Development of 2nd Generation Biorefineries – Production of Dicarboxylic Acids and Bio-based Polymers Derived Thereof

BioREFINE-2G Project Grant Agreement n°613771

Final Project Report
Part of D1.8

30.11.2017

SEVENTH FRAMEWORK PROGRAMME

About BioREFINE2G project

The BioREFINE-2G project - Development of 2nd Generation Biorefineries – Production of Dicarboxylic Acids and Bio-based Polymers Derived Thereof - aims at developing commercially attractive processes for efficient conversion of pentose-rich side-streams from biorefineries into dicarboxylic acids, which can be used as precursors for bio-based polymers including biodegradable polymers. Further information about the project and the partners involved are available under www.biorefine2g.eu.

Project coordinator

Project partners

About this document

This report corresponds to (number of deliverable) of the BioREFINE-2G project - (name of deliverable). It has been prepared by:

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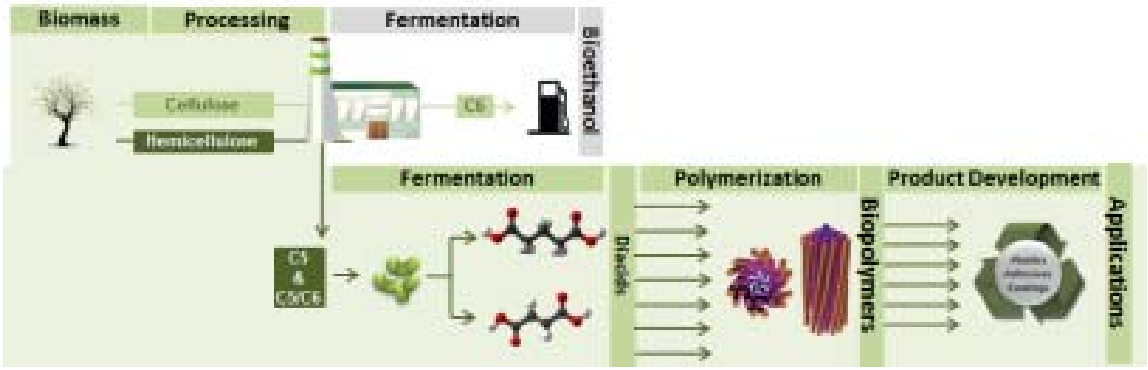
1 Executive Summary

The existing 2nd generation biorefineries utilize only a fraction of the biomass feedstock for ethanol production and major side-streams are produced, such as pentose and lignin streams, which are today used for biogas and energy production, respectively. Converting the carbon from these waste streams into higher-value products would increase the profitability and improve the environmental benefits of the biorefineries. In fact, an innovative process for bio-based chemicals production from bio-waste would represent a true paradigm shift with a huge impact on commercial viability and environmental issues such as waste reduction, pollution and greenhouse gas emissions. BioREFINE-2G aimed at developing commercially attractive processes for efficient conversion of pentose-rich side-streams from biorefineries into dicarboxylic acids, to be used as precursors for bio-based polymers including biodegradable polymers.

The project has covered the whole value chain, from characterization of side streams from forest industries and other uses of non-food feedstock, development of novel robust industrial yeast cell factories, fermentation and downstream process development, to polymerization methods development for the production of biodegradable polymers applicable as plastics, coatings or adhesives, scale-up and demonstration and to life cycle and economic viability analyses. The consortium involved eight distinguished industrial and academic partners. The strong industry drive was ensured by participation of 4 SMEs and one large enterprise, Borregaard biorefinery, which is directly interested in demonstrating and integrating the new technology into the current and future biorefinery plants. The involvement of industry partners assured demonstration activities on the technical and commercial feasibility of process concepts with regard to scale up to industrial production.

