BRIGIT

BRIGIT NEWSLETTER 3

March 2015

New tailor-made biopolymers produced from lignocellulosic sugars waste for highly demanding fire-resistant applications

What is **BRIGIT** project about?

BRIGIT aims to develop a cost-competitive and environmentally friendly process to produce biopolymers, polyhydroxy butyrate (PHB), polybutylene succinate (PBS), and their copolymers and blends from waste-derived lignocelullosic sugar feedstock liquor from the wood sulfite pulping process. The fermentation process to produce PHB and succinic acid for the production of PBS will be carried out "in-situ" in the spent sulfite liquor by means of a new fermentation technology without alteration of the composition of current lignosulfonates contained in the spent liquor.

The main innovations in BRIGIT are the use of an existing sugar-rich waste stream from the production of cellulose and the process integration with the existing industrial operation. The use of non-sterile (if possible) steps due to the production and selection of efficient microorganisms, and optimized fermentation and downstream technology will permit an overall reduction in resources consumption, in greenhouse gas emissions, and a substantial reduction of operational costs.

Starting from the obtained biopolymers, BRIGIT aims to develop bio-based composites for high-tech fire-resistant applications in the transportation sector (such as in trucks and buses among others). The bio-composites in combination with natural fabrics will be used to produce 3D sandwich panels, as an alternative to the current sandwich panels made of thermoset resins reinforced with continuous glass fibres. The new panels will be recyclable, lighter, and will be obtained by a continuous compression moulding process in contrast to currently available sandwich panels.

A scheme of the sandwich panel is depicted in figure 1 below:

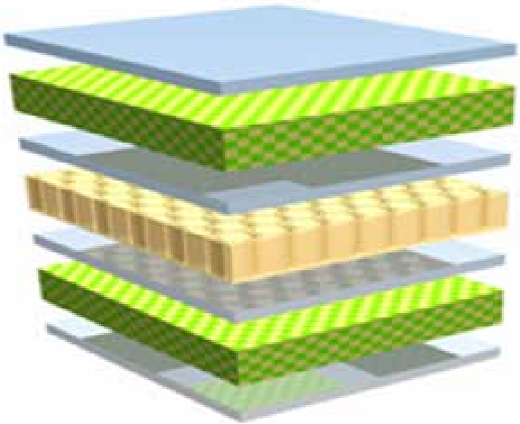

Fig. 1. Scheme of multi-layer sandwich panel to be developed within BRIGIT project

Project **PARTNERS**

The project consortium is formed by the following specialized centers:

AIMPLAS: Plastics Technology Centre in Valencia, Spain.
ULUND: Lund University in Lund, Sweden.
UNICAN: Universidad de Cantabria in Cantabria, Spain.
BIOTREND: Inovação e Engenharia em Biotecnologia, S.A. in Cantanhede, Portugal.
SILICO: SILICOLIFE LDA in Guimarães, Portugal.
AUA: Agricultural University of Athens, in Athens, Greece.
AVECOM: Avecom N.V. in Wondelgem, Belgium.
BANGOR: Bangor University in Bangor, United Kingdom.
NEXTEK: Nextek Limited in London, United Kingdom.
DLAB: Daren Laboratories & Scientific Consultants Ltd. in Ness-Ziona, Israel.
GSOUR: Green Source, S.A. in Cantabria, Spain.
ADDCOMP: Addcomp Holland BV, in Nijverdal, Netherlands.
PROFORM: Pro-form Ipari és Kereskedelmi Kft, in Budapest, Hungary.
XPA: X-perion Aerospace GmbH in Immenstaad, Germany.
SOLARIS: Solaris Bus & Coach, S.A. in Poznan, Poland.
CRF: Centro Ricerche Fiat S.C.p.A in Orbassano, Italy.

