughterhouses, rendering industry, and waste fractions of the biodiesel production. These wastes are upgraded to the role of resources for renewable raw materials, thus bringing together diverse waste producing players.

This envisaged process was designed based on the experimental input of all project partners. A detailed and complete process flow diagram, encompassing energy and material flows and balances, was elaborated and is available for industrial implementation.

- Improvement of the quality of biodiesel by removal of its saturated fraction.

The saturated fraction was removed from the animal-based biodiesel mix by a convenient technique and successfully applied as fermentative carbon feedstock. In addition, this saturated fraction was used for catalytic generation of precursors for biotechnological production of special PHA-building blocks (3-hydroxyvalerate).

- Assessment of the raw material sources (lipids from animal waste, saturated biodiesel fraction, surplus glycerol from the biodiesel production) for the fermentation process by selected microbial strains suitable for the production of structurally diversified PHAs under physiological stress conditions.

Different waste fractions were investigated regarding the fatty acid pattern of their lipid fraction. The lipids were converted to biodiesel which, together with glycerol as the second waste stream of the lipid conversion, was used as carbon source for various microbial PHA producers.

- Aiming at the improvement of microbial growth on the selected substrates and the quality and amount of the polymer produced; appropriate strains were studied by different approaches which include recombinant gene expression and host cell genome modification for enhanced expression of nuclease and deletion of intracellular PHA breakdown.

Strains were selected and assessed both for the production of thermoplastic scl-PHA and latex-like mcl-PHA.

- Set-up of microbial growth and production phase amenable to be scaled-up for a novel optimized directed production of structurally predefined PHAs. The experimental polymer production under efficient control strategies using fed-batch cultivation will be developed aiming at reproducible product quality. Predefinition of the polymer structure on a molecular level, hence the monomeric composition was accomplished by triggering the feeding of main- and co-carbon sources.

Reproducible fermentation protocols for up-scaling the process to industrial conditions were established. The fermentation experiments were kinetically analyzed and converted into mathematical models.

- Development of an environmentally safe and efficient downstream processing for recovery and purification of PHAs; separation of PHAs from microbial biomass by cheap and ecologically benign techniques avoiding harmful solvents, focusing on mechanical cell disruption techniques, were implemented, monitored, and assessed.

Novel recovery methods with restricted solvent requirements were assessed and compared to classical PHA extraction methods.

- Chemical, structural, biological, physical and mechanical characterization of the produced PHAs.

The biotechnologically produced materials were characterized regarding their material properties. New dependencies of the cultivation conditions on the material properties were recognized and described.

- Preparation of blends & composites of the PHAs with selected polymeric materials including synthetic analogues of PHA, inorganic and/or organic fillers like nanofillers. The organic fillers will be taken from renewable agro-waste (lignocelluloses, polysaccharides and surplus crops), either directly or after appropriate physical or chemical modification whereas organophilized inorganic fillers (clays, sepiolithes and calcium carbonate) will be used.

Blends and composites were produced based on PHA and various compatible materials (clays, hazelnut shells, pine shells, natural fibers etc.) and characterized.

- Engineering design of PHA production, extraction and purification unit operations combined with the analysis of the cost effectiveness of the industrial process as set up in the down-stream processing of slaughterhouses, medium and large rendering and biodiesel factories.

The process was designed based on the facility units and is ready for industrial realization.

- The cost effectiveness of the process for industrial scale production of PHAs will be assessed in terms of costs of raw materials, chemicals (additives, cofactors, solvents) and energy required for the production of PHAs and its blends. This issue is a key factor for the success of the project as the introduction and penetration into the market of new polymeric materials has to be competitive in terms of cost/performance balance.

The accomplished economic and ecologic process assessment was completed, indicating the environmental benefit of the new process in terms of the ecological foot print, and the possibility to reduce PHA-production costs by using the ANIMPOL-process in comparison to established methods based on expensive raw materials and even other carbon-rich waste streams. The cost appraisal indicated that ANIMPOL-BASED PHA CAN BE PRODUCED AT A PRICE OF LESS THAN 2 EUROS / kg which is below reported values for PHA production starting from pure substrates of nutritional value (glucose, edible oils), but also below the estimated PHA production price based on other agro-industrial waste streams like whey lactose (nearly 3 EUROS /

kg). Most importantly, this value is already approaching the production price for petrochemical plastics like polypropylene (about 1 EUROS / kg) to be replaced by biopolymers in the not too distant future.

- Assessment of eco-compatibility with evaluation of biodegradability under different environmental conditions (solid and aqueous media) of the obtained PHA formulations as well as of some selected prototype items based on relevant blends and composites. Validation of the selected items eco-compatibility was assessed by means of LCA and ecotoxicity tests.

The eco- and biocompatibility of the produced PHA was demonstrated by the accomplished experimental investigations.

- Assessment of biocompatibility of some selected PHA formulations and relevant items processed by means of in vitro cell toxicity and genotoxicity tests in respect of their potential value-added applications in food, packaging and biomedical fields.

Also the eco- and biocompatibility of the follow-up products was demonstrated by the accomplished experimental investigations.

- The utilization of novel biodegradable plastics, as attainable by means of environmentally sound process based on waste from renewable resources as raw material, for environmentally friendly plastic materials, meeting the EC directive 62/94 and the subsequent national regulations, will constitute the ultimate goal of the project.

This is the step that has to follow based on the elaborated results! The ecological footprint of the ANIMPOL process is compared to competitors like fossil based PE polymer and PHA based on 'classical' feedstocks of nutritional value, indicating the ecological benefit of this new technology. The effect of use of renewable energy as well as comparison of plant geographic location and CO2 emission per ton of PHA production was studied in details, indicating that the ecological foot print of ANIMPOL-PHA shows a bandwith from 373,000 to 956,000 m²/t PHA, depending on the applied energy source. This is a clear benefit to the ecological foot print of 2,508.000 m²/t polypropylene as the main petrochemical competitor.

Project context considering ecological and economical aspects

Based on ecological considerations, an increasing need for industry to switch from classical plastic materials to biobased alternatives like polyhydroxyalkanoates (PHAs) is nowadays undisputed. This can be underlined by the estimated amounts of about 280 Mtons of petrochemical plastics produced globally in 2013, with expected annual increases of 4%. Economic reasons are generally considered as the major obstacles for replacing conventional commodity and semi-commodity plastics by PHAs on a relevant scale. The project was needed as a viable strategy to enable production of these highly promising biopolyesters in Europe, to avoid that in the future PHAs have to be imported from outside the EU. If successfully implemented now, these seminal biopolymers will be decoupled from manufacturing in various global regions, where biopolyester and biofuel synthesis competes with human nutrition, and frequently lacks consideration of environmental requirements and acceptable human working conditions.

Currently, the production of plastic materials demands about 5% of the entire delivered amounts of fossil feed stocks. This percentage is forecasted to rise significantly during the next decades as a consequence of the increase of the economy and life level of emerging countries and countries in transition. In order to become competitive on the market, the price per kg of a biopolymer for a special application must be in the same range as the competing 'classical' plastic. Hence, the production costs of PHAs have to be minimized considerably despite the instable market price for mineral oil. The utilization of diverse waste streams which do not interfere with the nutrition chain but constitute severe disposal problems for the industrial branches where they accrue can be considered as the most promising approach in making PHAs more competitive.

ANIMPOL elaborated the sustainable and value-added conversion of waste from slaughterhouses, rendering industry, and waste fractions of the biodiesel production. Lipids from slaughterhouse waste were converted to fatty acid methylesters (FAMEs, biodiesel). FAMEs consisting of saturated fatty acids generally constitute a fuel that has an elevated cold filter plugging point (CFPP) which can be somewhat limiting in blends that exceed 20% (vol/vol) FAMEs. In the ANIMPOL process, these saturated fractions are biotechnologically converted towards high-value added biopolymers. As a by-product of the transesterification of lipids to FAMEs, crude glycerol phase (CGP) accrues in high quantities (about 1 kg per 10 kg biodiesel). CGP is also available as carbon source for the production of catalytically active biomass and the production of low molecular mass biopolymers. This brings together waste producers from the animal processing industry with meat & bone meal (MBM) producers (rendering industry), and with the bio-fuel industry, resulting in value creation for all players.

According to the European Biodiesel Board, the available saturated biodiesel fraction in Europe amounts to annually 50.000 tons; the entire amounts of animal lipids from the slaughtering process can be quantified with more than 500.000 tons per year. From the saturated fraction, the amount of PHA biopolyesters that can theoretically be produced amounts to approximately 35.000 tons annually, if calculated with a conversion yield of 0.7 g/g. The surplus CGP from the biodiesel production can be quantified for Europe with annually 265.000 metric tons (estimations for 2008; Oleoline glycerine market report). If this glycerol is applied for production of catalytically active biomass as foreseen in the project, one can expect 0.4 to 0.5 g biomass per g of glycerol.

Hence, ANIMPOL developed an integrated process, comprising the scientific fields of microbiology, genetic engineering, biotechnology, fermentation technology, chemistry, chemical engineering, polymer chemistry & processing, LCA and Cleaner Production studies, combined with feasibility studies for the marketing of the final products. The research was performed in close cooperation of academic and industrial partners. The outcomes can contribute to solving local waste problems which affect the entire European Union; the solutions were developed on local scales, but are meant to be applied to the entire EU.

Specific background regarding raw materials and products

Only a few technologies are found in current industrial processes that can successfully convert feedstocks that are made up of a high percentage of saturated fat. The most successful technology which has been developed

and implemented is the production of fatty acid methyl esters (FAME) that can be used as liquid fuel (biodiesel). The most prevalent issue with FAMEs is its operability at lower temperatures specifically with cold filter plugging (CFPP) point at blends over 20% saturated FAMEs on vol/vol basis. Considering blends greater than 20% would require modifications to the vehicle injection system. An operator that uses a saturated feedstock also requires a good understanding of handling as the raw material often solidifies at room temperature.

PHAs constitute a family of polyesters classified as bio-based, biodegradable and biocompatible materials. 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. Economic reasons are pointed out as the major obstacles for replacing common plastics by PHAs on a relevant scale.

Plastics that should be replaced by novel materials like PHA are those accruing at huge amounts, such as polypropylene (PP) or polyethylene (PE). These materials are utilized only during a relatively short time span. After that, they are often incinerated, elevating the atmospheric CO2 concentration. The main problem arising from incineration of plastics is the same as for energy recovery from fossil feedstocks: carbon that was fixed during millions of years and that within this time was not part of the natural carbon cycle, is converted to CO2 that can accumulate in the atmosphere, contributing to negative climatic effects.

Beside, more and more waste of the said highly recalcitrant plastics that are not incinerated is piled up every year, thus aggravating the landfill crisis. Recycling systems demand a sufficient degree of purity and a certain sorting accuracy. In addition, the collection costs are fairly high, and each recycling cycle has a negative impact on the mechanical properties of the materials, such as an increase in brittleness.

In order to become competitive on the market, the price per kg of a biopolymer for a special application must be in the same range as the competing 'classical' plastic. Hence, the production costs of PHAs have to be minimized considerably despite the increasing market price for mineral oil. According to the 'state of the art', these biopolyesters are produced on a larger scale starting from rather costly carbon sources like purified sugars. Classically, it is estimated that the PHA production costs from purified substrates are about five to ten times higher than the costs for petrochemically produced plastics like PP or PE, as indicated by the market prices for PHAs from China (companies Tianan, Tianjin & DSM), the USA (Telles, Tepha Inc., P&G), or Brazil (PHB Industrial S/A); this goes for the case of thermoplastic scl-PHAs. In the case of mcl-PHA to be used of elastomers, the market prices are even higher (Polyferm Canada; about 200 US-dollars per 10g of mcl-PHA).

On the one hand, recent studies indicate that this production of PHAs from such 'classical' carbon substrates has been economically optimized to a high extent. On the other hand, diverting materials that are of high importance for nutrition of mankind, towards biotechnological production of 'green plastics' raises serious ethical conflicts considering the enormous number of starving people worldwide and the increase in the world population, and hence in needs for food & commodities. Converting nutrients like edible oils or sugars to fuels or polymers cannot be regarded as an ethically viable strategy for the future (see contemporary

'food versus fuel' or 'plate versus plastic' controversy). In contrast, the utilization of diverse waste streams which do not feature any value for nutrition but constitute severe disposal problems for the industrial branches where they accrue can be considered as the most promising approach in making PHAs more competitive. In the past, successful attempts in utilizing waste materials for PHA production have been done based on surplus whey from dairy industry (WHEYPOL, Growth EU project GRD2-2000-30385) and more recently from wastewaters and perujo of olive oil pressing industrial units. (POLYVERY Graft FP6 EU Project COOP-CT-2006-032967). In the case of PHA production from whey, calculations show that it was possible to lower the production price to below 3 EUROS per kg pf PHA. In the proposed project, waste streams from slaughterhouses, rendering industry, and waste fractions of the biodiesel production are upgraded to feed stocks for biotechnological PHA production. These waste materials constitute innovative alternatives to the conventionally used carbon-sources and processes. The proposed utilization of the waste resources mentioned above contribute to the reduction of the production costs and feature a future-oriented approach of solving disposal problems for the involved industrial branches.

Biotechnological PHA production occurs in aerobic processes, therefore only about 50% of the 'classic' carbon sources like sugars, and even a lower percentage of the precursors used for generation of copolyesters end up in the desired products. Normally, 0.48 g poly-3-hydroxybutyrate (PHB) can be expected per g sugar (value without taking into account the sugar consumption for biomass formation). Based on sugar cane sucrose, the company PHB Industrial S/A in Brazil produces, on pilot scale, PHB at a mass conversion yield of 0.3 g/g. Based on the metabolic circumstances of the microbial PHA producers (beta-oxidation pathway for break-down of long alkyl chains to acetyl-CoA), this value can be doubled using biodiesel instead of carbohydrates like sugars. This was demonstrated by the results obtained in ANIMPOL.

During the last couple of years, the preparation of composites and blends has turned into one of the key research fields in biopolymer science. For enhancement of the material properties, PHAs or their derivatives like polyester urethanes 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), PLA, poly(e-caprolactone) (PCL) etc., including synthetic analogues of PHA (e.g. atactic PHB), inorganic fillers (clay materials or calcium carbonate), and organic fillers of agricultural origin such as bagasse, powdered or in form of fibers, was successfully tested. Concerning fillers from agriculture, the application of surplus materials like lignocelluloses such as sugar cane bagasse, hazelnut shells, wheat flour, fruit peels, crop fruit fibers, saw dust, wheat straw, or cellulose derivatives (e.g. cellulose acetate) is of major interest.

In general, nano-composites and natural fibers composites have to be distinguished. Nano-composites have the potential to enhance special polymer properties, such as gas permeability and thermal and mechanical characteristics. Novel nano-biostructured packaging materials are described especially for application in the food packaging sector. For creation of nano-composites, rather small amounts of the filler, usually an organophilic modified clay, are needed for efficient enhancement of the material performance. Natural fibers composites often display excellent mechanical properties, and, as desired for many applications,

they result in a lower 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 generally enhanced. Because such fibers do not feature a considerable price or even constitute waste materials, this normally goes in parallel with a reduction of the entire production cost of the marketable composite product. Therefore, ANIMPOL developed also a process for using agricultural waste as filler for production of PHA-based composites. This provides an additional solution for agriculture to upgrade surplus materials, and further lowers the overall costs of bioplastic items.

Project results:

The following section provides a summary of S & T results/foregrounds and details for each task in line with the structure of Annex I to the Grant Agreement on a WP and task-by-task basis; significant results are highlighted

WP1: feedstock and upstream processing (RDT)

Task 1.1: supply and costing of raw materials

This task is dedicated to the supply of raw materials and the examination of sources of raw materials. The deliverable of all raw materials was accompanied by the examination of the related costs. The allocation of animal waste was done by Partner 11 (RIX) and the saturated biodiesel fraction was delivered by Partner 5 (ARGENT).

A broad range of animal waste materials, saturated FAME (biodiesel) and its by-product crude glycerol phase was delivered by the beneficiaries RIX and ARGENT since the beginning of the project. The delivery occurred according to the requirements of the project partners doing further work with these materials (TUG, UNIPD, KFU) for other work packages. These requirements and allocation strategies for special waste fractions (e.g. porcine) where discussed during several direct meetings and via means of telecommunication. It can be underlined that the flow of raw material during the entire project period was fulfilling the needs for all involved project partners.

The responsible company RIX delivered the subsequent materials to the before mentioned project partners:

January 2010 (to get started!):

3.7 kg of pork belly with rind (residue from the bacon production) as lipid-rich reference material. Material analyzed in details by partner KFU

Delivered later during the first 6 months:

- Entire amount of more than 10 kg of 4 different porcine organs that clearly constitute waste materials for the meat processing industry without interference with food or feed purposes (intestines and guts, udder, spleen, heart)!!