During the 4 years of the project, promising achievements in strain and process development, both up- and down-stream, as well as on novel polymer development have been made. The development of diacid-producing strains and efficient purification methods will remain a major challenge to meet the required low production cost. Remarkably, the consortium developed novel tools for rapid engineering of robust industrial polyploid yeast, and purification methods for separation of diacids from various fermented biomass hydrolysates look very promising. The consortium designed and assessed novel polyester and polyurethanes, and new bio-based and biodegradable polymers with interesting properties were obtained. Life Cycle Sustainability Analysis (LCSA) to was used to assess and compare the impacts of the developed products within the BioREFINE-2G project. A methodological framework on environmental, economic and social aspects of Life Cycle Sustainability Analysis (LCSA) to guide all project partners during the whole project was created and adapted to the project context.



BioREFINE-2G – Activities are highlighted in green

2 Summary description of project context and objectives

The finite nature and raising price of fossil resources demand novel solutions to enable a transition from a petroleum-based to a sustainable petroleum-independent economy. Here, the production of bioethanol has played an important role. For decades, bioethanol has increasingly been produced in 1st generation biorefineries where the ethanol has been derived from glucose in corn, wheat or barley or from the sucrose in sugar cane or sugar beet. This has led to two major issues: Firstly, the increasing demand for bioethanol has led to competition between the food and fuel market, and the competition has been accelerated by food speculation on agricultural commodities including corn, wheat and other food. Inevitably, in some places of the world this has led to increased food prices. Secondly, it is more and more disputed whether 1st generation processes particularly based on corn, wheat and barley are actually environmentally friendly since some studies today seem to indicate that the process may not actually be energy positive.

Therefore, there has been an increased interest in Europe and in the US towards moving to 2nd generation biorefineries, aiming at utilizing the cellulose in non-food biomass for ethanol production more efficiently. However, while we see certain progress in the development of 2nd generation biorefineries, few technologies for production of cellulosic bioethanol have reached beyond the lab or pilot stage. Of the ones already at demo scale or commercial scale, less than 20% of the biomass results in bioethanol and high volumes of side and waste streams are produced in the process. Typically, two major side-streams evolve:

(1) a lignin rich side-stream with high energy value and (2) a pentose rich side-stream with low energy value.

The relative amounts vary with biomass feedstock and process efficiency. The lignin rich side-stream is used for CHP (combined heat and power) production and can also replace coal in power generation, and the pentoses are utilized for low value biogas production. Some of this energy can be used to run the biorefinery, but the usefulness of such a solution is often low because of low demand for heat locally, lack of infrastructure for use of the electric power and low local demand for biogas. Therefore, the profits connected to running biorefineries for low value fuel and energy production are minimal and biorefineries for fuel and energy production are associated to low commercial viability and high risk. As a consequence, financial commitment from private and public investors for biorefinery plants solely dedicated to energy production, such as ethanol, gas and electric energy is reduced or entirely absent. There is therefore an urgent need to develop more environmentally friendly 2nd generation biorefineries and a requirement to assure commercially sustainable 2nd generation biorefineries for the future. Particularly, there is a need to identify and execute strategies that aim at developing sustainable 2nd generation biorefineries.

The idea and rationale behind BioREFINE-2G stems from the scientific feasibility to facilitate and accelerate the development of 2nd generation biorefineries, and integrate chemical production into a commercially viable biorefinery concept. BioREFINE-2G aimed to develop novel yeast strains, fermentation and downstream processes for the production of higher value chemicals such as diacids from side- and waste streams in a biorefinery. These chemicals are presently produced either from petrochemicals, or possibly by "first generation bioprocesses" based on pure glucose or sucrose. By utilizing biomass derived C5 streams or mixed C5/C6 streams, the production base for biochemicals can be significantly increased, and the environmental footprint of chemical production can be reduced. Further, by coupling new bioprocesses into existing industrial biorefinery facilities, the investment and running costs required for the production of the new biochemicals is equally reduced due to the synergies with the existing production site. Another important aspect of BioREFINE-2G was to develop novel bio-based polymers derived from dicarboxylic acids for use as i.e. plastics, adhesives, coatings, etc.

The consortium involved a number of distinguished industrial partners within the biotechnology and chemical industry, directly targeted by the call. In combination with a cross-disciplinary team of leading European scientists as well as a group of enabling technology providers and biotechnology specialists, the group of five industrial partners and three world leading RTO (Research and Technology Organization) partners wished to address the major scientific potential and economic opportunity in achieving significant and tangible improvements throughout the development of a 2nd generation biorefinery concept through industrial scale demonstration of highly innovative and high-impact production of diacids and diacid derived biopolymers.