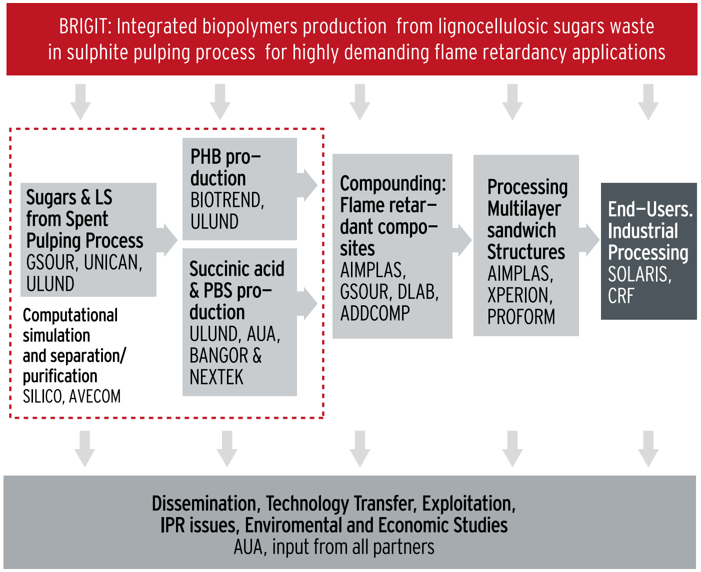

Fig. 2. Overview of the partners and their role in the project

The **KBBE (Knowledge-Based Bio-Economy)** programme plays an important role in a global economy, where knowledge is the best way to increase productivity and competitiveness and improve our quality of life, while protecting our environment and social model. It is a sector estimated to be worth more than 1.5 trillion per year.

KBBE addresses the following needs:

- Growing demand for safer, healthier, higher quality food;
- Sustainable use and production of renewable bio-resources;
- Increasing risk of epizootic and zoonotic diseases and food related disorders;
- Sustainability and security of agricultural, aquaculture and fisheries production;
- Increasing demand for high quality food, taking into account animal welfare and rural and coastal contexts and response to specific dietary needs of consumers.

The BRIGIT project has been funded by the European Union Seventh Framework Programme (FP7/2007-2013) within the KBBE programme in the Area 2.3.4 Biorefinery and the topic KBBE.2012.3.4-02: Biotechnology for novel biopolymers.

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Fig. 3. BRIGIT 30th month meeting at Proform facilities, Budapest (Hungary).

The 30th month meeting of the project took place on the 21st and 22nd January 2015 at PROFORM facilities, Budapest (Hungary). During the meeting, the latest scientific developments achieved by the project partners were discussed. One of the main topics was the optimization and the detoxification of the spent sulfite liquor (SSL) to increase the available sugars and reduce potential inhibitor compounds in the SSL for the subsequent fermentation process. Concerning the fermentation processes, different strategies for the improvement of the fermentation yield for PHB accumulation and succinic acid (SA) production at the laboratory scale were reported. These strategies included metabolic modeling, engineering of bacteria and yeast strains for PHB and SA production and deploying process engineering solutions. First results of PBS polymerization at lab scale were also reported. Regarding the production of multi-layer sandwich panels, flame retardant PHB/PBS blends were obtained and then extruded into sheets for the production of the skin layers of the panel. Finally, sandwich panels were obtained and characterized at lab scale, and the results were reported to the consortium.

The next project meeting will be held in June 2015 in Coimbra, Portugal. The meeting will be combined with the **2nd BRIGIT Open Workshop** on the topic of **"Industrial Biotechnology: A Systems Approach from Microbe to Process"**. The workshop will be organized on the 16th June 2015 by BIOTREND and SILICOLIFE, with the participation of BRIGIT partners AIMPLAS, AUA, and ULUND.

The workshop will cover the basics of in silico metabolic engineering for the rational design of microbes, the molecular biology methods for strain modification and providing case-studies and addressing the principles of fermentation-based bioprocesses. The workshop will also include a visit to BIOTREND's bioprocess development lab and pilot plant.

Fig. 4. Tentative program of the 2nd BRIGIT Open Workshop "Industrial Biotechnology: A Systems Approach from Microbe to Process"

Industrial Biotechnology: A Systems Approach from Microbe to Process
Workshop within BRIGIT, a EU FP7 collaborative research project
Location: Biocant Park, Cantanhede, Portugal

Start	End	
09:00	09:30	**Welcome and coffee**
09:30	10:00	**Industrial Biotechnology: A Systems Approach from Microbe to Process (AIMPLAS: Miguel Angel Valera; Biotrend: Bruno Sommer Ferreira) 0.5 h** Basics of industrial biotechnology (Biotrend) Overview of the BRIGIT project (AIMPLAS)
10:00	12:30	**Part 1: In silico metabolic engineering (SilicoLife: Paulo Maia / Isabel Rocha) 2.5h** Genome-scale metabolic models Simulation methods - Flux Balance Analysis, Mutant Phenotype Simulations In silico strain optimization Introduction to OptFlux
12:30	13:30	**Networking Lunch**
13:30	15:30	**Part 2: Molecular biology methods for rational strain design (SilicoLife: Rui Pereira; ULund: Marie Gorwa-Grauslund/Anders Sandström) 2h** Methods for gene deletion, insertion, over/underexpression (SL) Strain physiology vs. genetic engineering and case-studies (ULund)
15:30	17:30	**Part 3: Fermentation-based bioprocesses (AUA: Apostolis Koutinas; Biotrend: Bruno Sommer Ferreira / Frederik van Keulen / João Cavalheiro) 2 h** Products obtainable from fermentation (AUA) Principles of (submerged) fermentation (Biotrend) Recovery of bioproducts (Biotrend) A walk through fermentation-based bioprocess design (includes visit to Biotrend's labs)
17:30	18:30	**Part 4: Integrating everything: Raw-material, microbe and process (ULund: Gunnar Lidén)**
18:30	18:45	**Farewell**