Delivered later:

-High quantities (22 kg) of a variety of special organs and waste fractions (e.g. porcine liver with high amounts of odd-numbered fatty acids, porcine reticulum, lungs, epididymis [fatty acid pattern was unknown before!]

-Ca. 5 kg of chicken waste. The company optimized crushing of poultry bones by milling prior to drying via lyophilization.

-Crushed bovine head (including brain; crushing procedure optimized in the company; 17 kg)

-Bovine spleen (8 kg)

As an additional task, the company RIX contributed to the cost assessment of the materials.

Materials displaying a market value [EUROS/kg]:

Heart: 0.85

Lung: 0.60

Liver: 0.70

Spleen: 0.80 (Bovine: 0.70)

Reticulum: 0.60

Udder: 0.40

Materials that have to be disposed of (cost demanding!):

Porcine testicles: disposal 0.30 EUROS/kg.

Bovine residues: Spinal marrow, intestines and guts, Head plus brain: risk materials – disposal 60 EUROS/t.

Avian residues (poultry waste): real waste material normally to be disposed of!

Since the start of the ANIMPOL project there have been a number of significant changes in the marketplace. These changes will impact directly on the economics of the overall process especially when considering the overall production costs in the generation of PHA's from the saturated fraction of animal fats. The original idea behind the project was to split the saturated fraction from that of the unsaturated part. The saturated fraction deemed as the inferior part would be used in the PHA production process using biotechnology. The unsaturated fraction seen as the high quality part of the methyl ester would be used as a high quality fuel. There was also a more desired fraction which possesses odd number carbon chains that would add further value the final biodegradable plastic.

When tallow derived methyl ester was first introduced into the market overall volumes were scares as this type of material was not fully understood. This made the trading of this material somewhat problematic. The blenders and fuel distributors were nervous of utilizing products that possess higher levels of saturation as this was seen as inferior to the unsaturated vegetable based oil. Their concerns were focused to operational issues in the field, especially those related to the temperature and more specifically cold flow plugging point (waxing/crystallization of fuel).

Argent has been supplying the market since 2005 with both used cooking oil (UCOME) and tallow methyl esters (TME). Initially supplying a higher proportion of UCOME this has now changed to predominantly category I tallow. During this period Argent secured a route for a mix of UCOME and TME to a major distributed/ petrochemical producer this was the first major step to instill a degree of confidence. This continued over a period but never saw dramatic increases of TME being blended into main

stream fuels. Argent, were then able to supply TME to a major global producer/distributor that were confident that this product could at least be utilized over the summer operating period. This continued for a lengthy period until economics dictated that we change. At this period Argent were seeing increase selling prices, however the differential between vegetable based fuels and TME were still sizeable.

Increased lobbying and the participation from Argent employee/experts over the years to the present date have increased the awareness and the acceptance of more saturated products in the marketplace. Involvement/participation in the construction of new fuel standards both on a national and European basis how facilitated an increased scope of new products from different raw materials. The new revision of EN 14214 has seen the introduction of a new distilled grade which includes TME with lower saturated mono-glycerides which can be used in a much greater range of blend over winter periods. This is seen as a crucial step for increasing potential volumes of biofuels to the market place. Emphasis is always focused around quality and potential operating constraints to protect the fuel market. On this basis TME is characterized a fuel of the highest quality which is fit for purpose which is significant change in classification from a number of years ago when it was classified as inferior. TME in its saturated form has some major advantages over the non-saturated types in that the product is more stable and it possess a higher cetane numbers that improves combustibility. The cold climatic using tallow can also be met under the current mandate of a 7% biofuels inclusion into a good quality petroleum base diesel.

The use of TME has also found new applications in higher blends that go much further than the 7%, in fact Argent currently supply blends in excess of 20%. These blends are used in fleet application like buses and have proven to be highly successful.

Other areas that have further enhanced the profile and value of TME is the introduction of the certificate system for methyl esters that have been generated from waste products like category I tallow and used cooking oils.

The renewable transport fuel obligation (RTFO) order obligates fossil fuel suppliers to produce evidence showing that a percentage of fuels for road transport supplied in the UK come from renewable sources and are sustainable, or that a substitute amount of money is paid. All fuel suppliers who supply at least 450,000 liters of fuel a year are obligated. This includes suppliers of biofuels as well as suppliers of fossil fuel.

Owners of biofuel at the duty point are awarded one (RTFC) per liter of biofuel supplied, however the December 2011 amendment to the RTFO order introduced double rewards for some fuel types, including those made from waste materials such as CAT 1 tallow and UCO, together with a requirement to have data on the carbon and sustainability performance of fuels to be independently verified before renewable transport fuel certificates (RTFCs) are awarded.

RTFCs may be earned irrespective of the volume of biofuel owned, providing a potential revenue stream for even the smallest suppliers. RTFCs may be traded between participants in the scheme.

Currently RTFCs are trading at around 15 EUROS-cents which equates to 30 EUROS-cents per liter or for that biodiesel which is eligible for double counting.

Changes in EU legislation relating to biofuels are also beneficial to further the use of TME i.e., Renewable Energy Directive and possibly ILUC.

The sales differential between TME and more conventional fuels like SME, RME, and UCOME has lessened over the years on the basis that it is produced to the same high quality but also they can operate under regional climatic conditions. Prices a few years ago were as low as 70 EUROS-cents for TME and these have been as high as 97.5 EUROS-cents however this varies somewhat. There is also a differential of approximately 5-6 euro cents between UCOME and tallow however the raw material is higher than that of category I derived TME. It should also be stated that the cost of category I tallow has changed substantially from about 243 EUROS euro/t to 585 EUROS/t and edible grade category III has a premium of about 67 euro/t. As category III tallow is used as a feed this will not be eligible under the certificate system and would forfeit the enhanced value in the biodiesel. These values translated into cost in the manufacturing matrix would have major implications on the viability of the PHA production process.

In addition to the delivery of biodiesel to the involved project partners according to their requirements during the entire project time, the work carried out during the later period investigated catalytic transformations of tallow methyl ester to yield more odd numbered carbon chains. A literature search was first conducted followed by extensive reaction work using numerous catalysts. The material was cracked and isomerized to try to achieve goal. The work was productive in that the amount of C15 and C17 was considerably increased. The solubility of the final was an issue for the fermentation and we looked to find a suitable dispersant.

A lot of separation techniques were investigated with limited success especially in terms of yields and these processes would be very expensive on an industrial scale. Other industrial scale processes were also considered during this investigation.

Detailed information was pulled together relating to biodiesel production and rendering for the University of Graz to use this in their economic model for assessing feasibility. Other technical aspects were also considered and supplied to the University regarding the combustion of distillate and effluent treatment etc.

Finally an appraisal of the saturated methyl ester market was carried out which considered changes that might impact on the economics of the project going forward.

Task 1.2: Assessment of raw materials

Different slaughterhouse waste materials from pork, beef and chicken were delivered from partner 11 (RIX). Out of that, udder, honeycomb stomach, liver, heart, epididymis, testicle, lung, bacon rind, spleen from pork and brain, spleen and head bones from beef and waste material from chicken were extracted, the fatty acid composition was analyzed and the overall quality for the further purpose as a feedstock for

transesterification and fermentation was evaluated. All samples were extracted with hexane and the thus obtained fat fraction was determined.

Regarding the search for odd-numbered fatty acids like margaric acid (heptadecanoic acid), acting as 3-hydroxyvalerate (3HV) precursors in biotechnological PHA synthesis, the amount of margaric acid (the expected key co-substrate for 3HV production in the fermentation experiments) was very low. Hence, alternative strategies for production of odd-numbered compounds had to be assessed and carried out (see task 1.3).

Emphasis is put on the monitoring of the different feedstock qualities of waste animal fats and slaughterhouse products. Partner 5 (ARGENT) and Partner 11 (RIX) delivered different feedstocks. ARGENT delivered distilled biodiesel and the corresponding tallow feedstock and RIX animal waste material porcine, bovine and avian origin. The distilled biodiesel was analyzed according to EN 14214. Due to the fact that no specific European standard exists for the determination of the qualities of waste feedstocks for biodiesel production, parameters characteristic in fats and oil analysis where selected and determined. Based on the results a statement about the expected quality of the corresponding fatty acid methyl ester (FAME) can be made. The obtained results are given in detail in Deliverable 1.3 This analytical part was ongoing until the end of the project.

The obtained fatty acid alkyl esters were separated into a saturated and an unsaturated fatty acid fraction via low-temperature-crystallization. The unsaturated fractions had better cold flow properties than their corresponding original esters. For example the cold cold-filter-plugging-point from original fatty acid methyl esters (FAME) decreased from 10 °C to -26 °C in the unsaturated fraction. The different saturated fractions were delivered to partner 1 TUG and investigated as a carbon source for the fermentation process.

Task 1.3: Modification of raw materials

The extracted fat and the tallow feedstock were used for classical and enzymatic catalyzed transesterification reactions. As enzymes two commercial lipases from Candida antarctica and Mucor mihei as well as a sample from Partner 2 (UNIPD) were investigated. For the transesterification experiments methanol, ethanol, propanol, iso-propanol, butanol, tert-butanol and iso-amyl alcohol were used. The overall yields of fatty acid alkyl esters were between 80 – 90 %. The results with tert-butanol were insufficient. Therefore, the production of tert- butyl ester was carried out by the esterification of free fatty acids with isobutene. Also, the ester yields coming from enzymatic transesterification with the enzyme received from partner 2 were insufficiently low.

In addition to the transesterification of animal lipids to fatty acid esters containing odd-numbered alcohol moieties, odd numbered precursors for 3-hydroxyvalerate (3HV) production were produced by chemical catalysis. Here, the saturated fatty acid ethyl ester fraction was taken as starting material for the production of odd-numbered carboxylic acids. This fraction was used to produce fatty alcohol using sodium and abs. ethanol in a first step. Via dehydration at high temperatures and alumina oxide as a catalyst 1-olefins were produced thereof. Finally, a mixture of carboxylic acids (C9 to C17) was synthesized from olefins via

oxidative ozonolysis. Also these samples were delivered to TUG and successfully tested as a carbon source for PHA-production.

Task 1.4: Screening and utilization of lipases

Summary: Although Acinetobacter venetianus DSM 23050 lipase activity was found promising using different triglycerides (tributyrin, maize oil, commercial lard, bacon rind or tallow provided by P4) as substrates (both at 37 and 55°C), also other interesting PHA-producing strains were selected: Hydrogenophaga palleroni DSM 63, Pseudomonas oleovorans DSM 1045, Pseudomonas fragi DSM 3456, Cupriavidus necator DSM 545 and Diaphorobacter sp. DSM 13225. More than 50 new isolates were selected for assessing their lipase activity once isolated from environmental samples (water and soil) and from waste (water and mud) coming from a slaughterhouse, and then tested for PHAs production. After an exhaustive discussion with all the Partners a decision was taken to adopt for further studies the two following strains: C.necator DSM 545 and Ps. oleovorans DSM 1045.

UNIPD:

After an exhaustive discussion, the decision was taken to search for bacterial strains, culture collection strains or new isolates that show lipase activity. A bibliographic study was performed in the first period of these 6 last months for the use of bacterial lipases and Acinetobacter venetianus DSM 23050 (or Acinetobacter sp. RAG-1) was selected as promising lipase producer. Other culture collection strains and new isolates from water, soil or slaughterhouse were also studied.

Acinetobacter venetianus DSM 23050: This strain was previously described to produce an extracellular lipase (LipA) with the following characteristics (Snellman et al., 2002 Eur. J. Biochem. 269: 5771-5779): the enzyme LipA is released into the growth medium during the transition to stationary phase, and it was found to be stable at pH 5.8–9.0, with optimal activity at pH 9.0. This lipase remained active at temperatures up to 70° C, with maximal activity observed at 55° C. LipA is active against a wide range of fatty acid esters of p-nitrophenyl, but preferentially attacks medium length acyl chains (C6, C8). The enzyme demonstrates hydrolytic activity in emulsions of both medium and long chain triglycerides.

In the present study Acinetobacter venetianus DSM 23050 lipase activity was tested in solid DSMZ 92 (Trypticase soy yeast extract medium), nutrient or TRA medium containing different lipid substrates for this enzyme. Bacon rind and tallow were delivered by Partner 4. Halo around colonies was detected on solid medium containing different triglycerides (tributyrin, maize oil, commercial lard, bacon rind or tallow), indicating that there is a lipase activity present in all cases. Activity was revealed using the entire culture or only culture supernatants. The signal is stronger when rhodamine dye is used and observations are performed under UV-light.

Lipase activity was also tested in liquid medium containing different substrates for this enzyme. Medium contained triglycerides as carbon sources. Bacon rind and tallow were delivered by partner 4 (KFU). Lipolytic activity was determined at 30 °C, pH 8.0, by spectrophotometric method according to Winkler and Stuckmann (1979, J Bacteriol 138(3):663-670) using p-nitrophenyl palmitate as substrate. The quantitation was

carried out with a standard p-nitrophenol curve measuring absorbance at 410 nm. The enzymatic activity (U) was expressed as micromoles of p-nitrophenol released per minute and mL of crude lipase preparation (µmol min-1 ml-1).

Activity was detected in liquid culture in the supernatants of A. venetianus DSM 23050 containing different triglycerides (tributyrin, maize oil, commercial lard, bacon rind or tallow) as substrates.

Production of lipase rich crude extracts. With the aim to check if lipase rich crude extracts of A. venetianus DSM 23050 could be useful for the conversion by transesterification of rendering fats compared to classical chemical reaction or conventional enzymatic reaction, some crude enzyme extracts were prepared as followed: Acinetobacter venetianus DSM 23050 recovered from frozen stock (-80°C) was used to inoculate DSMZ 92 agar medium (Trypticase soy yeast extract medium). Bacteria were led to grow at 30°C. Single colonies were selected for inoculation of a primary culture in 100 mL liquid DSMZ 92 medium and grown at 30°C for 48 h with shaking at 150 rpm. A 1-L Erlenmeyer flask containing 300 mL of DSMZ 92 medium or minimal medium (LNPS) was inoculated with the primary culture at 5% inoculum. To produce lipase the liquid medium was supplemented with different kinds of triglycerides (2.5 % w/v), emulsified with arabic gum (1.0 % w/v). As triglycerides, corn oil (maize oil) or tallow from Partner 5 was used. After incubation for 48-72 h in DSMZ 92 or minimal medium amended with maize oil or tallow (2.5 % w/v), cells were removed by centrifugation 5000 x g at 4°C for 20 minutes and supernatants pooled. Ammonium sulphate was added to cell-free supernatant (crude enzyme) to a final concentration of 65 % w/v saturation with 10 % intervals. After that, the suspension was kept at 0°C for 30 minutes with gentle stirring. The precipitate was collected by centrifugation at 10000 x g and 4°C for 30 minutes, and further dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enzyme solution (65 % w/v ammonium sulphate precipitation) was dialyzed extensively against the same buffer at 0°C. The dialysate was then lyophilised and sent to Partner 4. Some supernatants were directly lyophilised and others were first dialysed and then lyophilised before sending them to Partner 4.

Other culture collection strains and new isolates: With the aim to find other interesting lipase activities, different bacteria were tested: (a) culture collection strains Hydrogenophaga palleroni DSM 63, Hydrogenophaga pseudoflava DSM 1034 and DSM 1084, Pseudomonas oleovorans DSM 1045, Pseudomonas fragi DSM 3456, Paracoccus denitrificans DSM 413, C. necator DSM 545, Azotobacter vinelandii DSM 86 and DSM 576, Diaphorobacter sp. DSM 13225 and new isolates deriving from water and soil; (b) new isolates deriving from slaughter-house.

(a) Other culture collection strains, soil and water isolates. Bacteria were grown first in a primary culture in liquid rich medium at their optimal growth temperature for 48 h with shaking at 150 rpm, and then transferred to a secondary culture in MSM liquid medium supplemented for induction of lipase with maize oil or tallow and as emulsified with arabic gum 0.7 to 1.0 % w/v. Bacon rind and tallow were delivered by partner 4 as well as lipids extracted from Udder and Lung. Assays were performed in the presence of rhodamine dye and visualized at visible- and UV-light.

With the aim to check if other lipase rich crude extracts deriving from some of the bacteria selected above could be useful for the conversion by

transesterification of rendering fats, enzyme crude extracts were prepared. Bacteria were grown first in a primary culture in liquid rich medium at their optimal growth temperature for 48 h with shaking at 150 rpm, and then transferred to a secondary culture in liquid minimal medium. The medium was supplemented for induction of lipase with maize oil and as emulsifier with Arabic gum 0.7 % w/v. Culture collection strains Pseudomonas oleovorans DSM 1045 and Pseudomonas fragi DSM 3456 and isolates from soil 3S and 7S were grown in DSMZ 81 medium. Water isolate 8H was grown in HM-I medium. After incubation for 48-72 h in liquid medium amended with maize oil, cells were removed by centrifugation 5000 x g at 4°C for 20 minutes and supernatants pooled. Supernatants were directly lyophilised and delivered to Partner 4.