In short, the envisaged BioREFINE-2G project directly addressed the objectives of the topic (KBBE.2013.3.4-01) to develop and demonstrate "...biotechnology approaches for the conversion of biorefinery by-products into added value biobased products, such as chemicals and chemical building blocks, biopolymers, materials. The feasibility of integrating the approach into a selected biorefinery value chain should be assessed. The project had a strong industry drive and included demonstration activities aimed at proving the techno-economic viability of the developed technologies, including a quantitative technological/economic viability analysis for up-scaling to industrial production. A life-cycle assessment was carried out in order to evaluate the environmental, economic and social performance of the developed technologies building upon existing and on-going LCA activities in the field of bio-based products and processes".

3 Description of main S&T results/foreground

BioREFINE-2G has reached significant progress in the areas of engineering of industrial yeast strains for applications in 2nd generation biorefineries, in development of downstream processing of dicarboxylic acids from complex fermentation broths, on obtaining novel polyesters based on diacids, and on life cycle analysis. The project results have been widely disseminated in the scientific community, among industrial stakeholders and general public.

The following tables give an overview of the peer reviewed publications from the project period and manuscripts in press and under preparations.

In the section after, descriptions of main results/foreground are given and snapshots of relevant publications of each sections presented. The section is divided between Strain engineering, Fermentation and Downstream Processing, Novel biopolymers, Life Cycle Analysis (LCA) and Dissemination & Exploitation