BRIGIT **website**

The BRIGIT website, available through the link **www.brigit-project.eu** contains updated information about the project. In addition, the website offers to visitors a Technological Watching Service with updated news, articles, patents and forthcoming events regarding biopolymers. Since the beginning of the project, more than 5,100 visitors from different countries have accessed the BRIGIT website.

www.brigit-project.eu

Fig. 5. BRIGIT project website

BRIGIT **dissemination** in the media

Many dissemination activities took place during this period involving conferences and workshops, various meetings with industrial partners, dissemination activities to public and scientific publications. BRIGIT was disseminated at:

- The 247th American chemical society national meeting and exposition, chemistry and materials for energy on the 16th-20th of March 2014 at Dallas, Texas, USA
- The Manchester polymer group seminar on plastic materials processing and performance “Plastics – the way forward?” in Manchester on the 20th October 2014, UK
- The 10th international conference on renewable resources and biorefineries, 4 to 6 June, 2014 in Valladolid, Spain
- The 36th symposium on biotechnology for fuels and chemicals in Florida, USA on the 28th April - 1st May 2014
- The 16th European congress on biotechnology on the 13th -16th of July, 2014 in Edinburgh, Scotland
- The 4th international conference on industrial and hazardous waste management in Chania, Greece in September 2 to 5, 2014
- The 4th international forest biorefinery symposium in Montreal Canada in February between 3 to 6, 2014.

In addition, BRIGIT was disseminated in an industrial meeting on the 7th of April 2014, in Cleveland, UK.

Finally, BRIGIT was disseminated also with scientific publications at international journals: (a) C. Rueda, et al., “Monosaccharide production in an acid sulfite process: Kinetic modeling” Carbohydrate polymers (2014), http://dx.doi.org/10.1016/j.carbpol.2014.05.006 (article in Press) and (b) A. G. Sandström et al., “Saccharomyces cerevisiae: a potential host for carboxylic acid production from lignocellulosic feedstock?”, Appl Microbiol Biotechnol, DOI 10.1007/s00253-014-5866-5.

The work carried out within BRIGIT project was mainly focused on the following areas of development:

Definition of case studies and requirements

CRF and SOLARIS proposed different case studies for transport and passenger applications/sectors. The material properties and standards requirements for the selected case studies were also defined.

Fig. 6. Examples of proposed case studies.
Left: door lower cover (CRF) - Right: side wall panel (SOLARIS)

Study of the sulfite pulping process

The optimization of the sugar content in the SSL was carried out by means of the digestion process simulation. The best results were obtained with *Eucalyptus globulus*, calcium based at pH=1.5 and 140ºC of maximum temperature, using 20:1 L/kg of fresh liquor to wood ratio, giving not only a 35% increase of the sugar content but also a desired dissolving pulp in only 300 minutes approximately.

SSL detoxification

Fig. 7. Membrane operation at pilot scale

SSL detoxification is one of the key points in the valorization of the SSL as a source of sugars for fermentation processes. SSL was detoxified prior to the fermentation stage. Different compounds present in the SSL have potential inhibitory effects over the microorganisms, thus reducing cell growth and fermentation yield. To improve the fermentation results strong collaboration between BRIGIT partners was necessary to identify and optimize the most efficient detoxification process. Different detoxification processes were studied, including: precipitation, evaporation, solvent extraction, adsorption and membrane treatment. The best inhibitor decreasing results are obtained using **anionic resin followed by liquid-liquid extraction with isopropyl alcohol**. Maximum LS separation was observed by **nanofiltration technology,** leading also to low losses of sugar.

SSL treatment via ultrafiltration is a promising pretreatment unit operation as improved succinic acid production was achieved using the permeate as fermentation medium. However, the cost-competitiveness of this pretreatment method should be evaluated as it is not possible to separate completely the sugars from the lignosulfonates. Further research will focus on the utilization of permeates produced via ultrafiltration and nanofiltration in succinic acid production.

Production of PHB

Naturally occurring strains with the ability of accumulating PHB were identified and isolated from a microbial consortium from waste water treatment plant. The performance of these strains was studied in comparison with reference strains.