(b) New isolates from slaughter-house. In order to find other bacteria able to use lipids coming from animal waste material, samples were taken directly from a slaughter-house. Five different types of samples were taken: Waste water, External well (liquid), External well (mud), Fresh stored Mud, Dry stored Mud. Two strategies were followed: (i) direct isolation of colonies; (ii) enrichment before colony isolation on different triglycerides delivered by Partner 4.

i. Direct isolation of colonies was performed on solid yeast-peptone medium or Beef meat-peptone medium supplemented with maize oil (2.5 % v/v) or commercial lard (2.5 % v/v) emulsified by Arabic gum (1.0 % w/v). Rhodamine dye was added to evidence colonies with putative lipase activity.

ii. Enrichment was performed in liquid beef extract medium supplemented with Arabic gum as emulsifier and bacon rind, tallow from Argent, lipids extracted from Udder, Heart and Lung as substrates. Growth was carried out for 96 h. Two consecutive cultures were performed before colony isolation. Colonies were then isolated in the respective substrate used for enrichment on solid Beef extract medium with Arabic gum (1.0 % w/v). Rhodamine dye was added to evidence colonies with putative lipase activity.

A number of rhodamine-positive colonies were found and kept for further analysis. In the meantime all the isolates were identified by 16S rDNA sequence analysis in order to exclude possible pathogenic strains.

KFU:

The extracted fat and the tallow feedstock were used for classical and enzymatic catalyzed transesterification reactions. As enzymes two commercial lipases from Candida antarctica and Mucor mihei as well as a sample from partner 2 were investigated. For the transesterification experiments methanol, ethanol, propanol, iso-propanol, butanol, tert-butanol and iso-amyl alcohol were used. The overall yields of fatty acid alkyl esters were between 80 – 90 %. The results with tert-butanol were insufficient. Therefore, the production of tert-butyl ester was carried out by the esterification of free fatty acids with isobutene. Also, the ester yields coming from enzymatic transesterification with the enzyme received from partner 2 were insufficiently low.

The conversion of rendering fats into biodiesel can be carried out by transesterification via classical or enzymatic synthesis. In that order, lipases can be used to perform this reaction. These enzymes are able to catalyze the hydrolytic cleavage and the synthesis of esters bonds in

glycerol esters. The transesterification of animal fat into biodiesel requires high temperatures, which are near to the lipases denaturation temperature. So this process requires thermal resistant enzymes.

The use of enzymes has several advantages. They enable conversion under moderate temperature-, pressure- and pH-conditions. Neither the ester product nor the glycerol phase has to be purified concerning catalyst residues or soaps which can be found if oils and fats are converted via alkaline catalysis. This means that phase separation is easier and higher quality glycerol will be formed. Furthermore, the transesterification of triglycerides and the esterification of free fatty acids can be carried out in one process step. Therefore, feedstocks with a high content of free fatty acids can be used without pre-treatment. Furthermore, lipases are able to catalyze transesterification reactions with longer or branched-chain alcohols, which can hardly be converted to fatty acid esters in the presence of alkaline catalysts. However, there are also some drawbacks. The reaction efficiency tends to be poor, so that longer reaction times and higher catalyst concentrations are necessary. Also catalyst (enzyme) recycling will be a challenging procedure. The main problem to use enzymes in the industrial biodiesel production is their high price, especially if they are used in high purified form.

Enzyme assays are laboratory methods for measuring the enzymatic activity. Screening for activity and quantifying activity are possible by using these assays.

Three methods commonly used for the activity measurement, a titrimetric, a colorimetric assay and a spectrophotometric method (Pinsirodom and Parkin, 2001). Two methods exploit the ability to measure the content of free fatty acids formed during lipase hydrolysis of native substrates. One method is based on using the substrate p-nitrophenyl butyrate, which is hydrolyzed to p-nitrophenol, a chromophore that can be determined spectrophotometrically.

By titrimetric determination, native substrates (triacylglycerols) are hydrolyzed to yield fatty acids. The amount of fatty acids released during the reaction is determined by direct titration with NaOH to the end point of indicator (phenolphthalein). Lipase activity is defined as the amount (in µmol) of acid released per minute per milligram of lipase.

Olive oil is used as substrate in the colorimetric method. The liberated fatty acids during hydrolysis by lipase can be determined colorimetrically using a cupric acetate/pyridine reagent. Fatty acids complex with copper to form cupric salts or soaps that absorbs light in visible range (max 715 nm), yielding a blue colour. A standard curve prepared using oleic acid can be used for quantification of fatty acid released by lipase.

The spectrophotometric method quantifies the level of p-nitrophenol (?max 400 to 410 nm) released following the hydrolysis of p- nitrophenyl butyrate substrate by lipase. A standard curve is prepared using p-nitrophenol. Activity of lipase can be calculated by comparing sample A410 values to those of standard curve.

Partner 2 (UNIPD) delivered six different lipases, which were investigated on their hydrolytic activity. Acinetobacter venetianus DSM 23050 is reported to have hydrolytic activity up to 70 °C (Snellman et al., 2002). Furthermore, the lipase is stable in the range of pH 5.8 -

9.0. Optimal activity was found at 55 °C and pH 9.0. This lipase is active against a wide range of fatty acid esters of p-nitrophenyl, but preferentially attacks medium length acyl chains like C6, C8. Due to these facts partner 2 choose this strain and prepared crude protein extracts. Moreover samples of two strains, Pseudomonas oleovorans DSM 1045 and Pseudomonas fragi DSM 3456, which are used for the PHAs production, were tested on their hydrolytic activity. Furthermore, partner 2 delivered crude lipase samples of isolates from soil (3S and 7S) and from water (8H). Furthermore, two commercial immobilized lipases from Candida antarctica and Mucor mihei were investigated on their hydrolytic activity and used for transesterification experiments.

The extracellular lipase of Acinetobacter venetianus shows a very low activity compared to the commercial lipase (0.200 µmol/min*mg), due to the fact that, the delivered samples are not purified. The samples PD-Av-2 (0.0319 µmol/min*mg) and PD-Av-4 (0.0301 µmol/min*mg) achieved the highest activity due to the protein precipitation step. Furthermore the values indicated that without the dialysis step (PD-Av-5 to 7) the activity will be much lower. That means the more purification steps the higher the activity.

As expected, these samples show a much lower activity compared to the samples of A. venetianus due to the fact, that only lyophilization and no dialysis of the samples has been performed they contain significant amounts of the medium as well as inorganic salts. Furthermore no precipitation step was carried out to enrich the protein.

The isolates from soil (S3 and S7) show a higher activity compared to the isolate from water (8H). Moreover the isolate from water has the lowest hydrolytic activity of all delivered samples. This value (0.0002 µmol/min*mg) is 1000-fold lower compared to the commercial lipase (0.200 µmol/min*mg). The low values are attributed to the less purification of the samples. These samples also contain amounts of the medium and inorganic salts.

The commercial lipase shows a 10-fold (PD-Av-1: 0.0213 µmol/min*mg) to 1000-fold (PD-8H-01: 0.0002 µmol/min*mg) higher activity than the delivered samples. Because of the few purification steps on the samples from partner 2, the activity is very low compared to commercial sources. The results of lipase activity determination indicated that, using precipitation and dialysis steps will lead to significant higher values. Nevertheless the samples with these purification steps (PD-Av-2: 0.0319 µmol/min*mg and PD-Av-4: 0.0301 µmol/min*mg) exhibit anyway a low hydrolytic activity. These crude lipase samples are not comparable with commercially available purified enzymes.

Lipases are able to catalyze hydrolysis and transesterification reactions. If the hydrolytic activity shows low values, also the synthesis of ester bonds will be very low. This means poor reaction efficiency for the transesterification process. So the reaction necessitates longer times and higher catalyst concentrations. Moreover it seems that the content of inorganic salts, coming from insufficient purification reduces the lipase activity. However, lipases investigated in this context within this project did not show any high efficiency comparable to commercial sources. This indicates that the assessed microorganisms will not be able to replace already available material. But it should be noted that within ANIMPOL the search for high efficient enzymes is mainly focusing on PHA production whereas the lipase/hydrolase

part is primarily targeting on getting additional experience with new strains.

Three different lipases were investigated as described subsequently:

Candida Antarctica: The yields of fatty acid alkyl esters were very high with concentrations of about 80 – 90 %. The results using methanol and iso-amyl alcohol showed with 92.3 % and 97.2 % the highest values. The conversion to fatty acid alkyl esters using tert-butanol achieved with 11.3 % the lowest yield.

The ester yield using iso-amyl alcohol showed with 98.5 % the highest value. Transesterification using methanol, ethanol, butanol, propanol and iso-propanol achieved yields about 89.8% to 62.6 %. The production of tert-fatty acid butyl ester by this lipase failed and had a very low result (11.3 %).

Mucor mihei: Due to the fact that the sample PD-AV-3 showed a hydrolytic activity, this sample was used for transesterification experiments. The yield with a value of 7.8% was insufficiently low and transesterification did not work.

Acinetobacter venetianus: Extracted fat of udder can be used also for enzymatic transesterification experiments. The yield of fatty acid methyl ester with Candida antarctica and Mucor mihei was very high and showed values of 88.0 % and 92.9 %.

In summary the conversion of triglycerides to fatty acid alkyl esters by enzymatic transesterification was successful and showed ester yields more than 90 % using the lipases from Candida antarctica and Mucor mihei. The sample from Acinetobacter venetianus cannot be used for transesterification experiments, because the result was very low.

WP2: Fermentation technology, genetics and mathematical modelling (RDT)

Task 2.1: Production of scl-PHAs

A broad number (about 100) of scl-PHA homo- and copolyesters with different compositions, produced by different feeding strategies and recovered by different techniques, was produced by TUG and UNIPD and send to the responsible partners for characterization. Concerning the co-polyesters, 3HB, 3HV and 4HB building blocks were inserted into the biopolymers. Here, media compositions were optimized, as well as the feeding strategies for the required substrates.

The impact of different concentrations of margaric acid on growth under balanced nutrient conditions and under nitrogen limited conditions provoking PHA accumulation was investigated for C. necator DSM 545 in the first phase of the project. As main carbon sources, the ANIMPOL-relevant substrates glycerol and biofuel were used and compared with the 'classical' substrate glucose. Based on the outcomes of these shaking flask experiments, the same production strain was cultivated under controlled conditions on bioreactor scale using biofuel as the main carbon source together with a co-feeding of margaric acid. It was demonstrated that a considerable amount of 3HV building blocks (4.5%) was incorporated into the polymer by supply of margaric acid. This fermentation provided kinetic data and material (PHA copolyester consisting of 3HB and 3HV) for characterization by the responsible

partners. Especially the productivities already obtained during these experiments (PHBHV from saturated biofuel plus margaric acid) can be regarded as promising for the final planning of a process. In the second half of the project, novel 3HV precursors were produced by partner KFU by chemical catalysis like ozonolyis (see results in WP1). Applying these materials as co-substrates for the production strain C. necator, it was possible to increase this value to up to 8.6% 3HV in PHBHV copolyesters!

On laboratory scale, a total of about 20 fermentations were accomplished using C. necator as production strain on biodiesel; an additional bioreactor fermentation was carried out using the archaeon Haloferax mediterranei DSM 1411. An example of the results obtained with C. necator is provided in the subsequent paragraphs:

This fermentation was accomplished by using animal-derived biodiesel as the sole carbon source both for biomass growth and PHA accumulation in aerobic fed-batch cultivation mode (repeated supply of biodiesel according to its conversion by the cells) in a 7.5 liter bioreactor (Labfors 3; Infors, CH) under controlled conditions of pH-value, oxygen supply and temperature (37°C). Nitrogen source was selected as growth-limiting factor for provoking PHA accumulation.

A maximum of 28.0 g/L PHA were obtained, corresponding to a PHA share in cell dry mass of 80.3 %. The specific growth rate of the production strain amounted to 0.17 1/h. Especially the high yield for biomass production from biodiesel of more than 0.6 g CDM per g biodiesel is exceptionally high if compared to well-known PHA substrates like sugars, where the theoretical yield do not exceed 0.48 g/g. This is due to the metabolic background of fatty acid catabolism by the cells that convert long-chain substrates like biodiesel via the ß-oxidation pathway. Considering the stage of process development (laboratory bioreactor scale), also the high volumetric productivity for PHA of 0.94 g/Lh for the entire process can be considered very promising if compared to available data for industrial PHA production from expensive substrates. Considering only the phase of predominant PHA accumulation after nitrogen limitation, volumetric productivity was as high as 1.36 g/Lh. Regarding the specific volumetric productivity during nitrogen limited conditions, a value of 0.19 g/gh was calculated. This is significantly higher than values reported before for utilization of whey lactose. During this phase, residual biomass (NPCM) concentration remained constant at about 7 g/L. In addition, the produced PHA was Poly-(3HB-co-0.84%-3HV) copolyester; here, odd-numbered fatty acids in the SFAE acted as 3HV-related precursor substrates. Analyzing the composition of the polyester during the fermentation and the concentration of 3HV in the fermentation broth it is well visible that, due to the identical composition of the added SFAE during the entire process, the share of 3HV in PHA (about 0.8%) is constant during the phase of nitrogen limitation.

Regarding the results from thermogravimetric analysis, Differential Scanning Calorimetry and molecular mass determination, results were in a range typical for this type of PHA (Td = 274.3 °C, peak of melting range Tm = 163.5 °C; glass transition temperature Tg = 3.6 °C, degree of crystallinity Xc = 34.8%, Mw = 311840, Mn = 208040, dispersity index Pi = 1.5; data for polymer characterization accomplished by partner UNIPI)

Also Glycerol as a by-product of the biodiesel production was successfully tested for scl-PHA production with Burkholderia sacchari, Haloferax mediterranei and C. necator on bioreactor scale. Also in the

case of this substrate, C. necator turned out to be the most promising candidate. The next paragraph provides the most significant results of this process.

Glycerol was utilized by C. necator DSM 545 for production of poly-3-hydroxybutyrate (PHB) in aerobic fed-batch cultivation mode in a 7.5 liter bioreactor (Labfors 3; Infors, CH) under controlled conditions of pH-value, oxygen supply and temperature (37°C). Again, nitrogen source was selected as growth-limiting factor for provoking PHA accumulation. The maximal specific growth rate was determined with 0.11 1/h, and the maximal specific non-growth associated PHB production rate with 0.17 g/g h. The maximum cell dry mass (CDM) obtained at the end of the process amounted to 45.3 g/L, the PHA concentration to 29.5 g/L, and the percentage of PHA in biomass to 65%. The volumetric productivity for the entire process was calculated with 0.98 g/L h, for the PHA-accumulation phase (provoked by nitrogen limitation), even 2.79 g/L h were obtained.

Regarding the results from thermogravimetric analysis, Differential Scanning Calorimetry and molecular mass determination, results were in a range typical for this type of PHA considering the applied substrate (Td = 265 °C, peak of melting range Tm = 169.5 °C; glass transition temperature Tg = 5.2 °C, degree of crystallinity Xc = 34.3%, Mw = 435800, Mn = 368060, dispersity index Pi = 1.2; data from partner UNIPI)

Task 2.2: Production of mcl-PHAs

UNIPD:

A lot of efforts were devoted to the production of mcl-PHAs, mainly using Pseudomonas oleovorans DSM 1045 as suggested by UNIPD according to their research results.

Pseudomonas oleovorans DSM1045 was investigated by as possible mcl–PHA producer in liquid E-medium using different carbon sources (see Tab. 1 of AnnexI-P2 of respective Deliverable 2.7).

A number of PHA samples obtained with the selected strain reported above were sent for chemical-physical analysis to P7, P8 and P9.

TUG

Three Pseudomas putida strains (Ps. putida ATCC29347, Ps. putida KT2442 and Ps. putida DSM1045 [= UNIPD strain Ps. oleovorans]) were analyzed for their ability to grow in the presence of alternative raw materials (biodiesel, gluconic acid and glycerol) and to produce various types of PHAs, constituents of fully degradable bioplastics to be applied as latexes or as special niche products (thermosensitive adhesives, staring materials for postsynthetic modification etc.). In the course of the experiments, different techniques of analytical chemistry (GC, HPLC, etc.) were applied to obtain as valuable and accurate results as possible.

The results showed comparable behavior of the investigated strains concerning the utilization of all three carbon sources. In the presence of glycerol and gluconic acid as sole carbon sources, the concentration of the initial cell density increased rather quickly compared to fatty acid methyl esters, components of biodiesel. However, during later growth stages, the cell density reached the same or even higher concentration

due to high energy reserves that are chemically bound within the long carbon chains of fatty acids. Special attention was devoted to the concentration of the carbon sources due to potential cell density fluctuation during growth.

