Project No: 318931

Project Acronym: BIOASSORT

Project Full Name: Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products

Marie Curie Actions

BIOASSORT Final Report
PUBLISHABLE SUMMARY

Period covered:
from 30/07/2012 **to** 29/07/2016

Date of preparation: September 2016

Period number: Final

Date of submission (SESAM): 11/10/2016

Start date of project: 30/07/2012

Duration: 48

Project coordinator name:
Prof. Dr. VINCENZA FARACO

Project coordinator organisation name:
UNIVERSITA DEGLI STUDI DI NAPOLI FEDERICO II.

Version: 1

A comprehensive summary overview of results, conclusions and the socio-economic impacts of the project.

The main research activities performed in the first two years of BIOASSORT project and the achieved results are summarized as following:

Task 2.1: Selection of microorganisms by screening of the microbial collections for their ability to produce enzymes cellulases, ligninases, xylanases and amylases, EtOH, PHAs, LA.

- A screening of DEBB microbial collection (partner 4) was carried out in order to enlarge the patrimony of microbes for waste conversion into high added value eco-friendly and cost-effective bio-products. Particularly, different fungal species (*Aspergillus sp.*, *Pleurotus sp*. and *Lentinus sp.*) belonging to DEBB collection were investigated for ligninolytic, cellulolytic and xylanolytic activities production. Screening on solid medium for ligninolytic activity was performed in the presence of the colored indicator Guaiacol, whilst the cellulolytic and xylanolytic activities were detected by Congo Red assay in the presence of carboxymethylcellulose and xylan, respectively, as sole carbon source. Starting from 32 fungal strains, the screening led to the selection of six strains producing ligninolytic activity, three strains producing cellulolytic activity and nine strains producing both cellulolytic and xylanolytic activities. The selected strains were grown in liquid media for the quantitative estimation of enzymes production. Time-courses of cellulase and xylanase activities production were evaluated in the presence of cellulose microcrystalline 1% as sole carbon source, whilst ligninolytic enzymes production was measured in the presence of glucose, as carbon source, and $CuSO_4$, as laccase inducer. Three strains belonging to the species *Lentinus* sp. and *Aspergillus* sp. were selected as the best cellulase activity producers. The maximum value of xylanase activity was produced by an *Aspergillus* strain whilst the highest laccase production level was achieved with a *Pleurotus* sp. strain.
- In order to select new microorganisms producing lactic acid and ethanol, six lactic acid strains and five yeast strains, belonging to DEBB collection (partner 4), were investigated for their performance in lactic acid and ethanol production, respectively. The screening led to the selection of one homolactic bacterium, namely *Lactobacillus acidophilus* LPB-04, and the strain *Saccharomyces cerevisiae* LPB-287.

Task 2.2: Isolation of new microorganisms able to produce enzymes, EtOH, LA and PHAs from natural habitats of India and Brazil.

- Isolation of new microorganisms from natural habitats was performed by exploring biodiversity of Western Ghat region (Kerale, India) to discover novel microbes producing cellulolytic and xylanolytic enzymes at high titer, thus improving the cost-efficiency of the industrial bio-processes. 50 soil samples of different nature (humic soil, grass land, inner part of rock, rock surface, excrement, laterite) were used for microorganisms isolation. 93 microorganisms were purified and then screened by Congo Red assay on xylan- and CMC-containing plates for xylanase and cellulase production, respectively. The screening led to the selection of seven xylanolytic and 14 cellulolytic microorganisms showing activity halos. Based on a preliminary morphological analyses and BLAST analyses of the 16S rRNA sequences, the new microorganisms showed high similarity to different species such

as *Bacillus* sp., *Streptomyces* sp., *Lysinobacillus* sp. and *Paenibacillus* sp. A further screening was carried out in liquid culture, using xylan- and CMC-containing media. This way, a total of four xylanolytic and eight cellulolytic microorganisms were selected.