A genome scale metabolic model was developed for *S. cerevisiae* modified to be able to uptake xylose and to produce PHB. Further model-guided modifications of the metabolic pathways are being performed for enhanced yields and productivities and validated through in vivo experiments.

In addition different feed strategies are being studied to optimize the fermentation process and prepare the transition to higher scales.

Production of PBS

Polybutylene succinate (PBS) is a valuable biopolymer with a growing area of applications worldwide. Succinic acid, which can be obtained from SSL via fermentation, is a building block for the production of PBS. In BRIGIT, a wild-type succinic acid producing bacterial strain has been chosen on a basis of its performance.

Fig. 8. Bioreactor for PHB production

Close collaboration between AUA, ULUND, SILICOLIFE and AVECOM allowed drawing critical conclusions regarding SA production via fermentation process. These conclusions related to the most efficient SSL detoxification technique, the optimum fermentation conditions and the most efficient mode of operation in the fermentation process.

BRIGIT partners use natural succinate producing bacteria. The SSL was initially pretreated via membrane ultrafiltration in order to separate the lignosulfonates (LS) from the sugar fraction. The sugars were then used as carbon source in fed-batch fermentation by bacteria, with yeast extract as nitrogen source. Further experiments aimed to optimise SA production via fermentation process by testing an alternative nitrogen source in order to replace the yeast extract and hence lower the cost of the nutrients required for SA production via fermentation.

Fermentation for SA production were carried out at AUA by using pretreated SSL (nanofiltrated and/or treated with solvent extraction) and results from fed-batch fermentations illustrated that SA productivity and final SA concentration were increased significantly. Moreover, immobilized cultures of wild-type microorganisms were also implemented in repeated batch and fed-batch fermentations (Fig. 9) Inmobilised cultures led to higher succinic acid production yield than the respective yield achieved when free cells were employed. This is very important as it will facilitate the purification of SA from the fermentation broth.

Fig. 9. (A) Immobilized A. succinogenes cells in alginate beads and (B) the bioreactor system used

Among central aspects of SA fermentative production is supply of CO_2. One molecule of carbon dioxide is fixated for each molecule of succinic acid produced. It has been shown that CO_2 can be provided either as carbonate or as a sparged gas. The second topic has been investigated for the inhibiting effects on the fermenting organism. These are caused by either the medium (SSL) itself or products formed during the fermentation, including the desired product succinate. Process engineering strategies, primarily fed-batch cultivations, were develo-

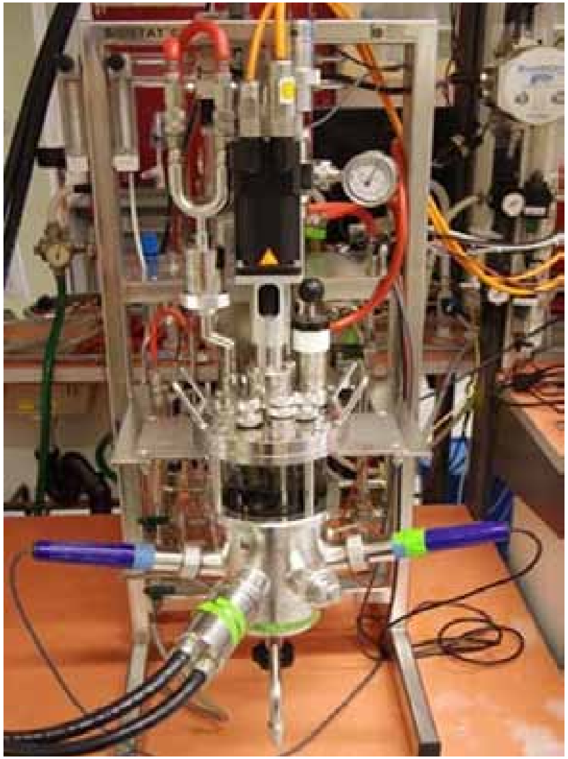
Fig. 10. Bioreactor setup for production of SA

ped to decrease the inhibiting effect of SSL, leading to higher final succinate titers. In a fed-batch process using immobilized cells and ultrafiltered SSL as medium it was found possible to reach the target succinate titer of 40 g/L.

AUA is also involved in the downstream separation and purification of SA. Research is focused in comparing different separation processes, including direct crystallization methods, solvent extraction and ion exchange resins. The different methodologies are compared in terms of SA yields and purities as well as for feasibility in large scale applications.