The highest biomass yield was observed using glycerol (2.5 - 4 g/L) as the carbon source followed by gluconic acid (2 - 3 g/L) and biofuel (2 – 2.5 g/L). Even though further analysis is necessary to clarify this phenomenon, one of the explanation can be attributed to better strain adaptation and metabolic processing of glycerol than of biofuel or gluconic acid. Another possible explanation might be the easy import of glycerol into the cells that does not necessarily require energy-demanding transport mechanisms as needed for the import of the other investigated substrates (transported through the cytoplasmic membrane through facilitated diffusion mediated by the glycerol uptake facilitator protein GlpF). One experiment with Ps. putida DSM1045 utilizing gluconic acid proved this to be true since the strain accumulated the most biomass recorded for gluconic acid only after a 10-day adaptation in the corresponding medium. Nevertheless, according to previous experiments claiming biodiesel to be a very cheap and effective carbon source, more studies should be conducted in the future with already adapted Ps. putida strains

Four different standards representing monomer units were analyzed for their presence in the PHA molecule in all three strains. The results showed that, independent from the carbon source or the strain, the preference of monomer integration was basically the same with minor differences. In almost all samples analyzed, the highest concentration of monomer was recorded for 3HDD followed by 3HD. Monomers 3HO and 3HHx were detected in only insignificant concentrations usually at the end of the cultivation period. The phenomenon of having 3HD and 3HDD as major monomer constituents when utilizing biofuel as a carbon source can be explained through the ß-oxidation process. In each turn of this metabolic pathway, two carbon atoms are cleaved from the fatty acid backbone forming new substrates for PHA synthase enzyme. However, even though the preference of 3HD and 3HDD incorporation in the presence of biofuel is not fully understood it can be caused by abundance of capric (10 C atoms) and lauric acid (12 C atoms) naturally occurring in biofuel. In the case of gluconic acid and glycerol utilization, the prevalence of 3HD and 3HDD in the polymer has also not been fully clarified. Nevertheless, based on the nature of the substrates, it is likely that the monomers were formed through de novo fatty acid synthesis and incorporated by specific Ps. putida PHA synthase preferring 3HD and 3HDD over the other monomers.

A detailed description of the outcomes using these strains is provided in the subsequent paragraphs:

Ps. putida ATCC 29347

When Ps. putida ATCC29347 was cultivated in glycerol containing medium, the lag phase was shorter than in the case of biodiesel. This finding was independent from the media composition. The faster initial growth with glycerol as a sole carbon source could have been caused by the fact that glycerol has a more simple chemical structure than fatty acids present in biodiesel and, hence, is more easily imported into the cells. Biofuel typically contains up to 14 different fatty acids that were chemically transformed into fatty acid methyl esters. Most of these fatty acids are saturated and thus do not possess any double bonds. Even though the fatty

acids are much richer in energy content than glycerol, they require longer metabolic processing than glycerol. This is the main reason why the cell density was increasing rather slowly at initial growth phase. Furthermore, a previous study with various species of Ps. putida growing in FAMEs and glycerol showed that glycerol was the preferred substrate as well as the energetically most favorable substrate for the formation of acetyl-CoA (Silva et al., 2009). Before acetyl-CoA is formed, glycerol is turned into dihydroxyacetone phosphate followed by a conversion to phosphoenolpyruvate and puryvate. Pyruvate is decarboxylated into acetyl-CoA which can then lead to PHA formation. For later growth phase however the cell density in the medium with biofuel equaled or even reached higher value than in the same medium with glycerol.

In addition, the distribution of biofuel droplets in the aqueous medium is a crucial item considering the availability of the substrate by the cells. Although an emulsifier was provided to the medium in order to improve the distribution of biofuel in the aqueous environment, the entire medium still constitutes a two-phase system. Hence, the availability of substrate for the microorganisms is only possible from the two-dimensional surface of the biofuel droplets. This is in huge contrast to the utilization of hydrophilic substrates like gluconic acid or glycerol that are completely dissolved in aqueous environments.

Similar results to glycerol were obtained for the same strain with gluconic acid as a carbon source. In this case, the lag phase was quite short allowing reaching relatively high cell density within the first 18 h of incubation. Exactly the same time was needed for the strain utilizing glycerol in the modified Küng media with the doubled concentration of buffer compounds. These findings confirm relatively similar level of conversion suitabilities of gluconic acid and glycerol. Like glycerol, gluconic acid is a highly polar molecule containing six carboxy groups. Via the Entner-Doudoroff pathway, gluconic acid is transformed into 6-phospho-D-gluconate which is then dehydrogenated to 2-keto-3-deoxy-6-phosphogluconate. Through successive chemical reactions, pyruvate and acetyl CoA are created eventually leading to PHA formation.

Refeeding the cultures with the carbon source usually resulted in higher optical density. The first refeeding occurred in both exponential and stationary phase depending on the speed of carbon and nitrogen utilization. In both cases, the excess of carbon source caused an increase in cell density either by promoting cell division in exponential phase or by supporting PHA accumulation in the stationary phase. However, with biofuel as a carbon source, the increase of OD420 was not as prominent and fast as with glycerol and gluconic acid. The results again proved the complexity of biofuel utilization in bacterial strains. Whenever the second refeeding took place, a similar pattern was observed. However, the third carbon source refeed performed with gluconic acid as the single carbon source resulted in the decrease of the optical density. Previous studies explained this effect by pointing out the fact that the higher concentration of the carbon source can become inhibiting or even toxic to the bacteria, lowering their numbers as a result.

The media composition was changed from the third experiment onwards containing half the amount of nitrogen source and doubled amount of KH_2PO_4 and Na_2HPO_4 for reaching faster growth limiting conditions by depletion of nitrogen and increasing the buffer capacity, respectively. Even though the level of the pH value remained constant throughout most or even the whole experiments, the final OD reached similar values

compared to the experiments where the pH value levels shifted from slightly alkaline or neutral to acidic. One possible explanation can be that this strain was resistant to the lower pH value and therefore not negatively influenced during growth. Furthermore, it is generally accepted that the pH value is lowered as a result of metabolic activities that lead to the formation of various acids. Based on this assumption, the next explanation could be that the metabolic by-products were partly reutilized in lower concentrations by the bacteria and thus the impact of low pH values was not so limiting in terms of bacterial growth.

Either way, one common feature was seen when comparing the effect of the pH value on growth characteristics. In the first two experiments with the highest pH value fluctuation, the level of ammonium ions never reached zero concentrations. This is not the case for experiments with doubled buffer capacity where zero concentration of NH4+ was observed within 41,5 h, 113,5 h and 26 h, respectively. The reason why the pH value could possibly influence nitrogen utilization has not yet been clarified but it is beyond any dispute that increased H+ somehow interacts with the nitrogen uptake.

Based on the reasons already mentioned, it is also understandable that the more cell biomass was accumulated at initial growth phase in the culture with glycerol and gluconic acid than in biodiesel. During later stages of growth, the weight of the biomass in biofuel reached corresponding level with glycerol and gluconic acid. The maximum biomass yield in the shaking flasks experiments with Ps. putida ATCC29347 were in the range of 2.5 – 4.0 g/L for glycerol, 2 – 2.5 g/l for biofuel and 2 - 2,5 g/L for gluconic acid. Quite analogous results were obtained by a previous group which used Pseudomonas resinovorans grown on triglyceride substrates such as oleic acid, tallow, lard and vegetable oils. The overall crude cell yield amounted approx. 3-3.8 g/L (Ashby and Foglia, 1998). Another study, focused on one- and two-stage chemostat cultivation system, reported the cell dry weight to be 2.14 g/L and 1.68 g/L when applying Ps. putida ATCC29347 using n-octane and citrate as carbon sources.

The maximum PHA yield was usually obtained by the end of the experiment equaling 15.8 mg/L in biodiesel, 14.5 mg/L in glycerol, and 12.0 mg/L in gluconic acid, respectively. These results showed similarities with a previous study conducted with Ps. aeruginosa ATCC 27853 cultured on various fatty acids (Ballistreri et al., 2001). The PHA yield in their experiments was in the range of 2-180 mg/l depending on the fatty acid used with or without magnesium-limiting conditions for each carbon source. Yields of analogous values were also obtained with Ps. putida ATCC29347 accumulating novel PHAs from various alkoxyalkanoic acids as demonstrated before by Kim D. Y. et al. (2003). However, no data were found for Ps. putida ATCC29347 utilizing either glycerol or gluconic acid and therefore the presented results could not be directly compared.

The monomers contained in PHA structure mostly involved 3HD and 3HDD. Only in the medium with gluconic acid, detectable amounts of other monomers (3HHx and 3HO) were found at the end of incubation time. As was explained earlier in this report along with other studies (Brandl et al., 1998), the nature of monomers incorporated depends on the carbon source and the type of microorganism. Through the process of beta-oxidation and de novo fatty acid synthesis, various monomeric units are arranged into the polymeric molecule. The preference for the different monomers is not yet fully understood. Nevertheless, a research done on Ps. putida

ATCC29347 growing on various n-alkanoic acids showed that intracellular PHA production was detected only on acids containing six or more carbon atoms (Brandl et al., 1998). Furthermore, a ten year old study with Ps. aeruginosa ATCC27853 experimentally proved that the most predominant units in the PHA are of the same length as the carbon source or shortened by multiple of two carbon atoms (Ballistreri et al., 2001). In relation to our findings, the most predominant fatty acids present in biofuel would be capric acid (with 10 carbon atoms) and lauric acid (with 12 carbon atoms) followed by acids with higher molecular weight with even number of C atoms. This would explain the monomeric composition with biofuel as a carbon source. In the case of gluconic acid and glycerol, the prevalence of 3HD and 3HDD is somehow connected to the activity of PHA synthase enzyme as described before with Ps. stutzeri 1317 utilizing glucose and various fatty acids (Chen et al., 2001).

The PHA content in biomass never exceeded 1% of the total biomass. The low amount of accumulated PHA is mainly due to low cell density as well as the fact the cells were grown in shaking flasks and not under controlled conditions in a fermentor. In fact, when pseudomonades are grown in regulated continuous culture, the PHA yields are much more significant (up to 63%) as previously demonstrated with Ps. putida ATCC29347 utilizing n-octane (Jung et al., 2001) or Ps. resinovorans utilizing triglyceride substrates (up to 51%) (Ashby and Foglia, 1998). In shaking flask experiments, the main deciding factor, besides the bacterial strain, for reaching high cellular PHA content is the choice of the carbon source. The study conducted by Chen et al. (2001) reported 65% PHA content in Ps. stutzeri 1317 when grown in glucose for 48 h whereas another one used our experimental strain under nitrogen limitation with octanoate as carbon source reaching PHA content of 36% (Wu et al., 2003). These previous findings suggest that either the carbon sources used in the scope of this thesis were not suitable for the strain or the culture conditions did not reflect the bacterial needs for higher PHA accumulation. Either way, it should be noted that not only high PHA contents are achieved in pseudomonas strains. PHA yields close to our observations were reported for Ps. putida KCTC 2407 in batch fermentation using undecenoic and methylphenoxyoctanoic acids as carbon sources (1.8% and 0.8%, respectively) (Kim D.Y et al., 2000). Analogous results were also obtained for Ps. putida grown in batch fermentation with various alkoxyalkanoic acids where the cellular PHA content was as low as 3% (Kim D.Y et al., 2003).

Ps. putida KT2442

Strain Ps. putida KT2442 was only tested in modified Küng media with gluconic acid as the sole carbon source. As with any other experiment conducted for the purpose of this thesis, two tests were run in parallel so the results could be compared. In this case, the growth patterns in both tests were in good correlation. The cell density increased quite rapidly just like in the case with glycerol mainly due to well-developed cellular tolerance as well as high accessibility by the bacterial cells. The NH4+ concentration was down to zero within 26 h after inoculation suggesting a start of accumulation phase at this point.

The maximum biomass in the shaking flasks was in a range between 2-2.3 g/l. These values correspond to the values obtained for Ps. putida ATCC29347 grown in biodiesel and gluconic acid as well as with the previous studies growing Ps. putida KT2442 in the presence of oleic acid (Lee et al., 2000) or Ps. cepacia utilizing glucose with addition of

small monocarboxylic and dicarboxylic acids (Ramsay et al., 1989). The correlation between the optical density and biomass - were not always proportionally equal. Although these dissimilarities occurred relatively often during the measurements, they were not so significant for determining the growth characteristics. However, the reason is most likely based on statistic errors in the experimental process.

The low biomass yield is reflected to the PHA yield. The level of PHA yield increased during the accumulation phase up to 19.3 mg/L taking up more than 1% of the biomass. However, later stages of accumulation phase were marked by PHA decrease from 19.3 mg/L to 10.4 mg/l equaling 0.7% of the cell weight. This phenomenon was described earlier by Brandl et al. (1998) and Cai et al. (2009) as a result of PHA degradation by PHA depolymerase to reutilize the accumulated PHA as carbon and energy source. The depolymerization usually occurs when a nutrient or growth factor becomes limiting. Nevertheless, the PHA utilization in the accumulation phase can take place to some extend even under the limiting conditions. This is due to the fact that the in vivo polymerization and depolymerization in native PHA granules is a dynamic process of cyclic nature. Hence, PHA chains in living organisms can always be considered to be 'under construction'.

The overall PHA content was a bit higher than in the previous case with Ps. putida ATCC29347 using all three carbon substrates. This suggests analogous metabolic pathways and efficiency of both strains to utilize the investigated carbon sources. However, the amount of polymer produced was still insignificant when compared with shaking flask experiments using 1.5% glucose (8.5% PHA content), 10 mM octanoate (22.3% PHA content) (Huijberts et al., 1994) or recombinant Ps. putida KT2440 utilizing gluconate (54-60% PHA content) (Rehm et al., 1998). The results confirm that more optimization in terms of culture conditions and strain adaptation is needed in order to improve biomass and PHA productivity.

Concerning the monomer composition, the produced PHA was predominantly composed of 3HD and 3HDD. The prevalence of 3HD (up to 70%) was described earlier with the same strain from unrelated substrates such as gluconate. The preference of 3HD was also demonstrated in this study for all other strains showing a close correlation between a specific substrate and PHA synthase enzymes.

Ps. putida DSM1045

Unlike Ps. putida ATCC29347 and Ps. putida KT2440, Ps. putida DSM1045 was grown in the presence of two carbon sources, biofuel and gluconic acid. When cultivated with biofuel as a carbon source, two media compositions were tested to determine which one is more suitable for growth. As for gluconic acid, only modified Küng was applied for the growth.

The biomass yield in the presence of biofuel was higher in the DSMZ81 medium than in the modified Küng medium even though in one experimental set up, the optical density reached almost twice the value of the cell cultures growing in DSMZ81 medium. The reason why high optical density corresponded to low biomass yield was due to formation of metabolic by-products causing a change in appearance of the culture broth. The culture broth was milky even after centrifugation which resulted in instable values obtained by the spectrophotometer. Concerning the growth in both media, it is not clear why more biomass was accumulated in the DSMZ81 medium. This occurrence can only be explained by the difference in media

composition where DSMZ81 medium contained a different nitrogen source with chloride ions (NH_4Cl) as well as compounds either totally missing in modified Küng medium or present in diverse concentrations.

However, the maximum biomass yield in both media (1.8 – 2.4 g/L for DSMZ81 and 1.3 – 1.8 g/L for modified Küng) did not match the cell biomass accumulated in the medium with gluconic acid reaching almost 3 g/L. Different observations were reported for Ps. putida ATCC29347 where the biomass yields produced from biofuel and gluconic acid almost equaled (2 – 2.5 g/L). The most likely factor causing higher biomass yields in this case was the application of an efficiently adapted strain. As was explained earlier in the text, it took more than 10 days before the strain started to grow in the presence of gluconic acid. Afterwards, the same bacterial cells were used to utilize the gluconic acid. Although the fatty acids comprising biofuel offer more energy than gluconic acid, they also feature a higher metabolic burden to the bacterial metabolism, sometimes they are even toxic at higher concentrations (Du and Yu, 2002). Along with the adapted strain, it is thus justified that the highest biomass yield was obtained in the medium with the gluconic acid as a sole carbon source.

The GC analysis of the PHA yield was only performed for the strain growing in DSMZ81 and modified Küng media containing biofuel. In both cases, the correlation between growing biomass and PHA yield was well observed. The maximum yield was 16.6 mg/L for the strain in modified Küng medium whereas in the second case, it reached 69.3 mg/L at the very end of the experiment. The value of 16.6 mg/L mostly corresponded to the values measured for both other strains along with the study using Ps. aeruginosa cultured on various fatty acids (Ballistreri et al., 2001). Even though high PHA yields with fatty acids as carbon sources were previously reported (Ashby and Foglia, 1998), the second value of 69.3 mg/L was found to be incorrect. The explanation is based on miscorrelation between the optical density of 14.8 and the PHA yield which was shown to be too high for such low OD420. This result misreading was probably caused by a mistake in the sample preparation for the GC as well as the fact that the second sample, run in parallel for comparison, was not measured by the GC due to sample destruction during transesterification process. Due to time restrictions, the PHA content in the medium with gluconic acid remains to be determined.