Task 3.1: Formulation of wastes and optimization of pre-treatment processes to prepare mechanically pre-treated wastes to the conversion by comparing AFEX, diluted acid treatment and/or biological systems.

- Brewers' spent grain (BSG), provided by the brewery Bier Hoff Curitiba-PR (Brazil) and by the micro-brewery Maneba (Striano, Naples, Italy) were subjected to two different pretreatment methods: by use of acid and alkaline and aqueous ammonia soaking, respectively. The BSG pretreated by use of acid and alkaline was saccharified with commercial cellulase and β-glucosidase and the hydrolysate used for ethanol and lactic acid (LA) production by the microorganisms selected in WP2.1 (in WP3.3 and WP3.5, respectively). The BSG pretreated by aqueous ammonia soaking was saccharified with different commercial enzymes used in combination with *B. amyloliquefaciens* xylanase activity (in WP4.3).
- Newspaper waste was collected in the recycle station of International Center (OISS) in MSU (partner 5). This Newspaper waste, mainly composed by free weekly newspapers distributed in Lansing area, was subjected to AFEX pretreatment testing different conditions. The composition analysis before and after AFEX process was performed according to the National renewable Energy Laboratory (NREL) protocols. Optimal conditions able to give the maximum glucan and xylan conversions upon saccharification of AFEX-treated Newspaper waste with commercial enzymes were identified.
- With the aim of defining a model substrate, Kitchen waste (KW) was prepared by collecting food wastes (Potato peelings, Salad, Tomato, Pineapple, Onion, Carrot) from Indian Restaurant and subjected to Solid State Fermentation (WP3.2).

Task 3.2: Optimization of mixed SSF

- The cellulolytic fungus *Trichoderma reesei* (from collection of NIIST-CSIR, partner 3) was used for setting up the SSF experiments on Kitchen waste (KW). In the experimental conditions, the fungus was shown able to colonize KW, used both alone or in combination with wheat bran, and produce cellulase activity.
- Solid state fermentation of the strain *Aspergillus niger* LPB-328 (from collection of DEBB, partner 4) was set up using soybean husk as substrate and xylanase production was detected.
- The analysis of the effect of several compounds such as cellulose, carboxymethylcellulose, xylan and xylose as inducers of cellulase and xylanase production by *Ganoderma applanatum* MR-56 (from collection of DEBB, partner 4) and the optimization of their production in liquid cultures by statistical methods were performed. The Plackett-Burman screening design was applied to identify the most significant inducers of xylanase and cellulase production by *G. applanatum* MR-56. The most significant inducer effect on xylanase and cellulase production was exercised by cellulose, even if xylose and CMC were also effective at some times. The combined effect of cellulose, yeast extract and pH was analyzed by a 2^3 factorial experimental design with four central points, showing that the maximum cellulose (1%) and yeast extract (5 g/L) concentrations tested gave the maximum production of xylanase (8.24 U/mL) and cellulase (3.29 U/mL) activity at pH 6.0 and 4.0, respectively.

- The analysis of the effect of different carbon and nitrogen sources on cellulase production by the fungal strain *Aspergillus niger* RMV-02 (from collection of DEBB, partner 4), isolated in Brazil from the residue of Palm (*Elaies guineensis*), was investigated. Optimization of cellulase production in liquid cultures by statistical methods was performed. The best carbon source and nitrogen sources were identified. The Plackett-Burman screening design was applied to identify the best concentration of the selected carbon and nitrogen sources for cellulase production.

Task 3.3: Production of EtOH.

- The strain *Saccharomyces cerevisiae* LPB-287 selected from DEBB collection (partner 4) for production of ethanol (WP2.1) was analyzed for its ability to grow on the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated Brewers' spent grain (BSG) from WP3.1, with or without yeast extract addition, and its ethanol yield. The strain was able to grow and produce ethanol.