Reconstruction of a genome-scale model for the selected strain for the production of succinic acid has been finalized by SILICOLIFE and experimental data were generated to support and validate the model. Besides the application of the model to evaluate the metabolic phenotype under different growth conditions, the model is also being used to design feeding optimization strategies to improve the production of SA. Moreover, an unstructured input-output model was developed by AUA for predicting the SA production by considering both substrate and product inhibition. All obtained results will be further used for the scale up of the SA process from the lab to the pilot scale.

A series of polybutylene succinates (PBS) and random PBS copolymers, such as poly(butylene succinate-co-butylene adipate)s (PBSA) were prepared at Bangor University, through a two-step one batch polymerisation process. The synthesis was scaled up to 1 kg and its conditions were optimized. More efficient equipment setup and process optimisation allowed production of

PBS and PBS copolymers with improved molecular weights, thermal and mechanical properties. PBS with Weight Average Molecular weight (Mw) of 112 kDa and PBSA with Mw of 142 kDa have been produced and characterised by a variety of analytical methods.

Modelling of the polymerisation process based on the most efficient two reactor technology, shows that the cost is inversely proportional to scale of production, and the production costs reduce significantly when made at higher outputs.

Modification of lignosulfonates (LS) and PHB to improve flame retardancy:

Lignosulfonate (LS) particles separated from the SSL were modified to improve the compatibility and reactivity with biopolymers, and to use them as flame retardant additive in biopolymer formulations. Hydroxymethylation, acetylation and incorporation of maleic anhydride reactions were carried out and optimized. In addition, enzymatic modification in order to increase molecular weight of LS was successfully performed (up to 12 times).

PHB oligomers grafted with phosphorus and nitrogen compounds were developed and assessed as potential FR additives for biocomposites. Furthermore modified LS particles were also incorporated to the FR formulation.

The thermal stability of PHB has been identified as an important limitation on the compounding and processing of the plastic materials. The parameters that influence the thermal stability of PHB will be investigated in the coming months.

Compounding, extrusion & panels development:

The compounding of PHB/PBS blends was studied and optimized at pilot plant scale in AIMPLS. Afterwards, the compounding process was scaled-up to 400kg in ADDCOMP, as well as the sheet production in PROFORM. Hundreds of meters of flame retardant biopolymer foil were obtained to be used in the production of sandwich panels.

The obtained foil and woven flax fibres were used to develop the skin layer of panels, whereas the core was made of natural cork. A second round of panel production was carried out in AIMPLAS to obtain panels (420x320x3mm) by compression moulding at lab scale. Panels with a flexural modulus of 7.9 GPa and density of 1.3g/cm^3 were obtained.

Fig.11. Picture of the compounding process at industrial scale for a blend of PHB/PBS with FR additives.

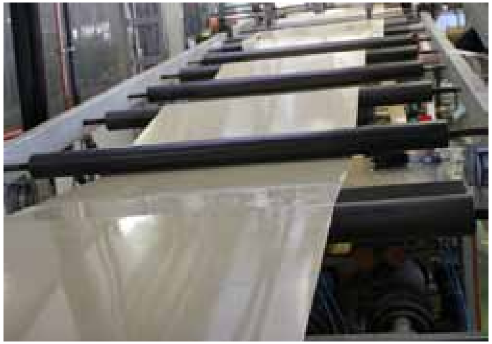

Fig.12. Picture of the sheet extrusion process at industrial scale for a blend of PHB/PBS with FR additives.

Fig. 13. Pictures of biocomposite panel prototypes.

Economic assessment of the project

An intermediate costing estimate has been completed on the synthesis of the intermediate and final PHB and PBS polymers. While a number of systems are still being optimised, these initial results show that cost effective polymers can be produced by these processes, in line with project objectives. The economic assessment has assisted to identify areas of higher cost that can now be revised and further improved.

A Life Cycle Analysis (LCA) study of the synthesis stages has commenced to quantify the environmental impact of the new processes and materials. This study can assist to identify materials and stages of the process that can be targeted to reduce the environmental impact and make comparisons with existing commercial products.

Regarding the compostability of developed materials, PHB/PBS blends were positive tested and further testing is underway with formulations with flame retardants and multi-layer sandwich panels.

Future work

The work in the following months of BRIGIT project will be focused on:

- The optimization of the fermentation processes to produce PHB and succinic acid.
- Pilot scale manufacture of PHB and PBS polymers.
- The improvement of flame retardant properties of PHB/PBS biocomposites. This will be achieved by the compatibilization of LS with PHB plus the use of non-halogen flame retardants.
- The production and characterization of biocomposite panels at pilot plant scale.
- Expansion of the economic modelling to include the downstream converting and manufacturing processes
- Addition of new process data to the LCA study to more fully describe the scale up synthesis process for PHB and PBS polymers.

Fig. 14. SOLARIS bus and tram

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