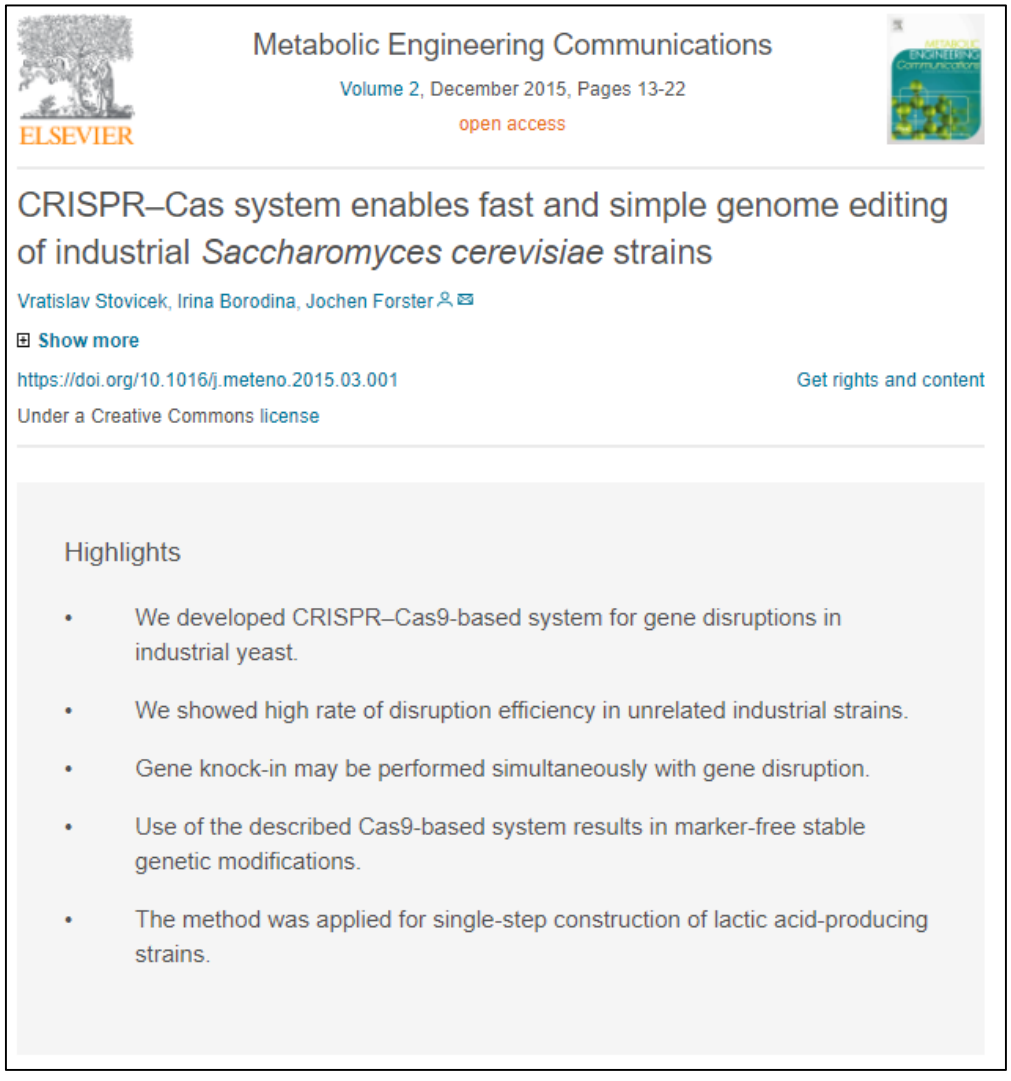
No	D.O.I. (Digital Object Identifier)	Title*	Author(s)*	Journal*	Publisher	Publisher location	ISSN	eISSN	Volume/ Issue*	Date of publication (dd/mm/yyyy)	URL	Rel. pages*	Open access[1] Yes / No
1	10.1016/j.meteno.2015.03.001	CRISPR–Cas system enables fast and simple genome editing of industrial Saccharomyces cerevisiae strains	Vratislav Stovicek, Irina Borodina, Jochen Forster	Metabolic Engineering Communications	Elsevier				Volume 2 (2015)	20/03/2015		13-22	Yes
2	10.1007/s10295-015-1684-8	"EasyClone 2.0: Expanded toolkit of integrative vectors for stable gene expression in industrial Saccharomyces cerevisiae strains"	Stovicek V, Borja GM, Forster J, & Borodina I	J Ind Microbiol Biotechnol,	SpringerLink				42(11) (2015)	16/09/2015		1519-1531	Yes
3	10.1002/biot.201600147	EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into Saccharomyces cerevisiae	Jessop-Fabre MM, Jakočiūnas T, Stovicek V, Dai Z, Jensen MK, Keasling J, Borodina I	Biotechnol J	WILEY-VCH				11(8) (2016)	23/06/2016		1110-1117	Yes
4		Redirecting the carbon flux towards the glyoxylate cycle in Saccharomyces cerevisiae: alpha-ketoglutarate as a case-study	Diogo J. Portugal-Nunes, Lisa Wasserstrom, Adrien Guibert, Basti Bergdahl, Gunnar Lidén, Marie F. Gorwa-Grauslund										

No	D.O.I. (Digital Object Identifier)	Title*	Author(s)*	Journal*	Publisher	Publisher location	ISSN	eISSN	Volume/ Issue*	Date of publication (dd/mm/yyyy)	URL	Rel. pages*	Open access[1] Yes / No
5	10.3390/fermentation3020013	Purification of Polymer-Grade Fumaric Acid from Fermented Spent Sulfite Liquor	Diogo Figueira, João Cavalheiro and Bruno Sommer Ferreira	Fermentation 2017, 3, 13	Licensee MDPI	Basel, Switzerland				01/04/2017	www.mdpi.com/journal/fermentation	1-11	Yes
6	10.1080/09168451.2017.1292839	A rapid method for analysis of fermentatively produced D-xylonate using ultra-high performance liquid chromatography and evaporative light scattering detection	Almqvist, Henrik; Sandahl, Margareta; Lidén, Gunnar	Bioscience, Biotechnology, and Biochemistry, 2017	Taylor and Francis				Online Feb. 2017		http://www.tandfonline.com/doi/full/10.1080/09168451.2017.1292839	1-3	Yes
7	10.1093/femsyr/fox030	CRISPR/Cas system for yeast genome engineering: advances and applications	Vratislav Stovicek, Carina Holkenbrink, Irina Borodina	FEMS Yeast Research, Volume 17, Issue 5, 1 August 2017, fox030	Oxford Academic	Oxford, UK			Volume 17, Issue 5,	15/05/2017 (advance access) 01/08/2017		1-16	Yes
8	10.3390/fermentation3030046	Carboxylic acid production	Gunnar Liden	Fermentation	MDPI	Basel, Switzerland	ISSN 2311-5637		3		http://www.mdpi.com/journal/fermentation/special_issues/carboxylic-acid	46	Yes
9		Exploring xylose oxidation in Saccharomyces cerevisiae through the Weimberg pathway	Lisa Wasserstrom, Diogo Portugal-Nunes, Henrik Almqvist, Anders G. Sandström, Gunnar Lidén and Marie F. Gorwa-Grauslund	Manuscript in preparation									Yes