Task 3.5: Production of LA

- The strain *Lactobacillus acidophilus* LPB-04 selected from DEBB collection (partner 4) for production of lactic acid (WP2.1) was analyzed for its ability to grow on the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated Brewers' spent grain (BSG) from WP3.1, with or without yeast extract addition, and its lactic acid yield. The strain was able to grow and produce lactic acid.

Task 4.1: Purification and characterization of lignocellulolytic enzymes.

- The enzymes responsible for xylanase produced by *Aspergillus niger* LPB-328 (from collection of DEBB, partner 4), at time of the maximum activity level by SSF on soybean husk (developed in WP3.2) were purified, characterized and identified. Proteomic analyses performed on the enzymatic mixture responsible for the maximum value of xylanase activity in SSF revealed the presence of two xylanases. This xylanolytic mixture was partially purified and characterized.
- Proteins responsible for xylanase activity of the most productive xylanolytic strain *Bacillus amyloliquefaciens* XR44A selected in WP2.2 were identified by an approach based on combination of zymography and proteomics and subsequently characterized.

Task 4.3: Test of enzymes in waste conversion for biofuel production.

- The xylanase from *B. amyloliquefaciens* XR44A, identified and partially characterized in the WP4.1, was adopted in the saccharification of the brewer spent grains (BSG) pretreated by soaking in aqueous ammonia solution (in WP3.1), in combination with different commercial enzymes.

The main research activities performed in the last two years of BIOASSORT project and the achieved results are summarized as following:

Task 2.1: Selection of microorganisms by screening of the microbial collections for their

ability to produce enzymes cellulases, ligninases, xylanases and amylases, EtOH, PHAs, LA.

In the last two years of the project, a further screening of microorganisms for lactic acid production was conducted using 4 strains of *Lactobacillus* (*L. agilis* LPB 14856, *L. pentosus* LPB227, *L. amylophillus* LPB4437 and *Lactobacillus spp.* LPB 07). The screening of strains with greater capacity for LA production was conducted using as substrate hydrolyzed potato pulp waste (HPPW). All strains produced a great amount of LA, more than 40 g/l and the yield was above than 84%. The strain producing the highest amount of LA (47.2 g/l) was *L. pentosus*, with a productivity of 0.65 g/l.h. Therefore, the following activities of improvement of LA production (task 2.3), production of LA (task 3.5) and recovery of LA and production and characterization of PLA (task 4.5) were performed with this strain.

Task 2.3: Improvement of lignocelullolytic abilities (A), PHAs (B) and LA production (C) by genetic engineering of microorganisms

In order to develop a one-step consolidated bioprocessing of lignocelluloses to bioethanol (Category II CBP), a strategy to engineer a *Saccharomyces cerevisiae* industrial strain was developed. This strain was transformed with the endoglucanase CelStrep produced by *Streptomyces sp.* G12 and the α-L-arabinofuranosidase PoAbf produced by Pleurotus ostreatus.

Moreover, the optimization of lactic acid production with the strain L. pentosus LPB 227 selected in the task 2.1 was carried out using experimental designs.

Task 2.4: Preparation of a database of BIOASSSORT microbial patrimony.

A database of the microorganisms isolated and selected during Bioassort project was created.

Task 3.1: Formulation of wastes and optimization of pre-treatment processes to prepare mechanically pre-treated wastes to the conversion by comparing AFEX, diluted acid treatment and/or biological systems.

Extrusion pretreatment was performed on BSG provided by Grupo Cuauhtémoc Moctezuma (Monterrey, México) using both standard conditions and conditions optimized through statistical analysis. The pretreated BSG was then subjected to hydrolysis (task 4.3) with both commercial enzymes and enzymes from the strain *Aspergillus niger* LPB 334 previously selected in the project (task 2.1) and produced by SSF on BSG (task 3.2).