Just like strains Ps. putida ATCC29347 and Ps. putida KT2442, the strain Ps. putida DSM1045 mostly incorporated 3HD and 3HDD monomers in the polymeric structure. As was described with the other strains, the monomer composition mainly depends on the substrate used. For instance, when grown on gluconate, Ps. putida strains most preferentially incorporated 3HD (Timm and Steinbüchel, 1990) whereas with octanoate, the cells prefered 3HO integration, up to 86% depending on the Ps. putida strain (Diard et al., 2002). Based on the monomer preference, it is likely that out of the four monomer standards used, the corresponding capric acid (10 C atoms) was the most predominant compound in the biofuel followed by lauric acid (12 C atoms). The other monomers were present in small percentages except for the last sample in DSMZ81 medium where 3HO took 26% share out of all four monomers. Even though caprylic acid (with 8 C atoms) is normally present in biodiesel, only a minimum concentration of the corresponding 3HO was detected in all other samples. This points out to the fact that the content of caprylic acid in the biofuel was either very low or the strains had difficulties processing the acid into 3HO monomers. Nevertheless, the higher 3HO prevalence in the last sample

along with detectable amounts of 3HHx (no C6 compound present in the biofuel) was attributed to beta-oxidation of higher fatty acids in the biofuel.

The maximum PHA content in biomass was reached at the end of the experiments equaling 1% for the strain in modified Küng medium and almost 2.9% in DSMZ81 medium, respectively. In the first case, the PHA content was within the same range as the other Ps. putida strains, independent of the carbon source. This is of course due to similar values obtained for PHA yield and the cell biomass. Relatively high PHA accumulation in the second case was in most parts caused by abnormally high PHA yield (69.3 mg/l) as explained earlier. Even though values of this kind can be easily achieved by Ps. putida strains (Diard et al., 2002), its occurrence under the applied conditions is unlikely. The indications confirming it were not just related to the overall PHA yield but also to the cell biomass and optical density reached. Besides, during the course of the experiment, the PHA accumulation never exceeded 1%, suggesting the final PHA content was within 1.3 – 2.0%. Under these circumstances, it can be concluded that Ps. putida DSM1045 demonstrated resembling metabolic activities with the strains Ps. putida ATCC29347 and Ps. putida KT2442 in terms of PHA productivity and monomeric preference.

For all the strains, the PHA content never exceeded more than 1% of the total biomass; hence additional search was devoted to screen powerful mcl-PHA producers:

Partner TUG performed fermentation experiments on bioreactor scale using the strains Ps. oleovorans DSM 1045, and the novel strains Ps. citronellolis DSM 5033 and Ps. chlororaphis DSM 50083.

In the case of Ps. citronellolis, the process development results in excellent data if compared to other mcl-PHA producing strain on expensive substrates. The subsequent paragraph summarizes the most significant data obtained by this organism, comparing two fermentations in laboratory bioreactors. The polymer characterization was accomplished by partner PLIPOC.

A maximum specific growth rate, μmax. of 0.10 and 0.08 1/h, respectively, was achieved in two different fermentation set-ups. Volumetric productivity for mcl-PHA amounted to 0.036 g/L h and 0.050 g/L h, final intracellular PHA contents calculated from the sum of active biomass and PHA to 20.1 and 26.6 wt.-%, respectively. GC-FID analysis showed that the obtained biopolyester predominantly consists of 3-hydroxyoctanoate and 3-hydroxydecanoate, and, to a minor extent, 3-hydroxydodecanoate, 3-hydroxynonanoate, 3-hydroxyhexanoate, and 3-hydroxyheptanoate monomers. This was confirmed by 1H- and 13C-NMR, also evidencing the occurrence of low quantities of unsaturated and 3-hydroxyvalerate building blocks. High purity of the recovered materials was proofed by elemental analysis. Regarding the results from thermogravimetric analysis, Differential Scanning Calorimetry and molecular mass determination, results were in a range typical for this type of PHA (1st fermentation: decomposition temperature Td = 296 °C, peak of melting range Tm = 48.6 °C; glass transition temperature Tg = -46.9 °C, degree of crystallinity Xc = 12.3%, Mw = 66000, Mn = 35000, dispersity index Pi = 1.9; 2nd fermentation: Td = 295 °C °C, Tm = 53.6 °C, Tg = -43.5 °C, Xc = 10.4%, Mw = 78000, Mn = 196000, Pi = 2.5). Considering the fact that the cultivations were accomplished in discontinuous fed-batch mode, the obtained results are already in a reasonable range if compared to similar, comparable

processes for mcl-PHA production on expensive carbon sources. Considering the concentrations of active biomass, the novel process even surpasses the performance of comparable processes using related Pseudomonad strains.

A novel description of mcl-PHA biosynthesis from animal-based biodiesel was also accomplished using by Ps. chlororaphis. Maximum specific growth rates (μmax.) of 0.08 1/h, 0.10 1/h and 0.13 1/h, respectively, were achieved in three different fermentation set-ups. Volumetric productivity for mcl-PHA amounted to 0.071 g/L h, 0.094 g/L h and 0.138 g/L h, final intracellular PHA contents calculated from the sum of active biomass and PHA from 22.1 to 29.4 wt.-%, respectively. GC-FID analysis showed that the obtained biopolyester predominantly consists of 3-hydroxyoctanoate and 3-hydroxydecanoate, and, to a minor extent, 3-hydroxydodecanoate, 3-hydroxynonanoate, 3-hydroxyhexanoate, and 3-hydroxyheptanoate monomers. The overall distribution of the monomers remained similar, regardless to working volumes, biodiesel concentrations and pre-treatment of the inoculum. The study demonstrates the high potential of the strain Ps. chlororaphis for mcl-PHA production from by-products of the animal-based biodiesel industry. Compared to Ps. citronellolis DSM 5033, the biomass production was about equal, but the mcl-PHA content was much higher in the case of Ps. chlororaphis. Referring to Ps. oleovorans, Ps. chlororaphis as well as Ps. citronellolis surpassed the capabilities of Ps. oleovorans GPo1 in a fed-batch process using the same inexpensive carbon source. Furthermore, the obtained results are already in a reasonable range if compared to similar processes for mcl-PHA production on expensive carbon sources.

Task 2.3: Microbiology and genetics

At the 6- and 12-months meetings in Portorož (Slovenia) and in Swierklaniec (Poland), the bacterial strains isolated and/or tested were proposed. A summary with the main information for each strain was designed as reported in previous reports.

Criteria for strain selection were:

No pathogenicity (only risk group I organisms)

Genetic stability

Growth rate on ANIMPOL-substrates

PHA-production rate on ANIMPOL-substrates

Copolyester production possible? (for scl-PHAs)

After an exhaustive discussion with all the Partners a decision was taken to use C. necator as possible scl–PHA producer. C. necator DSM 545 was grown firstly in DSMZ81 solid media using crude glycerol phase (CGP) and biodiesel (both delivered by Partner 5), pure glycerol, stearic acid methyl ester, palmitic acid methyl ester and myristic acid methyl ester, and in triglycerides as bacon rind and tallow (delivered by Partner 4).

scl-PHAs: Growth and production of PHAs were also tested in liquid DSMZ81 medium using polymer accumulation conditions (high carbon and low nitrogen sources). Determination of PHAs from stearic acid methyl ester,

palmitic acid methyl ester and myristic acid methyl ester, bacon rind and tallow, were carried out.

mcl-PHAs: At the 6- and 12-months meetings, after a fruitful discussion with all the partners, a decision was taken to use Ps. oleovorans DSM1045 as possible mcl-PHA producer. During the last 6 months of the project, new experiments were performed to check biomass and polymer production by Ps. oleovorans in liquid E* medium and PHAs were determined after 72 h using different carbon sources. The results indicate that Ps. oleovorans can be considered as a good mcl-PHA producer from glycerol, although this carbon source cannot efficiently support biomass development. On the other hand, biodiesel was found to be a good substrate for obtaining PHAs or it can be used as co-substrate to produce co-polymers.

In addition to Ps. oleovorans DSM 1045, two additional strains of Ps. oleovorans (a.k.a. Ps. putida), Ps. citronellolis and Ps. chlororaphis were selected by partner TUG (see also detailed description of experiments, task 2.2).

Reduction of cell lysate viscosity by cloning Staphylococcus aureus nuclease gene

During downstream processing of biomass, high viscosity of the cell lysate frequently represents a problem; this phenomenon is caused by the high content of nucleic acids released by cell disruption. To reduce viscosity during this stage, a nuclease enzyme could be used. The aim was to introduce in the selected bacteria (C. necator DSM 545 and Ps. oleovorans DSM 1045) the nuclease gene (nuc) from S. aureus. The nuc gene encodes for staphylococcal extracellular thermostable nuclease (SNase). The plasmid pNuc was kindly provided by Prof. Schleifer (Technische Universität München, Germany). From this plasmid the nuc gene was obtained as an amplified fragment of 700 bp using the primers nucA (5'-TTCTCTAGAATTCAGGAGGTTTTTATGGCTATCAGTAATGTTTCG-3') and nucB (5'-GCCGGTACCTTATTGACCTGAATCAGCGTTG-3') and the following reaction conditions: 50 cycles of incubation at 95°C (30 s), at 55°C (45 s), and at 72°C (45 s) (Boynton et al., 1999. App. Environ. Microbiol., 65:1524-1529).

Cloning of the amplified nuc gene in the plasmid pGEM-T was performed; this plasmid was designed pGEM-T-nuc.

The construct pGEM-nuc was digested with two endonucleases NdeI and SacII and the fragment containing the nuc gene was separated. This fragment was introduced in the broad host range plasmid pHM2 between the sites NdeI and SacII. This plasmid was designed pHM2-nuc.

Constructs of C. necator and Ps. oleovorans harbouring the plasmids are now available!

Reduction of intracellular PHA degradation in C. necator

In order to reduce intracellular PHA degradation for increased PHA productivity, one of the intracellular PHA-depolymerases could be inactivated by chromosomal integration of a cassette containing a kanamycin resistance gene (Kmr). In a previous work (Povolo et al., 2010), part of the depolymerase phaZ1 was cloned in the suicide plasmid pSuP102 obtaining plasmid pSUP102-phaZ1. The interruption of the fragment of the phaZ1 with the Kmr cassette was the strategy adopted. This

construct was further transferred from E. coli to C. necator DSM 545 by conjugation and chromosomal insertion was then obtained. The effect on reduction of PHA depolymerisation was tested in a bacterial culture previously grown under accumulation conditions. Under carbon starvation culturing, while the wild type C. necator reduced the intracellular PHA to 30% of the initial content, the transconjugant strain C. necator-phaZ1-Kmr maintained its PHA content to 85% after 96 hours incubation. The newly constructed strain is now available at P4.

Task 2.4: Mathematical modeling and kinetic analysis

Partner 3 (UNZA) have fulfilled their obligation for the ANIMPOL project. During the project time they were successful in modeling and optimization of different types of fermentation performed by C. necator DSM 545 and Pseudomonas chlororaphis DSM 500083 on different substrates (glucose, glucose with glycerol, glycerol, biodiesel, and biodiesel with addition of odd-numbered fatty acids as 3HV-related co-substrate) and different cultivation modes (fed-batch or continuous mode). The feedback of UNZA by the established models help partner TUG to a high extent to improve their fermentation strategies (inoculum preparation, concentrations of nutrients), especially in the case of scl-PHA production by C. necator on the ANIMPOL-related substrates biodiesel and glycerol.

In total 5 different low structured mathematical models and 2 high structured mathematical models were established. Simulations of technologically-biological system (low structured models) have been performed by Berkeley-Madonna quick solver software version 8.3.14 using numerical integration methods for solving differential equations. The mentioned five low structured mathematical models were based on the subsequent fermentations: a five stage continuous cultivation performed by C. necator DSM 545 on glucose (with validation of the mathematical model by two additional fermentations accomplished by TUG), fed-batch cultivation of C. necator DSM 545 on glucose with glycerol, fed-batch cultivation of C. necator DSM 545 on glycerol (followed by experimental validation of mathematical model by an additional fermentation), fed-batch cultivation of C. necator DSM 545 on biodiesel combined with valeric acid (with validation of model by additional fermentations) and fed-batch cultivation of Pseudomonas chlororaphis DSM 500083 on biodiesel (with validation of model by additional fermentation).

In order to design those models and to reduce the experimental work, modeling principles have been established for each low structured model, model equations and values of model parameters (part of them was experimentally estimated and the rest were developed by help of mathematical software tools using curve fitting and optimization procedures from Berkeley-Madonna software and applying the sum of square differences between measured and simulated data as objective functions). For all established low structured models simulation of processes were performed in order to optimize the feeding strategy (nutrient supply, nutrient limitations), and product yields of processes. High structured mathematical models have been established for 5-stage continuous cultivation by C. necator DSM 545 on glucose and for C. necator DSM 545 growth on glycerol. For established those two high structured metabolic models (for C. necator DSM 545 growth on glucose and on glycerol) metabolic analysis, elementary flux modes and yield space analysis were performed with a software package Metatool (see http://pinguin.biologie.unijena.de/bioinformatik/networks/ online) which is a software deployed as tool for MATLAB. Elementary modes for 5-stage

continuous cultivation by C. necator DSM 545 on glucose were further utilized to match the experimental data (linear combination of elementary modes with weighting factors for each elementary mode). For this purpose, the simplex method was implemented, and a set of mass balance equations was established along with kinetic equations. Intracellular metabolite concentrations, flux equations and kinetic constants were tested and, thereafter, applied to the whole 5-stage bioreactor system by using Berkeley-Madonna software.

In addition, P3 (UNZA) has contributed in one scientific article printed in Appl. Microbiol. Biotechnol. and created another one together with P1 (TUG) that was printed in Bioprocess and Biosystems Engineering. In addition three other scientific papers were prepared in cooperation with P1 and one in cooperation with P1 (TUG) and P4 (KFU). They are in the process of editing, reviewing or rewriting.

The scientifically paramount and most significant modeling achievements to be highlighted:

- low structured mathematical model for 5-stage continuous cultivation performed by C. necator DSM 545 on glucose

- high structured metabolic models for C. necator DSM 545 growth on glucose as well as on glycerol.

Task 2.5: Preliminary characterization of raw polymers

An exhaustive number of characterization analyses was carried out by the responsible partners for determination of the properties of delivered polymer samples; the results indicate the impact of the different applied PHA production strategies on the PHA quality and properties. In total, 57 samples from TUG, 12 samples from ARGUS and 18 samples from UNIPD were analyzed and characterized.

Summray: The polymers produced in the project have undergone detailed characterization. The extraction method has been confirmed to be adequate and does not need improvement. Composition control on the other hand to achieve the desired variation in properties appeared to be insufficient during the first phase of the period; this problem was overcome by the action of the biotechnological group in optimizing the feeding strategy for odd-numbered co-substrates acting as 3HV-precursors, hence by adjusting the co-feeding of the main carbon source (biodiesel) and the 3HV precursors. This is related to the choice (new precursors provided by partner KFU!) of and timing of substrate addition in the fermentation procedure. It was shown by the accomplished measurements that higher variation (different amounts of 3HV) significantly influences thermal properties and, as a consequence, mechanical properties like the degree of crystallinity.

The measurement of molar masses needed improvements during the first project period to give more reliable data. At this point the polydispersity was quite high which could confirm the production of polymer blends. Also this problem was overcome by optimizing the fermentation parameters that impact molecular mass and it´s distribution.

Partner UNIPI: The preliminary characterization of the raw polymer samples was carried out on samples received both by the Technical University of Graz (Partner P1) and the University of UNIPD (Partner P2).

By following a concise summary of the characterization done on the submitted PHA samples, whereas detailed information on the analyses performed are reported in an Annex that is available on demand. As a general comment it can be stated that the contribution given by Partner P7 (UNIPI) required an effort in term of man power, higher than that expected. The major effort was due to the very large number of samples produced by partners P1 & P2 and supplied for structural characterizations.

Partner PLIPOC: Received samples for testing were analyzed by elemental analysis (EA), gel permeation chromatography (GPC), Nuclear Magnetic Resonance (1H and 13C) (NMR), Thermogravimetric analysis (TGA) and multistage mass spectrometry (ESI-MS). Results and methodological details are provided in Deliverable M.20.

Partner NIC: Start-up actions were focused on collecting and reviewing the latest state of the art situation in the areas of NIC activity – mainly PHA characterization, and setting up the instrumental conditions and procedures. The conclusion of these activities was the establishment of practical procedures for sample analysis. A key aspect was ensuring complete solubility of samples for chromatographic analysis. This was done using acid-free chloroform. The process involves 60 min reflux boiling, followed by filtration. Other aspects were linked to the selection of analytical procedures: NMR sequences and quantification limits, DSC heating/cooling rates and scanning range, chromatographic conditions (flow rate) and column/pre-column selection.