No	D.O.I. (Digital Object Identifier)	Title*	Author(s)*	Journal*	Publisher	Publisher location	ISSN	eISSN	Volume/ Issue*	Date of publication (dd/mm/yyyy)	URL	Rel. pages*	Open access[1] Yes / No
10	10.1039/C7SM01569K https://doi.org/10.1039/C7SM01569K	Molecular weight prediction in polystyrene blends. Unprecedented use of a genetic algorithm in pulse field gradient spin echo (PGSE) NMR	Arrabal-Campos, Francisco M. Álvarez, José D. García-Sancho, Amador Fernández, Ignacio http://orcid.org/0000-0001-8355-580X	Soft Matter					Soft Matter, 2017,13, 6620-6626	18/09/2017			Yes
11		Sustainability of bio-based chemicals: State and challenges	Ólafur Ögmundarson, Markus J. Herrgård, Jochen Forster, Michael Z. Hauschild & Peter Fantke	Nature Biotechnology	Nature Publishing Group	New York, USA				Was submitted to the journal on 29 August 2017			Yes
12		Development of industrial yeast cell factories for biorefinery applications: From lignocellulosic biomass to dicarboxylic acids	Vratislav Stovicek, Henrik Almqvist, Laura Dato, Ksenia Chekina, Lasse Emdrup Pedersen, Anna Koza, Gunnar Lidén, Jochen Forster, Irina Borodina							In preparation			Yes
13		Rational and evolutionary engineering of an industrial yeast strain for efficient consumption of xylose	Vratislav Stovicek, Laura Dato, Ksenia Chekina, Lasse Embrup Pedersen, Anna Koza, Henrik Almqvist, Gunnar Liden, Jochen Forster, Irina Borodina							In preparation			Yes

3.1 Strain engineering - main S&T results/foreground

BioREFINE-2G has developed an efficient and versatile genetic toolbox for engineering industrial *S. cerevisiae* strains. The toolbox includes the usage of CRISPR/Cas9 technology and allows creating stable strains without antibiotic selection markers, which is important for large-scale fermentations. Four peer-reviewed articles have been published and two manuscript are under review. BioREFINE-2G has created industrial yeast strains capable of efficient utilization of xylose and tolerant to Borregaard hardwood hydrolysate even at low pH. We also created proof-of-concept industrial strains capable of producing smaller amounts of dicarboxylic acids (fumaric, succinic, and malic) from xylose. In the engineering of alpha-ketogluratic acid production from xylose via the Weimberg pathway, we have identified bottlenecks in xylonic acid conversion, which are addressed and discussed in relevant deliverables and peer-reviewed publications.



ELSEVIER

Metabolic Engineering Communications

Volume 2, December 2015, Pages 13-22

open access

CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains

Vratislav Stovicek, Irina Borodina, Jochen Forster

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https://doi.org/10.1016/j.meteno.2015.03.001

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Highlights

- We developed CRISPR–Cas9-based system for gene disruptions in industrial yeast.
- We showed high rate of disruption efficiency in unrelated industrial strains.
- Gene knock-in may be performed simultaneously with gene disruption.
- Use of the described Cas9-based system results in marker-free stable genetic modifications.
- The method was applied for single-step construction of lactic acid-producing strains.