Moreover, Rice straw (RS) was used as a further raw material and it was subjected to combined pretreatmement by sonication and enzymatic hydrolysis. Optimization of various process parameters affecting combined pretreatment and hydrolysis of RS was carried out by adopting a Box-Behnken design

Task 3.2: Optimization of mixed SSF

The solid state fermentation on BSG was set up using the three ligno-cellulolytic fungal strains *Aspergillus niger* LPB-334, *Pleurotus sajor-caju* LPB-368 and *Lentinus edodes* LPB-373 belonging to the "Strain Collection of the Bioprocess and Biotechnology Division" from the Department of "Engenharia de Bioprocessos e Biotecnologia", University Federal do Paraná (DEBB), Curitiba-PR, Brasil (partner 4), selected during the previous year of the Bioassort Project for producing an efficient enzymatic mixture (cellulases, xylanases and laccases) that can be used in the bioconversion processes. Time courses of cellulases and xylanases production by *Aspergillus niger* LPB-334 and of laccases production by *Pleurotus sajor-caju* LPB-368 were evaluated. On the other hand, the experiments of SSF with *L. edodes* LPB-373 revealed the incapacity of the fungus to colonize the waste quickly and extensively.

The SSFs of *A. niger* LPB 334 were also performed on the BSG provided by Grupo Cuauhtémoc Moctezuma (Monterrey, México) [Task 3.1] in the same conditions. Scale-up of SSF of *A.niger* NRRL 3312 was performed in 1L Erlenmeyer flasks and the enzymes produced by A. niger LPB 334 were used in the hydrolysis of BSG pretreated by extrusion in task 4.3 .

Task 3.3: Production of EtOH.

- The strain *Saccharomyces cerevisiae* LPB-287 previously selected in WP2 in the first two years of the project (task 2.1, section B3) was evaluated for its ability to grow on the carbohydrates mixture (obtained after supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) of high value metabolites from *Arthrospira platensis* biomass (provided by CBTM) for the production of ethanol and lactic acid respectively.
- A strategy of Consolidated Bioprocessing (CBP) for direct production of bioethanol from Brewer spent grain through *Trametes hirsute,* a white rot fungus, which produces efficient ligninolytic enzymes and is capable of producing ethanol from sugars was developed.
- The non-detoxified hydrolyzate obtained after combined pretreatment and hydrolysis of rice straw (task 3.1) was also evaluated as raw material for ethanol fermentation using *Saccharomyces cerevisiae*

Task 3.4: Production of PHA

The hydrolysate obtained from the optimized conditions of combined pretreatment and hydrolysis of Rice Straw (WP 3, task 3.1) was directly used in PHB production using *Comamonas* sp.

Task 3.5: Production of LA

- The strain *Lactobacillus acidophilus* LPB-04 selected from DEBB collection (partner 4) for production of lactic acid (WP2.1) was analyzed for its ability to grow on the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated Brewers' spent grain (BSG) from WP3.1, with or without yeast extract addition, and its lactic acid yield. The strain was able to grow and produce lactic acid.
- The strain *Lactobacillus acidophilus* LPB-04 previously selected in the first two years of the project (WP2, task 2.1 section B2 of the first periodic report, deliverable *D2.3 Report on screening of microbial collections*) was evaluated for its ability to grow on the carbohydrates mixture obtained after supercritical fluid extraction (SFE) and microwave-assisted extraction (MAE) of high value metabolites from *Arthrospira platensis* biomass (from WP3, task 3.1, section B1 of the present report) for the production of lactic acid.
- The strain *L. pentosus LPB 227* belonging to the collection of the Department of "Engenharia de Bioprocessos e Biotecnologia", University Federal do Paraná, Brasil (DEBB, partner 4) and selected in WP2 (task 2.1 of the present report) was used for lactic acid production by fermentation in Erlenmeyer flasks and in a 7 l stirred tank reactor (STR) (Laboratory fermentor model MDL, B.E. Marubishi, Thailand) adopting conditions selected in 2.3.