WP3: Downstream processing, LCA and engineering (RDT)

Task 3.1: Downstream processing

For recovery of PHA, solvent extraction, cell disintegration by ultrasound technique, combined with the separation of released PHA granules via a dissolved air flotation (DAF)-unit, was investigated. Using solvent extraction, focus was devoted to the utilization of non-toxic solvents (Dichloromethane, lactic acid esters, methyl acetate) and in minimizing the solvent quantities. Concerning the classical PHA-solvent chloroform, continuous (Soxleth) extraction was compared with discontinuous extraction (batch). Different pre-treatment methods for PHA-containing biomass regarding the removal of lipids were assessed. In addition, non-solvent cell-disruption using SDS was optimized for scl-PHA containing biomass. All details are available in Deliverable 3.9 and in the experimental part of the Deliverable M.20.

As most promising strategy, the utilization of High-pressure-homogenization (HPH) was applied successfully as a solvent-free approach. The disruption performance of HPH was studied in PHA-rich culture broth of C. necator with biomass concentrations in the range of 1 - 6% of CDM in cultivation broth. The fermentation broths have directly been used with and without pretreatment with NaOH and SDS solutions. 2 cycles at 800 kg/cm2 were carried out. The efficiency for cell disruption was studied by plate counting of viable colony forming units (CFU/mL). In order to make the process more efficient, the fermentation broth was concentrated in later experiments up to 20% of CDM by means of ultrafiltration.

The results indicate a very good performance of HDH in cell disintegration. Best results (greater than 99.99%) were achieved by

combined pretreatment with NaOH (strongly alkaline conditions of pH 12) and SDS (1%). Even the results without any pretreatment step are very promising regarding the degree of cell disruption of 91 - 99%.

In addition, dissolved air floatation (DAF) was tested for PHA recovery. DAF experiments have been accomplished under the same conditions published by van Hee et al. with cultures of C. necator after treatment by HPH for cell disruption.

The digestion of non-PHA biomass and separation of the PHA granules is an alternative to the solvent extraction process for PHA recovery. Different methods can be used to separate the dissolved biomass from the PHA granules. DAF is a very interesting separation method with a number of advantages compared to the more established processes of filtration and centrifugation. DAF, which is widely used in big scale wastewater treatment, uses air under pressure dissolved in water. When the pressure is released, small air bubbles are generated that attach to particles and float them to the surface for removal. In contrast to centrifugation and filtration, DAF is also efficient, when the density of the particles is similar to water (like it is the case with mcl-PHAs) or when the particle size distributions of the particles to separate overlap. DAF is easy to scale up and can easily be integrated in technical processes. The floating cell (FC) applied in the ANIMPOL experiments has a total height of 40 cm with an active working height of 30 cm and an internal diameter of 6 cm. The dissolved air vessel (DAV) is a modified 6 L pressure tank which is connected to an air compressor. Working pressure amounts to 5 bar and has to be maintained constant during the experiments. The functionality of the process was demonstrated with water. Very fine bubbles are produced to separate cell debris from PHA granules.

In this case, C. necator was cultivated on saturated biodiesel as carbon source. Probably due to remaining droplets of biodiesel used as fermentation substrate no separation of PHA granules was observed. The complex particle-interface interactions were inhibited although they were also of hydrophobic character.

Nevertheless, HPH as a solvent-free method for cell disintegration is a valuable step towards sustainability in downstream processing for PHA recovery. As a subsequent step as alternative to DAF, classical methods like centrifugation can be applied for separation of the released PHA granules from the surroundings, as also proposed for the process Design by partner 1 (see ANNEX).

Purification of the finally obtained PHA was accomplished by subsequent washing steps and peroxide application; the product was of high purity, especially regarding the absence of lipid (biodiesel) residues).

As a method for disruption of non-PHA biomass without bringing the biopolymer in solution, sodium dodecylsulfate (SDS) was utilized as digestion agent. SDS is a rather cheap and non-toxic digestion agent and, as mentioned before, is of interest especially at high intracellular PHA contents. Due to the fact that the PHA granules remain more or less unscathed after the application of SDS, this method does not negatively affect the molecular masses of the polymer.

SDS, this method does not negatively affect the molecular masses of the polymer.

Fourteen flasks containing 500ml of the fermentation broth had been prepared. In each flask, different amounts of SDS had been added at the following ratios to biomass (SDS / g/L): 0.15; 0.20; 0.25; 0.30; 0.35; 0.40; 0.45; 0.50; 0.55; 0.60; 0.70; 0.80; 0.90 and 1.00 (sample 1 to sample 14, respectively).

All flasks were agitated with a magnetic stirrer for 1 hour. The solutions were further heat-treated at 121 °C for 20 min in an autoclave.

The resulting solutions were centrifuged at 13 000 rpm for 10 min; the remaining solids were washed with distilled water, centrifuged again and dried at 60 °C for 5 hours.

Observing the resulting polymer, it is well visible that from the sample 1 (0.15 ratio) until sample 5 (0.35 ratio), a very dark coloration remained, very close to the colour of the biomass. It turned out that all of the investigated ratios between SDS and biomass result in purity level around 100%. This indicates that the extracted polymer is highly pure, without a significant influence of the amount of SDS added.

Task 3.2: LCA and cleaner production studies

In this study SPI methodology was the preferred life cycle impact assessment (LCA) method. It results in an ecological footprint, calculating the area necessary to embed the whole life cycle to provide products or services sustainably into the ecosphere. The sustainable process index (SPI) is based on the assumption that the only energetic income of our planet is solar energy. This income drives all natural processes and global material cycles (e.g. the global carbon cycle). The key resource to transform this income into utilisable material (e.g. biomass) or energy is area, e.g. using the techniques like photovoltaic, thermal solar energy or the indirect utilization of solar energy via conversion of biomass. Productive land, air and water have to be retained in a condition that allows them to remain the key production factors in a sustainable economy, therefore all emissions into the three compartments air, water, and soil are considered for the ecological footprint calculation following the principles that global material cycles must not be changed and that the local qualities of these compartments must not be changed either. Therefore the SPI value is a sum of seven different sub-areas (area for land occupation, area for non-renewable material, area for renewable material, area for fossil carbon, area for emissions to soil, water and air).

The sum of all areas to provide raw materials, energy and to absorb emissions is the ecological footprint of the life cycle of the product or service.

The SPI may be used to compare different technologies, optimize the environmental performance of a single product (ecodesign) or to optimize the environmental performance of a company. Especially the latter is of importance in the case of utilizing energy from bagasse for PHA production from sugarcane (Harding et al., 2007). The SPI realized as software tool SPIonExcel (Sandholzer and Narodoslawsky, 2005) is able to design the whole product-service chain of PHA production and provides concrete and encompassing information about the environmental impacts of the processes in question.

A particular tool to minimize waste and emissions during the PHA production process based on environmental assessment and to maximize the product output can be identified by the strategy of Cleaner Production. Cleaner Production is a preventive ecological protection methodology that may be applied to a particular company and product. Cleaner Production helps a company to intelligently selecting adequate materials, save energy and to avoid waste streams, waste water generation, gaseous emissions, noise and unused heat. Cleaner Production is also based on the strong belief that solar energy has to be applied wherever possible (Schnitzer et al., 2007; Schnitzer and Ulgati, 2007).

The sustainable process index (SPI) methodology describes an LCA (life cycle impact assessment) method which can be used for interpretation of LCA results. SPI is part of the ecological footprint family and uses square meters of land as ecological indicator. To calculate the SPI for production processes a freeware tool SPIonExcel (see http://spionexcel.tugraz.at online) is used. The ecological footprint for ANIMPOL process is compared with footprints of competitor polymers like PE-LD fossil based polymer and PHA_RSO (Rape Seed Oil) based PHA.

To take the best available technologies into account, a rendering plant with different product lines and an integrated waste water treatment was visited. A set of key figures according to the integrated processes is partly already analyzed and used as a basis of decision.

Realizing a sustainable development of our planet requires a reduction of waste production, harmful emissions and higher energy efficiency as well as utilisation of renewable energy sources. One pathway to this end is the design of sustainable biorefinery concepts. Utilizing waste streams as raw material is gaining great importance in this respect. This reduces environmental burden and may at the same time contribute to economic performance of biorefineries. PHA is the target product while production of high quality biodiesel along with meat and bone meal (MBM) as by-products improves the economic performance of the process.

Focus was devoted to ecological comparison of different production scenarios and the effect of geographical location of production plants taking different energy production technologies and resources into account; Ecological Footprint evaluation using sustainable process index (SPI) methodology was applied. Keeping in mind that the carbon source for PHA production is produced from waste through energy intensive rendering process, the effect of available energy mixes in different countries becomes significant. Ecological Footprint results from the current study shows a bandwidth from 372,950 to 956,060 m2/t PHA production, depending on the energy mix used in the process which is compared to 2,508,409 m2/t for low density polyethylene (PE-LD).

Details:

Animal residues:

As explained in the economic analysis (deliverable D 3.23) it is assumed that waste cost is equivalent to transportation cost for material collection from different slaughtering facilities. Similarly waste materials from slaughterhouses are considered with an SPI value of 0 m^2/t. This allocation is made because the footprint is already included in the main product of slaughtering which is meat. Nevertheless, the transportation of the waste material to the rendering plant is taken into account. For 1 t of PHA production 13.64 t of waste material will be transported within 75 km radius causing 150 km distance per trip. The total freight transportation amounts to 2,046 tkm. The SPI value for transportation using 28 t transportation trucks is 84.95 m2/tkm. Thus the calculated SPI value for waste is 173,855 m2.

Hydrolysis:

SPI for offal hydrolysis is the sum of calculated SPI values for offal transportation, electricity consumption for chopping and acid reclamation, heat consumption for heating and acid consumption. The Ecological assessment result for hydrolysis carried out using SPI Methodology using inventory inputs data for 1 t equivalent of organic nitrogen production through offal hydrolysis. It turned out that reveals that mineral acid and base are the key footprint producers and contribute to about 83 % to the overall footprint of the sub process, while transportation and energy sectors also have significant shares. The higher share of mineral acid and base footprint is due to fossil fuel based highly energy intensive production processes of these chemicals.

Rendering:

SPI calculations for the rendering process are also divided into two parts depending on the material to be processed. Contaminated waste material is exclusively used for heat production for the Rendering II process. In contrast Rendering II processes the main part of the waste stream to produce meat and bone meal (MBM) and tallow.

As explained in the rendering process description (D 3.23), rendering I material constitutes contaminated material which is not allowed for the processing of tallow. The SPI for Rendering I is calculated using inventory data for 1 MWh heat production as shown in D 3.23. Rendering II is the main rendering process which processes non risk material having extra fat, bones and animal viscera as the main constituents to produce tallow and MBM. Tallow is further utilized to produce biodiesel while MBM will be sold on the market. Inventory data for 1 t of tallow production by rendering process is given in D 3.23.

It was calculated that energy (electric and heat) and transportation are the main contributors with 87.25 % and 12.23% shares. This shows the potential to minimize the footprint by utilizing energy from cleaner technologies utilising renewable resources.

Biodiesel production:

Biodiesel is produced by transesterification of fat with methanol. Inventory data for 1 t of biodiesel production are provided in D 3.23 and

indicate that tallow obtained from rendering process, KOH, H2SO4, CH3OH, heat, electricity and waste water are main energy and mass flows for the process. The raw material (fat from rendering process) along with methanol and electricity are the main contributors to the overall footprint of the process. Fat production is a highly energy intensive process along with being the main material input for biodiesel process accounting to 1.02 t/t biodiesel production. It shares about 69 % of total footprint along with 17 % of methanol and 11 % of electricity. Heating have a little share of 1 % because heating requirements are fulfilled to a great extent by utilising heavy glycerol material having calorific value equivalent to heavy fuel oil, obtained during biodiesel distillation.

PHA production:

PHA production, including the downstream processing is comprised of fermentation process, PHA separation and the purification process. Inventory data for 1 t of PHA production in fermentation process are provided in D 3.23. Hydrolysate is a source of organic nitrogen and mixture of essential amino acids while ammonium hydroxide serves as a source of inorganic nitrogen and also helps to maintain reaction conditions. Biodiesel and glycerol are the main raw materials acting as carbon source for bacteria to produce PHA. Inorganic chemicals are a mixture of essential chemicals and biochemicals required for the fermentation process. Energy consumption comprises stirring during the fermentation process, pumping in and out of the fermentation media from the reactor and maintenance of the fermentation media temperature at about 37 °C. Similarly, water is consumed for fermentation media and downstream processing.

The footprint distribution of 1t PHA production according to the given inventory data shows that the raw materials (biodiesel and glycerol) are the major footprint contributors along with electricity consumption and contributes about 68% and 19% to the overall footprint respectively.

Impact of geographical context:

The comparison of ecological footprint for biodiesel production using electricity mixes from different countries with renewable energy mix for EU, using rape seed oil for biodiesel production (bio_RSO) and diesel production was provided. It turned out that diesel production has the highest footprint with 715,469 m2/t among all these processes, while biodiesel production using the renewable energy mix has the lowest value of footprint 310,771 m2/t.

The differences in the footprint value for different countries are based on energy mix composition; the higher the share of renewable energy production, the lower the footprint value of the product or service will be. The categorical comparison of footprint reveals that 'area for fossil C' occupies more than 75 % of total footprint area except in the case of France, where emission to water are also high. This high emission to water is representative for the high share of nuclear power generation of 72 % in the net electricity mix for France. Similarly, the highest share of 'area for fossil carbon' is occupied in case of Poland energy mix which is representative for the high share of coal powered electricity production, amounting to 84%.

The conventional energy production technologies are usually relying on fossil resources like crude oil, coal and natural gas. Due to this reason these technologies exert largest pressure during operation by emitting enormous amounts of CO2 in the atmosphere. It's a common perception that Energy technologies based on the renewable resources such as solar radiation, biomass and wind power are environmentally friendly and can cope with the global warming problem. It was calculated that the footprint comparison per t PHA production using energy mix for different countries with the EU mix, the renewable energy mix, rape seed oil as carbon source (PHA_RSO) and fossil based polymer competitor low density polyethylene (PE-LD). It can be seen that PE-LD has the highest footprint value of 2,508,409 m2/t while PHA produced using renewable energy mix have the lowest footprint value of 372,950 m2/t.

Similarly, in the case of Norway, PHA production has a significantly lower footprint of 567392.860 m2/t which is almost equal to 'renewable E+NG' 750684.674 m2/t (representing renewable electricity mix and natural gas for heating), because more than 91% power is generated utilizing hydropower production system.

In PHA production emissions to water category have increased for PE-LD and France to a significant amount as compared to biodiesel production results PHA production from EU energy mix scenario has 62 % less footprint compared to PE-LD while renewable scenario has 85 % and 62 % lower footprint compared to PE-LD and EU energy mix scenario, respectively. Similarly renewable E+ NG scenario has 70 % lower footprint than PE-LD and 21 % lower than EU energy mix one. The life cycle CO2 emissions for different scenarios have been calculated based on the subcategory 'area for fossil C'.

As expected PE-LD has the highest 7.4 t CO2 emission per t of PHA if compared to renewable energy resources with only 1.5 t CO2 emissions per t of PHA production. Similarly, in the case of Poland, significantly higher CO2 emissions (5.6 t per t PHA) are calculated if compared to other countries, especially Norway, with only 2.97 t CO2 emissions. Renewable E+NG have quite high CO2 emission which is due to natural gas consumption as well as the use of fossil fuel in the production of solar photovoltaic plants and biomass burning. Germany and the People's Republic of China also have moderately higher CO2 emissions of 4.41 t and 4.45 t, respectively, which is caused by the rather high share of coal fired power generation systems in their energy mixes.

Task 3.3: Engineering and costing

It was necessary to perform analysis of economic feasibilities of technology to be transferred to the industry for successful implementation. The cost for any product can be divided into three main categories: direct costs, fixed cost and general costs. Direct costs comprise of the expenses which have direct effect on the production rate i-e. raw materials, operating labor and utilities costs. Fixed costs are independent of the production rate and are effective even there would have been interruptions in the operation process. It consists of facility or plant depreciation, taxes and insurances etc. The last but not the least cost section is general expenses which include overheads for maintenance of the plant operations, administrative costs and research and development funds (C.G. Pereira et al. 2007).

A detailed economic analysis of the sub processes has been made in deliverable 3.23. The economic analysis is updated by calculating investment costs, operating costs and making sensitivity analysis. In the last 6 months of ANIMPOL project the responsible partner has finalized the process design and development. The design has been optimized via a Pinch analysis, based on a model of the entire process with the tool CAPAD. Detailed economic analysis based on scenarios about organic nitrogen sources and effect of possible market price fluctuations for by products (biodiesel and MBM) has been studied. Using these scenarios sensitivity analysis feasibility of the process has been calculated. Based on the pre-requisites for the PHA-production, the process design was developed over the time by obtaining novel experimental data from the different project partners. From the slaughterhouse-waste to the final product PHA, there are several sub processes, which were analyzed. The final process design consists of following main sub-processes i.e. offal hydrolysis as source of organic nitrogen, rendering process for tallow and MBM production, biodiesel production from tallow and fermentation process. Every decision in the process design is influenced by the fundamental principle to create an ecologic and economic efficient process.