Task 3.6: Design of optimized production processes by Cost Benefit Analysis (CBA) and Life Cycle Assessment (LCA) analyses

Activities to support the design review, development improvement and practical validation of a computer tool (SOST-TOOL) for assessing the environmental sustainability of new process technologies in biorefineries, using different biomass wastes and residues were performed.

Task 4.1: Purification and characterization of lignocellulolytic enzymes.

The identification of both cellulases and xylanases produced by the strain *Aspergillus niger* LPB-334 and laccase produced by *Pleurotus sajor-caju* LPB-368 (selected from the Collection of DEBB partner in the task 2.1, during the previous year of the project) through solid state fermentation (described in WP3 task 3.2 section 2 of the present report) was reported.

Task 4.2: Directed evolution of lignocellulolytic enzymes.

A strategy for improving the enzymatic properties of the endoglucanase CelStrep by direct evolution was developed. It was previously identified in the laboratory of Prof. V. Faraco (UNINA, partner 1) as the enzyme responsible for the cellulase activity produced by the strain *Streptomyces* sp. G12 isolated from mature compost obtained from agro-industrial wastes.

Task 4.3: Test of enzymes in waste conversion for biofuel production.

The enzymatic hydrolyses of AFEX and EA pretreated newspaper waste (NW) (previously prepared in WP3, task 3.1, I periodic report) were carried in 5 mL vials. The enzymes endocellulase rCelStrep, α-L-arabinofuranosidase rPoAbf and its evolved variant rPoAbf F435Y/Y446F were tested for the hydrolysis of pretreated NW.

In particular, a mixture of purified enzymes containing cellulases, xylanases and accessory hemicellulases, was chosen as reference mix and rCelStrep and rPoAbf or its variant were replaced to the commercial endoglucanase and arabinofuranosidase respectively. The results showed that these enzymatic mixes are not suitable for the hydrolysis of NW after AFEX or EA pretreatment. On the other hand, when the enzymes rCelStrep, rPoAbf and rPoAbf F435Y/Y446F were tested for their effect in hydrolysis of pretreated NW by addition to a commercial enzyme mixture, it was shown that the total polysaccharides conversion yield reached 37.32% for AFEX pretreated NW by adding rPoAbf to the mix whilst the maximum sugars conversion yield for EA pretreated NW was achieved 40.80 % by adding rCelStrep.

The samples of BSG extruded according to the conditions reported in task 3.1 were enzymatically hydrolyzed using commercial enzymes. Comparison between hydrolysis of BSG extruted without and with alkali using commercial enzyme complex and a substitution of enzymes generated from the strain Aspergillus niger LPB 334 (task 3.2) was also evaluated.

Task 4.4: Recovery and characterization of PHA.

The PHB polymer produced in task 3.4 by fermentation of the hydrolysate obtained from the combined pretreatment and hydrolysis of rice straw (WP 3, task 3.1) with *Comamonas* sp. was extracted and purified by sodium hypochlorite- chloroform dispersion method followed by non solvent precipitation. Optimization of different parameters was carried out to enhance the PHB recovery from the biomass. The PHB obtained after fermentation was characterized by FTIR and NMR

Task 4.5: Recovery of LA and production and characterization of PLA.

Separation and recovery processes of LA from broth of fermentation performed using the strain *Lactobacillus pentosus* LPB-227selected in WP2 (task 2.1.) were developed for using it in further studies of polymerization and PLA production.

Task 4.6: Design of optimized recovery processes by CBA and LCA

Activities to support the design review, development improvement and practical validation of a computer tool (SOST-TOOL) for assessing the environmental sustainability of new process technologies in biorefineries, using different biomass wastes and residues were performed.