Based on this principle, the process design is evaluated during the changes caused by the new research results. A rough design of the equipment is the basis for the optimization of the energy demand. The entire energy situation is taken under consideration, the supply as well as the demand. One step is the calculation of the energy recovery potential with pinch analysis, but also cleaner production studies, process integration and process intensification are taken into account. After this increase of energy efficiency, the sources to supply this minimalized energy demand were reviewed. One essential part of the energy supply system is the rendering two. In this rendering process only risk material is treated. The tallow of this process is used, as fuel, to provide the thermal energy; a further step could be to burn this tallow in CHP (combined heat and power) plant.

This resulted in an economic assessment of the elaborated ANIMPOL process resulted in a cost appraisal indicating that ANIMPOL-based PHA can be produced at a price below 2 EUROS / kg (ca. 1.7) which is below reported values for PHA production starting from pure substrates of nutritional value, but also below the estimated PHA production price based on other agro-industrial waste streams. This economic assessment is taking into account the allocation of animal-based raw materials, the actual situation on the biofuel market, the optimized data for the bioprocess, a downstream processing without excessive use of chemicals, and enhanced energy-input scenarios. Especially in the case of downstream processing, the new method developed by the consortium, based on high-pressure homogenization, is an additional benefit of the ANIMPOL process, not only in terms of economics, but most of all for environmental soundness.

Details:

According to data obtained from project partner Argus, the investment cost for microfiltration plant having a capacity of 1 m3/h, considered for acid reclamation, amount to 60,000 EUROS. According to data obtained from a commercial chopper producer company based in Italy, the chopper price varies from 30,000 EUROS to 55,000 EUROS depending on the chopping capacity which varies from 3000 kg/h to 5000 kg/h.

Investment cost data for a rendering plant and biodiesel production plant are obtained by personal communication with Mike Scot serving as Technical Director at 'Argent Energy (UK) Ltd'.

Fermentation plant investment costs are calculated by measuring the plant size and calculating its building cost, with rule of thumb from the Christoph group by using Guthrie factors. (Koncar,2010; Christoph Group,2011).

Downstream processing investment cost consists of installation for membrane filtration unit, homogenization unit and centrifugation unit. This data is provided by the project partner 'Argus'.

Waste water plant feasibility

In order to accomplish a feasibility study of a waste water treatment plant, it is necessary to calculate the annual water consumption and discharge. In the current project cumulative waste water to be treated is sum of waste water produced from rendering unit, biodiesel production unit, fermentation and downstream processing unit. This calculation is in accordance to the basic assumption of an annual PHA production of 10000 t.

Annual waste water consumption: 227,863 m3/y

Waste water treatment per day: 690 m3/d

Assumption:

It is assumed that 'waste water composition is similar to the waste water produced in the breweries'.

According to the literature survey, the Biological Oxygen Demand (BOD) value for brewery waste water is about 1.8 kg/hl of beer which is equivalent to 9 kg of BOD/m3 of waste water (Inyang, 2012). This value has been utilized to calculate the BOD load per day, which is 6,214 Kg of BOD/day. The volume of waste water is normally expressed in the units of Population equivalent (P.E.). It is defined as 'per capita' waste water generation (0.2 m3/day) or BOD load for sewage water (54 g/inhabitant .day). BOD load value is used to calculate waste water input for. The P.E value for the current study is 115,082.37 inhabitants (Henze et al, 2002; Sperling and Chernicharo 2005). Based on P.E value a comparative analysis of WWTP size is made using Vienna Waste water treatment plant (WWTP) as standard.

Investment cost for extra units to be installed for industrial WWTP, e.g. oil separator, dissolved air flotation units and polishing steps such as advanced oxidation and activated carbon adsorption cost about 35 % of the main investment cost which is 1,244,063 EUROS.

Other costs such as site preparation, construction (civil, mechanical, Instrumentation and electro-technical equipment, piping, central process control system) start up (equipment and supplies) and additional costs (initial studies, design and engineering, project management, construction management etc.). Rough estimation of these costs is about 50% for large capacity plants and 100 % for small capacity plants of the total construction cost. This plant is considered as a small operating

unit and 100 % of the main cost is added to the investment cost. The total investment cost for the WWTP system is 8,352,994 EUROS.

Biogas plant feasibility

According to the available data 4,808,525.36 m3 of biogas is estimated to be produced annually at a production rate of 601 m3/h. Investment costs for the current case is calculated by comparing investment costs for biogas plant having 250 Nm3/h biogas production capacities (BIOGAS Netzeinspeisung 2013). For 601 m3/h biogas production, it is assumed that all the parameters will be calculated using a linear function while a factor of 0.70 is used to calculate fermenter cost.

Infrastructure:

Infrastructure cost for the industrial facility has been considered for an area of about 900 m2 internal floor area. It includes cost break down from feasibility report to turnkey construction facility (Langdom and Wilkes 2006). The calculated infrastructure construction cost is about 896,000 EUROS.

Depreciation:

Life span of the facility is considered 20 years. Using a linear function for all sub processes, the investment cost annual depreciation is calculated.

Operational costs:

Operational cost for the overall process is calculated according to the method described by Haandel and Lubbe (2007). Operational cost for rendering process and biodiesel production process has been obtained from the partner groups. For other sub processes operational cost is calculated according to the described method. The required investment cost is obtained by subtracting rendering and biodiesel production investment cost from the overall investment resulting in 30,253,879 EUROS.

PHA production costs:

PHA production cost is the sum of overall operating costs for all sub processes to produce input materials and chemical cost for 10,000 t PHA production and annual plant depreciation. It counts for 1.68 EUROS/kg of PHA production.

Revenue:

The revenue is calculated using selling prices for the main product PHA and the byproducts, namely high-quality biodiesel and MBM. The selling prices are 4 EUROS/kg PHA, 0.97 EUROS/l biodiesel and 300 EUROS/t MBM.

Biodiesel selling shows net revenue gain per year while MBM, energy production in rendering I and energy production from fermentation biomass shows net negative revenue. Overall revenue obtained from the byproducts is about 1,96 MEUROS/y. It makes the overall process economically more feasible, decreasing the PHA production cost from 1.68 EUROS/kg to 1.48 EUROS/kg.

As PHA is a renewable polymer it has better price than conventional fossil based polymers (PE-LD). According to data obtained by personal communication with Dr. Martin Koller, a selling price 4 EUROS/kg of PHA is decided. The calculated recovery time for the overall investment is about 3.5 years.

Details for Calculations see Deliverable 3.23.

WP4: characterization, processing of PHA formulations and relevant blends and composites (RDT)

Task 4.1: Preparation and processing of PHA formulations and relevant blends and composites

TERMO in close cooperation with UNIPI carried out processing trials on pilot scale of two different grades of PHA (PHB homopolyester and Poly(3HB-co-4HB) according to lab-scale protocols. The selected materials (PHAs) were scaled-up to kg quantities and submitted to granulation in double screw extruders upon processing of the various powder-formulates in turbomixer. It was demonstrated that, using the available processing equipment, the utilization of Poly(3HB-co-4HB) was beneficial for processing in comparison to PHB.

Regarding ANIMPOL- based marketable products, a formulation was developed for production of PHA-foils with enhanced material properties such as decreases crystallinity and improved processability. In total, 14 formulations were developed for processing PHA with bio-compatible materials; among them, 5 were used for preparing prototype items via melt extrusion or compression molding.

Task 4.2: Characterization of PHA formulations, relevant blends & composites and processed items

The blends PHB/PS/P(S-MMA) where the PHB was the matrix and the P(S-MMA) the compatibilizing agent showed mechanical and thermal similar to the pristine polymer.

The PS and the PVAc increase the onset degradation temperature of the PHB when are blended with the PHB IN 33% in wt. and 10% in wt, respectively. The blends PHB with PVAc showed good compatibility.

The composites done with lignin extracted from pine nuts showed a poor thermal stability compared with the pristine material. Instead the composites makes with the lignin derived from hazelnuts shells without purification increase the thermal stability of the PHB and its composites as well as the disintegration mechanism. In particular, the functionalized lignin shows a positive effect on the biodegradation of the sample observed by the higher weight loss; however the thermal stability and the processing of the PHB having functionalized lignin show a significant decrease of these properties.

Details see respective Deliverable D 5.28, D 5.29, and D 4.34

WP5: Ecocompatibility and biocompatibility of the prepared PHAs and relevant processed prototype items (RDT)

Task 5.1: Determination of biodegradability of PHA Formulations and relevant typical prototype items (Blends& Composites) under different

environmental conditions as ecocompatibility indicator- biodegradation tests in solid media and in Aqueos Medium Blends and Composites

After an exhaustive discussion, the decision was taken to search for bacterial strains, culture collection strains or isolates that show depolymerase activity. The aim was to develop a method useful to rapidly test biodegradation capacity at lab-scale. The decision to test Diaphorobacter sp. DSM 13225 was taken. The first results obtained after growing these bacteria in minimal DSMZ457 agarose medium with both polymers PHB or PHBHV indicated that the biodegradation activity can possibly be rapidly detected at lab scale. A test in liquid media was also performed in order to verify if a film of copolymer can rapidly be degraded by the selected strains.

Finally, several samples of PHAs produced by all the selected/constructed stains were delivered to P7 for ecocompatibility and biocompatibility tests. These results are directly provided by P7.

The work accomplished for biodegradation is collected in M.20 and in Deliverable 5.19. Degradation experiments have successfully been accomplished in the said environments. A comprehensive search on the propensity to biodegradation of different PHAs has been carried out. The methods that will be applied are based on the utilization of respirometric apparatus as specifically designed for monitoring the CO2 developed during the aerobic incubation of the PHA formulations of choice. The amount of CO2 developed in trapped in a solution of KOH solution under equilibrium conditions and its evaluation is made by back-titration procedure. As trial sample PHB (Mw = 425 KDa) was used for the assessment of its biodegradability in solid media (soil and mature compost) and in aqueous media (river fresh water) and specifically aimed at the validation of the applied procedures.

Task 5.2: Eco-toxicity tests of PHAs and relevant items

PHA formulations and relevant prototypes have been screened for cytotoxicity and genotoxicity assessment by using murine balb 3T3 clone A31 cell line, which is usually considered a sensitive cellular model to verify in vitro toxicity. The possible leaching of toxic compounds from the PHA formulations was evaluated by mean of polymer extracts assay by measuring cell proliferation and Colony Forming Efficiency (CFE), following the ISO-10993/5 guidelines. Carcinogenic potential was evaluated by mean of cell transformation assay (CTA) as indicated in ISO-10993/3. Results highlighted good values of cell proliferation and colony forming efficiency for all the tested formulations and prototypes confirming a complete absence of toxicity. Moreover, no evidence of cell transformation has been detected from the carcinogenic potential evaluation carried out on the tested formulations and prototypes.

The formulations F2 and F6, in powder form, have been selected for the preliminary biocompatibility studies. Formulation components and concentrations (wt%) are reported in Deliverable D 5.32. The formulations F2 and F6 processed into film have been selected as prototypes to be tested for preliminary biocompatibility studies. As binary system, the prototype of PHB-lignin with lignin percentages of 5, 10 and 20% was also tested.

In conclusion, a preliminary screening of toxicity was carried out on PHA formulations and relevant typical prototype items, as well as on binary

systems. Cytotoxicity and genotoxicity in vitro studies were carried out using murine balb 3T3 clone A31 cell line. After extracts exposure, the results showed good values of cell proliferation and a good ability of balb 3T3 to form colonies starting from a low density seeding procedure. Moreover, no evidence of cell transformation was detected, allowing for a validation of all the selected formulations and prototypes in respect of their potential applications in food, packaging and biomedical fields.

Task 5.3: Evaluation of LCA

This task is closely related to the activities in task 3.2. Based on the actual process design, material – and energy balances, an ecological footprint was calculated with the SPIonExcel tool.

The initial footprint for PHA at the beginning of the calculations is about 1,960 m^2/kg which is compared to Polyethylene LD (2,500 m^2/kg) better in terms of environmental impact. Improvements during the project revealed a footprint for the ANIMPOL-PHA of less than 400 m2/kg PHA, taking into account scenarios of sustainable energy input, and the positive outcomes of the biocompatibility tests.

Task 5.4: Determination of biocompatibility of PHAs and selected relevant prototype items by cytotoxicity and genotoxicity tests

This task is closely related to the activities accomplished in Task 5.2, using the same methodology, but investigating complete PHA-prototype items.

Three-dimensional porous scaffolds based on Poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx – HHx=12%) were fabricated by using an in-house (UNIPI) modified additive manufacturing (AM) technique, in the view of exploring the possibility to employ PHAs as polymeric biomaterials for tissue engineering applications. In particular attention was devoted to the preparation of 3D micro-structured scaffolds with defined geometry for bone tissue engineering applications. The manufacturing of scaffolds was performed by means of a ROLAND MDX 40A (Roland MID EURORE, Italy) modified in-house in order to allow for the production of computer controlled 3D scaffolds with the principle of wet-spinning.

Scaffolds architecture was changed with 45° and 90° angle steps between two successive layers (called 0-45 and 0-90 configurations, respectively). Different fiber spacing (1mm, 0.5mm, 0.2mm) were employed in 0-90 configuration mode (see M.20). The optimized parameters for scaffolds fabrication are resumed in M.20.

Morphology of the prepared scaffolds including top-view and cross section was investigated by Scanning Electron Microscopy (SEM). Analysis confirmed the optimal overlapping of the fibers and highly porous surface that appears optimal for cells colonization (M.20)

To investigate the ability of the prepared microstructured scaffolds to support cell growth for bone tissue regeneration, mouse calvaria-derived pre-osteoblastic MC3T3-E1 (CRL 2594) cell line from American Type Culture Collection [ATCC] was selected as cell model. Cells were propagated as indicated by the supplier using MEM containing ribonucleosides, deoxyribonucleosides, sodium bicarbonate and supplemented with 2 mM of L-glutamine, 1 % of penicillin:streptomycin solution (10,000 U/ml:10 mg/ml), 10% of fetal bovine serum and antimycotic (complete MEM).

Cell adhesion and proliferation onto the prepared scaffolds was investigated by using WST-1 tetrazolium salts as previously described. Results highlighted the ability of the PHBHHx based scaffold to sustain a significant cell adhesion and proliferation (see M.20). Moreover in order to assess the behavior of the preosteoblasts cell grown onto the prepared scaffolds in terms of osteoblastic differentiation, the production of Alkaline phosphatate was also investigated. The bone isoform of alkaline phosphatase is considered an early marker of the expression of osteoblastic phenotype. ALP is a glycosylated membrane-bound enzyme that catalyses the hydrolysis of phosphomonoester bonds and may also play a physiological role in the metabolism of phosphoethanolamine and inorganic pyrophosphate. Alkaline phosphatase (ALP) activity was determined in cultured MC3T3-E1-scaffold on days 3, 7, 14 and 21. The measurement was assessed with a colorimetric method that is based on the conversion of p-nitrophenyl phosphate into p-nitrophenol by the ALP enzymatic activity. The results showed satisfying ALP activity for all the investigated scaffolds, with a more pronounced production in the case of the 0.2mm (0-45) sample.

Potential impact:

What are the major outcomes of the project?

It has to be underlined that nowadays about 280 Mt of plastics are produced globally with an expected annual increase of about 4%. Thereof, only a minor share of about 5% is attributed to biobased and biodegradable polymers. Nevertheless, currently, we are experiencing a remarkably dynamic biopolymer market with an impressive increase of the volume and range of products: in 2010, the market value reached a magnitude of 10^10 US-dollars with a clearly upwards trend. Only from 2008 to 2015, the global production of biopolymers is estimated to increase from 180 to 1710 ktonnes. Since the launch of ANIMPOL, the price for petrol, the raw material for production of 'classical', highly recalcitrant polymers, has fluctuated up and down. At the same time, the political situation in many petrol-exporting countries like Bahrain, Libya etc. became highly instable, and the future in other important petrol exporting countries is not predictable. In addition, the public awareness and sensibilization for ecological concerns like the greenhouse effect, global warming, was continuously increasing since the start of ANIMPOL, indicated by the strong presence in media and the controversial discussions dealing with these topics. The ANIMPOL consortium is convinced that, based on the elaborated results, a strategy can be presented that at least to a certain extend provides a solution for the discussed concerns.

In accordance with the topic and the original project aims, the ANIMPOL consortium converted agricultural and industrial waste stemming from the animal processing and biodiesel industry by upgrading them to raw materials for value-added bio-products by application of new technologies. Novel and economically viable solutions were elaborated for industrial waste treatment, biotechnological polymer production and processing the polymers to prototype plastic items.