Potential impact and use (including the socio-economic impact and the wider societal implications of the project so far) of the expected final results

Expected impact to the scientific area, for the ERA and collaboration with the Third country partner organisations According to the Mid-term Progress Report (LMI, EC

2009), there are already several bio-based products on the market in Europe. However, the chemical industry currently uses only 8-10% renewable raw materials to produce various chemical substances. Europe has a few small companies specialised in bio-based products and several major chemical companies developing bio-based applications. This project aims at improving competencies of partners from different Countries through international mobility of researchers and knowledge transfer. The proposal focuses on valorisation of wastes largely generated in Europe exploiting micro-organisms from wide culture collections and the biodiversity of the microbiota from different regions to transform these wastes into commercially valuable bio-products. This programme responds to ERA - **Communication Towards a European Research Area** (Brussels, 18 January 2000. COM (2000)6)-, as specifically described at page 30. It supports the **Environmental Technology Action Plan** (ETAP, EC 2004) that claims an investment of 3% of GDP for research for development, demonstration and dissemination of environmental technologies, to promote sustainability of the biomass processing industry. It is in accordance with the guidelines established in the **Waste Framework Directive** (EC 2008a) and the **Lead Market Initiative** in the field of bio-based products (LMI, EC 2007).

Economical impact on the different sectors

Regarding the market sectors within the scope of this project - enzymes for biomass conversion into ethanol, biopolymers PHA and PLA for the plastic sector and ethanol for fuel industry- this project will provide new bioproducts for a market in expansion with the advantages of positive environmental impact in relation to the currently existent products and lower cost. The current production technologies have some disadvantages, that will be overcome within this project, i.e. the raw material costs and water input of the current processes of enzymes production by submerged fermentation in synthetic media; the non-biodegradability, non-biocompatibility, petrol-based origin and utilization of chemical raw material of the plastics sector; the high GHG emissions of petrol-derived fuels.

Industrial enzymes. The global market for industrial enzymes was estimated at $3.3 billion in 2010. This market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period (Market Research, 2011). According to CGEE (2006), the world demand for cellulases and amylases was estimated as US$ 295 million and US$ 374 million, respectively, in 2009. Recent prices have been estimated as US$ 260/1,000,000 IU for laccase (MycoEnzyme Ltd.); US$ 287.00/10g - 25U/mg for cellulase (Worthington Biochemical Corporation). As a prospection for 2014 by Center for Global Environmental Education (CGEE) in 2006, the world demand of cellulases will be of US$ 430 million.

Bioplastics. According to European Bioplastics, the global market for biopolymers reached 766,000 metric tons in 2009 and a forecast of 1,5 million metric tons in 2011. In 2007, the most important products in terms of production volumes were starch plastics (0.15 Mt) and PLA (0.15 Mt). Consumption of biopolymers will grow to approximately 100 million metric tons/year by 2020 in Europe. Based on the company Toyota announcements, it is projected that the most important representatives by 2020 will be starch plastics (1.3 Mt), PLA (0.8 Mt), bio-based PE (0.6 Mt) and PHA (0.4 Mt). Furthermore, due to the fact that PLA and PHAs can be technical substitutes for polyolefins, polyesters and polystyrene (accounting for the 69% of the EU thermoplastics market) the impact of the project findings could be even higher (data from PRO-BIP 2009). Recent prices have been estimated as €12/kg for PHB from Biomer (Germany); € 10-12/kg for PHB-co-3HV from Metabolix (USA); € 2.2-3.4/kg for Polylactic acid from Cargill Dow (USA).

Biofuels. Bioethanol is the most produced biofuel worldwide with almost 74 billion litres in 2009, EU ranking third behind United States (54%) and Brazil (34%), with a production of 3.7 billion litres in 2009 (eBio 2010). Fossil fuel reserves depletion, global warming, costly and problematic waste recycling have induced development of renewable energy sources aimed at a sustainable development. European Directive 2009/28/CE (EC 2009) is the reference for EU renewable energy targets. One of the main difficulties for achieving

economical feasibility of lignocellulosic ethanol is the high cost of enzymes for biomass hydrolysis. This project aims at developing an advantageous process for enzymes production from wastes, thus reducing their production costs. A significant economic impact can be accrued, since the current costs of EU-produced biofuels make it difficult for them to compete with fossil fuels. The abatement of the costs of second-generation ethanol production, facilitating its commercialization, would contribute to achievement of targets of EU Directive 2009/28/CE (EC 2009).