Now, value is added to the different waste streams by using enzymes and microorganisms for production of valuable bio-products (PHAs), and, for the first step of biodiesel production, also for transesterification of waste lipids to biodiesel; further, after separation of the separated fraction that is used for the bioprocess, the quality of the remaining biodiesel (unsaturated fraction) as a 2nd generation fuel is improved. The project elaborated local solutions for local problems, but due to the fact that the problems to be solved are common or at least very similar for many regions of the European Union the solutions developed, the consortium is convinced that these solutions are now applicable for all Europe.

Utilization of waste-streams directly in the regions where they are produced will have a positive impact on such rural areas, will create new jobs in fields of advanced technologies (this already happened to a high extent during the ANIMPOL project!), will have positive impacts on environmental protection, and will minimize the quantity of mineral-oil-derived plastic packaging in use by replacing them with high quality biodegradable polyesters. Finally, the reduction of waste will contribute to changing the public perception of food industry as a heavily polluting one.

As the major outcomes, calculations accomplished by the responsible consortium partners indicate as well the environmental benefit of the

ANIMPOL process as determined by means of life cycle assessment, and also demonstrate the economic progress if compared to common biopolymer production strategies based on prized substrates.

Who wants the elaborated solutions?

The project will bring together the players of the waste resources from slaughterhouses, rendering industry, and waste fractions of the biodiesel production with polymer producing and/or processing industry. Large companies and SMEs involved in the meat-processing sector from Austria, Germany, Slovenia and Italy have already addressed their interest in the elaborated results and solutions.

The project is expected to create transnational added value due to the fact that the European community will gain future benefit for industrial development by the created successful network of partners from different European countries having complementary expertise. This expertise was used in a synergistic way for the utilization of two different waste streams (materials directly resulting from animal waste and the saturated fraction of biodiesel) for sustainable production of value added and ecologically benign materials. According to the ideas of physiological material cycles, the organic waste streams lead to bio-products (PHAs), which after their life-span can be composted, recycled, or hydrolyzed to their monomeric building blocks to be used as synthons for fine chemicals (interesting for the pharmaceutical and cosmetic industry).

Two different branches of European industry will be able to handle their tremendous waste streams, and polymer producing and processing industry will be enabled to switch to alternative, sustainable products. Although tasks of the polymer-specialized partners are mainly aimed to help to develop production technologies for a range of different grades of biodegradable PHA polymers, important progress was accomplished also in the field of preparation of blends and composites based on PHAs as either matrix or dispersed phase. Various procedures of making pairs of immiscible polymers compatible were improved, with special emphasis on PHAs based blends. Various low-value filler materials, mainly of agro-industrial origin, were successfully applied for production of PHA-based composite materials. Further on, progresses in characterization techniques were achieved by the synergistic expertise of the specialists in the Consortium with profound knowledge in various techniques; thus it was possible to compare different parameters, resulting in more reliable conclusions on the morphology, structure and special properties of different PHAs. These materials are applicable in various fields.

Summing up, the elaborated solutions are of high interest for the meat-producing industry, the rendering companies, plastic converters and chemical companies, but also for local and regional authorities engaged in sustainable regional development. At the moment, several companies from Austria and Germany are already interested in implementing the ANIMPOL results.

Transnational impacts

Already now, transnational partnership between industry and academic institutions in the participating countries is facilitated with balanced benefits between scientific partners and the companies. The project outcomes provide new input to European industrial and scientific institutions and create economic and ecological benefits by converting

waste into value added, environmentally benign materials. For synthesis of progress in environment conservation and economic value addition by reduction of pollutants, biotechnological polymer production features a future-oriented solution with a high public acceptance. The created network will be of strategic value as well for the academic institutions as for the companies involved. For example, the ANIMPOL network was integrated into the PLASTiCE project that encompasses the most relevant European players in the field of biopolymers. The project will connect European research institutions with large companies, opening the door for co-operations in future. Further, research input coming from the research institutions is forcefully aspired by the European industry as precious impulse for product and process development. The cooperation between ARGENT, TERMO, ARU, RIX (SMEs) and the academic partners provides the required scientific input for the European industry to establish itself in the field of biopolymers and enables ARGENT to improve quality of its product (biodiesel) and to create value from by-products (saturated biodiesel fraction). For the research institutions, the cooperation with industry opened the door for future projects with these partners that are now in close contact.

Socioeconomic impacts

All industrial partners in the project can profit from the highly qualified scientists and technicians to be educated within the planned cooperation at the research institutions as valuable human resources. Grace to ANIMPOL, jobs was created both at the research institutions and at the industrial partners. A total of 72 jobs were created grace to ANIMPOL, corresponding to about 35 person/years; among those, about 20 people were exclusively recruited for the project. Many of these people maintained their employments at the companies and universities even after the end of the project.

The cooperation between researchers from different European countries was strongly intensified, e.g. by exchange of young researchers, especially exchanges between TUG, KFU, UNIPD and NIC, and the frequent project meetings. By the created network of academic and industrial partners, research results coming from the research institutions can now be verified on industrial scale; in the case of processing of PHA, this was already accomplished during the project time. Moreover, this project provides a competitive advantage to the European meat converting and rendering industry by providing substrates for biotransformation as well as for the European biodiesel industry.

If implemented now, the project will contribute to socioeconomic benefit by job creation directly in meat converting factories and biodiesel factories, in further research activities and distribution of the created value-added products. Although a significant part of the knowledge on PHAs (microbiology, physiology, and engineering) has been acquired in the EU, R&D activities towards industrial production of PHAs are mainly situated outside Europe (North- and South America, Asia).

The ANIMPOL Consortium believes that the results obtained during by the ANIMPOL project constitute a viable strategy to enable production of these highly promising biopolyesters in Europe, to avoid that in the future PHAs have to be imported from outside the EU.

Successful gender balance

Gender balance, as promised at the negotiation process for granting ANIMPOL, was realized. Among the about 20 positions exclusively created for ANIMPOL, more than half were devoted to female co-workers. Although all work package leading positions were occupied by men, 10 women with highest qualification (doctoral degree) were involved in the research.

The consortium was able, by addressing especially female student by the actions of open labs etc., to achieve a share of female PhD students active for ANIMPOL of 56%, and a similar value for students having accomplished their master thesis on ANIMPOL tasks.

What further development steps will be needed?

Now, an up-scaling of the developed processes should be done in a pilot plant. This should be accomplished by interested lead-users of the project results, of course with the help of the academic partners involved. Several interested industrial companies from different European countries already contact the consortium leader. Especially the implementation of ANIMPOL-derived plastic materials for food packaging seems to be the most likely application in the next few years.

After a successful pilot phase, the final step has to be towards the market launch of bio-polymeric products produced by the strategies elaborated in the project.

Dissemination and exploitation

The development of dissemination and exploitation strategies required the highest degree of collaboration between all participants in the consortium in order to achieve a common consensus about the 'conversion' of the results obtained during the research and to make them accessible for the lead companies and users and finally to the broadest possible and sustainable use. Due to the efforts done by all partners, the representation of ANIMPOL in public, the number of scientific publications and the contributions to conferences and fairs by far exceeded the expectations of the Consortium at the beginning of the project. Dissemination of the results as a central item of ANIMPOL was done by a huge number of written and oral publications in research journals, industrial journals and books as well as at diverse conferences. Especially the huge number of national and international conference contributions (oral and poster presentations) presenting the concepts of and data from the ANIMPOL project has to be underlined.

The conferences with ANIMPOL contributions of major importance should also briefly be mentioned here:

- Renewable Plastics 2010 (Brussels, Belgium) 2010

- Renewable Plastics 2011 (Brussels, Belgium) 2011

- BIT´s 3rd Annual World Congress of Industrial Biotechnology 2010 (Dalian, People´s Republic of China) 2010

- 14th International Conference on process integration, modelling and optimisation for energy saving and pollution reduction (Firenze, Italy) 2011

- Highlights der Bioenergieforschung: Nationale und internationale Ergebnisse zu den IEA Schwerpunkten. (Wieselburg, Austria) 2011

- 4th ICPB-IUPAC - International Conference on Polymer Behavior 2010 (Lódz, Poland) 2010

- 53rd Annual Meeting of Polish Chemical Society 'PTChem-SITPChem' (Gliwice, Poland) 2010

- 3rd Conference The Future of Biodegradable Packaging (Warszawa, Poland) 2010

- 'Medical Device Polymers 2011' (International Conference on Polymer Materials and processing Technology for the Medical Device Manufacturing Industry) (Köln, Germany) 2011

- 19th European Biomass Conference and Exhibition (From Research to Industry and Markets) (Berlin, Germany) 2011

- 15th European Congress on Biotechnology- ECB15. Istanbul, Turkey, 23.09.2012

- Biopolymer World Congress. Venezia, Italy, 22.04.2012

- Europe for Sustainable Plastics (PLASTiCE). Bologna, Italy, 24.10.2011

- European Symposium on Biopolymers 2011. Dublin, 27.09.2011

- BiPoCo 2012, an International Conference on Bio-Based Polymers and Composites. Siófok, Hungary, 27.05.2012

- E-MRS 2012 Fall Meeting, Symposium B, Renewable polymers as multifunctional materials: properties, processing, applications. Warsaw, Poland, 17.09.2012

- 'TeamBILDUNG' von JO!N, ÖH der FH Joanneum. Graz, Austria, 17.10.2012

- 15th European Round Table on Sustainable Consumption and Production 2012. Bregenz, Austria, 02.05.2012

- 20th Jubilee Conference on materials and technology. Portoroz, Slovenia, 17.10.2012

- EUPOC 2011: Biobased Polymers and Related Biomaterials. Gargnano, Italy, 29.05.2011

- Bio-Alternatives 2013. Graz, Austria, 03.04.2013

- Siebtes Expertenbeiratstreffen klima:aktiv nawaro markt. Schwerpunkt: Stoffliche Nutzung von agrarischen Reststoffen and alternativen Rohstoffen. Vienna, Austria, 12.12.2012

- SD Symposium 2012, Graz, Austria, 15-17.02.2012

- CAPE Forum 2012, Vezprem, Hungary, 26-28.03.2012

- IAMAW 1st international workshop, 5-8 June 2012, Santarém, Portugal

- I sottoprodotti animali: conosciamoli meglio. Verona, May 25th, 2012, Italy

- 103rd AOCS Annual Conference and Expo in Long Beach, USA, April 29th to May 2nd, 2012

- Joint Conference of the Polish Mass Spectrometry Society and German Mass Spectrometry Society 2012, Poznan, Poland, 4-7.03.2012

- IEA Bioenergy Task 42 Biorefinery, 04 April 2011, Tortona, Italy

- POLYOR 2011, Opole, 6 July 2011

The entire consortium, especially the industrial partners, recognized the duty of the academic partners to publish their results in high ranked scientific journals. This has to be considered as a measure of the scientific impact of the ANIMPOL project and the scientists involved in it. Most of all, publishing is of high importance for the young scientists, especially PhD students who have to get their feet on solid scientific ground based on their efforts for ANIMPOL. The Consortium had two central points of focus when publishing:

- Does the publication endanger eventual patenting? If yes, the Consortium resigned from the idea of publishing.

- Quality of the journal based on impact factors and indexing

- Open access

- Synergistic effects by involving several ANIMPOL partners to compile a publication (prime examples: Muhr et al., 2013, Reactive and Functional Polymers; Koller et al., 2013, Engineering in Life Sciences; Povolo et al., New Biotechnology, 2013)

Journals with ANIMPOL contributions:

- Chemical Engineering Transactions (Kettl et al., 2011; Kettl et al., 2012)

- Applied Microbiology and Biotechnology (Atlic et al., 2011)

- Nachwachsende Rohstoffe (Koller et al., 2010)

- Engineering in Life Sciences (Koller et al., 2011)

- International Journal of Mass Spectrometry (Bednarski et al., 2011)

- Bioresource Technology (Vrana Spoljaric et al., 2013; Kwiecien et al., 2013)

- European Polymer Journal (Adamus et al., 2012)

- Bioprocess and Biosystems Engineering (Horvat et al., 2013)

- Reactive and Functional Polymers (Muhr et al., 2013)

- Journal of Biotechnology (Muhr et al., 2013)

- Clean Technologies and Environmental Policy (Titz et al., 2012; Shahzad et al., 2013)

- Resources, Conservation and Recycling (Koller et al., 2013)

- Journal of Polymers and the Environment (Povolo et al., 2012)

- New Biotechnology (Povolo et al., 2013)

- Materiali in Tehnologije (Koller et al., 2013)

In addition, the concepts of ANIMPOL were manifold presented in newspapers (e.g.: 'Der Falter'; 'Kleine Zeitung', 'Profil', 'Bayrisches Landwirtschaftliches Wochenblatt', 'Industrial Technology', 'der Standard', 'Salzburger Nachrichten', 'Food Trade Review', 'Kurier', 'Technology Reviews', „Metro', „Der Grazer', „Recycling Magazine', 'Bioplastics Magazine'; 'TrenntMagazine')

In the Austrian, German and Swiss television (ORF2 ['Report'; 'Steiermark heute']; 3 SAT ['Nano']; SF2 ['Einstein'])

In several German (rbb, Detektor.fm, Radio Köln) and Luxembourgian (RTL Radio Lëtzebuerg) radio stations.

In addition, lectures are available on youtube, where ANIMPOL is presented in details:

You tube: lecture 'The ANIMPOL Project: From Animal Waste to PHA-Bioplastics' in: Europe for sustainable plastics PLASTiCE, Bologna, October 24th to 25th 2011, is available on You tube in two parts since April 4th, 2012 (together 19 min 17 sec).

(see http://www.youtube.com/watch?v=PUnaZDCT7jA and http://www.youtube.com/watch?v=TP4RWn9y8i4&feature=relmfu online)

You tube: Lecture 'Polyhydroxyalkanoates: Biodegradable polymeric materials from renewable resources', online since November 20th, 2012 (36 min sec) (see http://www.youtube.com/watch?v=5b5uNTxq6S8 online)

Until today, an entire number of more than 110 publications accrue from ANIMPOL. In addition, the development of the ANIMPOL website is still in permanent progress. This is in absolute agreement with the activity descriptions as provided in Annex 1 of the Grant Agreement.

A total of 5 articles are already submitted or in status of preparation for submission to high-ranked journals (Biochemical Engineering Journal, Journal of Polymers and the Environment, Journal of Theoretical Biology, Journal of Biotechnology, Bioresource Technology).

To a broader public, ANIMPOL was also spreat by several activities:

- Open Labs (2011, 2013. TU Graz): demonstration of biotechnological biopolymer production and ANIMPOL prototypes to a broad public

- Invitation of School Classes (School Ferdinandeum, Graz)

- Lecture at 'TeamBILDUNG' von JO!N, ÖH der FH Joanneum, Graz on 17.10.2012; adressed mainly students

Patentable know-how from ANIMPOL

No option for the ANIMPOL consortium is to manage intellectual property (IP) merely to exclude rivals (and by doing so blocking innovation) or merely to limit the exploitation by piling IPRs for potential cross licensing of single partners or to put technology without an explicit business case consideration to the public domain. The subsequent section provides an overview of accomplished and envisaged patenting activities.

Accomplished patenting (partly from ANIMPOL activity):

Scandola M., Focarete L., Mazzocchetti L., Kowalczuk M., Kurcok P., Adamus G., Kawalec M.: Process for controlled degradation of polyhydroxyalkanoates and products obtainable therefrom. US Pat. Appl. US 2011/0275729 (2011).

The following outcomes and results of ANIMPOL are at the moment considered for additional patenting:

UNIPI: The partner is finalizing a series of experiments aimed at setting up the formulation of innovative hybrid blends based on PHAs obtained in the implementation of the ANIMPOL project as well as on commercially available PHA samples. As second component of the blends poly(hydrocarbon)s samples such as Polyethylene(PE) and Polypropylene(PP)containing pro-degradant/pro-oxidant additives will be used. The use of recently produced PHAs in the ANIMPOL implementation as consisting of monomeric units containing in 3 position long chain alkyl substituents should guarantee for a good chance of compatibility with appreciable amounts of low cost poly(hydrocarbon)s. An additional approach aimed at reducing the drawbacks of PHA processing and at limiting the production and hence retail costs of PHAs is to formulate also composites based on ligninolytic fillers derived from shells of pine seeds, chestnut and walnuts

TUGRAZ and other partners: PHAs for defined applications from animal waste streams (process patent covering the entire production chain with the final aim to design a pilot plant to be integrated into production lines of biodiesel companies).

TERMOPLAST: The partner is involved in a series or processing trials on a pilot plant of PHAs formulation set up in collaboration with UNIPI aimed at reducing the deleterious degradation effects caused by temperature and residual moisture present in PHAs. These trials will be also extended to PHAs blends and composites designed by UNIPI.

UNIPD: culture supernatant to be directly utilized as an enzymatic formulation for lipid transformation into biopolymers

List of websites:

http://www.anipol.tugraz.at