BIOASSORT contribution to European industries

The impacts of this project to improve **the competitiveness and potential innovation of European industries by exploiting industrial biotechnology for developing bioproducts** will comprise the following aspects.

- *Development of novel biosystems for enzymes, ethanol and polymer production.*

•**Novel biosystems for enzymes, ethanol, polymer (PHB) and polymer precursor (LA) production** will be developed by i) **Isolation of novel strains** with optimised microbial function exploring Indian and Brazilian biodiversity from natural and industrial areas; ii) **Screening of the huge microbial patrimony consisting an assortment of thousand microorganisms** belonging to 2 different collections provided by DEBB and NIIST; iii) **Development of Engineered PHA producing organisms** by introduction of growth related genes to improve PHA yield by increasing biomass and thus reducing the total cost of PHA production; iv) **Development of Engineered microorganisms for LA production** with the aim to enhance the optical purity of L-lactic acid (which is the most important biodegradable form), by inactivation of undesired genes (especially the *ldhD*) and overexpression of the desired gene (*ldhL*).

•*Discovery, characterisation and development of novel enzymes* ***with optimised biocatalyst function for improved production of bioproducts by discovery of cellulases from novel microbes, directed evolution of cellulases and genetic engineering of cellulolytic microbes for improved biomass conversion*****.**

- *Development of improved bio-processes with increased yield, quality and purity***.**

•**Improved bio-processes for obtaining of PHA** will be achieved through: **Optimisation of microbial processes** for PHA production by optimizing nutritional, biological and physical parameters by statistical analysis and using of response surface method; **Media engineering by** evaluating the effect of adding different substrates and inhibitors to the culture media to check yield and the nature of PHA produced. **Metabolic Engineering for PHA production** by introduction of growth related genes to improve PHA yield by increasing biomass; **Fermentation science** by fed-batch studies to get high cell density and high PHA content. •**Improved bio-processes for obtaining of LA** will be achieved through**: Optimisation of microbial processes for LA** by statistical analysis of experiments to obtain a mathematical model of the process; **Metabolic Engineering for LA production** to enhance the optical purity of L-lactic acid by the inactivation of undesired genes and overexpression of the desired gene. **Fermentation science** by optimizing the pH, pH control, temperature, composition of the medium and inoculum during fermentation; **Media Engineering** by determining the best proportion of BSG and OFMSW hydrolysate and the need for supplementing the media with organic and inorganic nitrogen sources and mineral sources to optimize lactic acid production. **Downstream processing** by testing numerous methods (e.g. alkalinisation and decantation; precipitation/filtration of the solid lactate salt; ion exchange; evaporation; vacuum distillation; etc.), in order to obtain pure LA at the lowest cost.

- **Environmental aspects.**

• **Recovery, reuse and recycle of wastes and conservation of natural resources.** The utilization of OFMSW, BSG and OPR as the raw materials for the processes to be developed contributes to waste sustainable management providing a raw material at zero cost and having a positive environmental impact by concomitantly absorbing residues and preserving natural resources. Exploiting as raw materials a diversified range of wastes

selected in accord with locally prevailing conditions, the new process will ensure the greatest long-term security of supply benefit (in accord with SET-Plan COM 723-2007). By using only residues as raw materials that are already conveniently used in other industrial processes, the project will contribute to avoid shortage of renewable raw materials commonly used by the chemicals, construction and packaging industries, responding to recent (14 December 2007) **EU member states call** (16620/07) for a new EU action plan to "promote the material and industrial recovery of renewable resources in the EU". The development of processes based on SSF minimizes the water input to the process. Use of chemicals will be avoided preferring biological pre-treatment.

•**Use of environmentally safe materials.** All the developed bio-based products (enzymes, biopolymers, polymer precursor and ethanol) will be tested to ensure the absence of biological and chemical contaminations. The advantages of these products, that will have a positive impact on their productive sector, are the non-fossil origin and their acceptability by consumers due to their biodegradability and biological origin and promotion of the substitution of petrol based- plastics/fuels with bio- plastics/fuels.

•**Reduction of landfill and GHG emissions from landfill.** The utilization of municipal and agro-industrial wastes prevents the utilization of land. Since the raw material proposed in this project is renewable, composed of “green carbon”, this is a positive point as far as carbon emission is concerned. Adoption of low-carbon and energy new processes will support the EC plan “Towards a low carbon future” (SET-Plan COM 723-2007) and, being in accord with the European GHG emission trading system (ETS) that puts incentives for adopting energy (and carbon) saving technologies, will give a driver for innovation to companies in Europe and in the other candidate Countries.

•**Reduction of the amount of biodegradable municipal waste going to landfill.** According to the European targets, the biodegradable municipal waste disposed in landfills must be reduced to 35% until 2016 with 1995 as the reference year. This project will promote the differentiate collection of MSW and the valorisation of the biodegradable fraction thus reducing the amount of waste to be directed in landfill or to incineration. The flowchart of the process to be developed is based on the concept of a bio-refinery and plans to use all of the fractions of biomass residues and wastes to produce high value products in an eco-efficient way.

•**Savings in limited fossil resources and reduce greenhouse gas emissions.** A key challenge of Europe is moving to a competitive low carbon economy in 2050 (8.3.2011 COM(2011) 112; A Roadmap for moving to a competitive low carbon economy in 2050). This project will promote production and use of both biodegradable plastics and biofuel ethanol, thus contributing to reduction of GHG emission expected to be of 34 -40% by 2030 and 83-87% in 2050 in industrial sector. As a rule of thumb, starch-based plastics can save between 0.8 and 3.2 tons of CO_2 per ton compared to one ton of fossil fuel-derived plastic, the range reflecting the share of petroleum based copolymers used in plastics.

•**Plastic Waste Management: high biodegradability and lower toxicity of the new materials.** In 2008, total generation of post-consumer plastic waste in EU-27, Norway and Switzerland was 24.9 Mt. Packaging is by far the largest contributor to plastic waste at 63%. Average EU-27 per-capita generation of plastic packaging waste was 30.6 kg in 2007. Moreover, the projections show a 23% increase in the overall generation of plastic waste of between 2008 and 2015, driven largely by the packaging sector. High biodegradability is a key property of PLA and PHA developed within this project.

➢ **Social-economic aspects.**

Since the waste management is a great challenge in Europe and all over the world, the European Commission expressed the high growth rate of jobs and business opportunities in the field of waste management and recycling sector (turnover of over €100 billion for EU-25, between 1.2 and 1.5 million jobs). The new processes for transformation of wastes into valuable products will provide a sustainable strategy for waste management as an alternative to highly polluting conventional treatment, supplying basis for a concrete

evolution in the European management of wastes. It will obviously lead to a positive impact on health and quality of life of European citizens.

- **Development of Key Enabling Technologies.**

This project aims at developing Key Enabling Technologies (KET), in the field of advanced materials and biotechnology, which are energy efficient and minimize the environmental impact, to improve the industrial capacities of the EU and enhance the competitiveness and sustainability of the EU's economy. Development of the biorefinery concept, contributing to boost innovation and sustainability and to increase the international competitiveness of the European enterprises overseas in the renewable materials, chemical, and biotechnological sectors, will be in accord with "Horizon 2015: Perspectives for the European Chemical Industry", and will support the EC SET-Plan, COM 723-2007.