Journal of Industrial Microbiology & Biotechnology
November 2015, Volume 42, Issue 11, pp 1519–1531 | Cite as

EasyClone 2.0: expanded toolkit of integrative vectors for stable gene expression in industrial *Saccharomyces cerevisiae* strains

Authors | Authors and affiliations

Vratislav Stovicek, Gheorghe M. Borja, Jochen Forster, Irina Borodina

Open Access | Metabolic Engineering and Synthetic Biology
First Online: 16 September 2015

Abstract

Saccharomyces cerevisiae is one of the key cell factories for production of chemicals and active pharmaceuticals. For large-scale fermentations, particularly in biorefinery applications, it is desirable to use stress-tolerant industrial strains. However, such strains are less amenable for metabolic engineering than the standard laboratory strains. To enable easy delivery and overexpression of genes in a wide range of industrial *S. cerevisiae* strains, we constructed a set of integrative vectors with long homology arms and dominant selection markers. The vectors integrate into previously validated chromosomal locations via double cross-over and result in homogenous stable expression of the integrated genes, as shown for several unrelated industrial strains. Cre-mediated marker rescue is possible for removing markers positioned on different chromosomes. To demonstrate the applicability of the presented vector set for metabolic engineering of industrial yeast, we constructed xylose-utilizing strains overexpressing xylose isomerase, xylose transporter and five genes of the pentose phosphate pathway.

Biotech Method

EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae* via CRISPR-Cas9

View Issue TOC
Volume 11, Issue 8
August 2016
Pages 1110–1117

Mathew M Jessop-Fabre, Tadas Jakočiūnas, Vratislav Stovicek, Zongjie Dai, Michael K Jensen, Jay D Keasling, Irina Borodina

First published: 23 June 2016 Full publication history

DOI: 10.1002/biot.201600147 View/save citation

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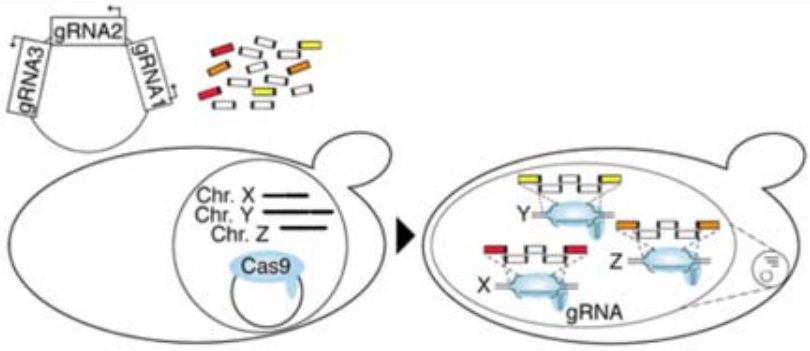
12

Abstract



Homologous recombination (HR) in *Saccharomyces cerevisiae* has been harnessed for both plasmid construction and chromosomal integration of foreign DNA. Still, native HR machinery is not efficient enough for complex and marker-free genome engineering required for modern metabolic engineering. Here, we present a method for marker-free multiloci integration of *in vivo* assembled DNA parts. By the use of CRISPR/Cas9-mediated one-step double-strand breaks at single, double and triple integration sites we report the successful *in vivo* assembly and chromosomal integration of DNA parts. We call our method CasEMBLR and validate its applicability for genome engineering and cell factory development in two ways: (i) introduction of the carotenoid pathway from 15 DNA parts into three targeted loci, and (ii) creation of a tyrosine production strain using ten parts into two loci, simultaneously knocking out two genes. This method complements and improves the current set of tools available for genome engineering in *S. cerevisiae*.

Keywords: **CRISPR/Cas9**; **DNA assembly**; **double-strand break**; **metabolic engineering**;

CRISPR/Cas system for yeast genome engineering: advances and applications

FREE

Vratislav Stovicek, Carina Holkenbrink, Irina Borodina

FEMS Yeast Research, Volume 17, Issue 5, 1 August 2017, fox030,
https://doi.org/10.1093/femsyr/fox030
Published: 15 May 2017 **Article history** ▾

Abstract

The methods based on the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system have quickly gained popularity for genome editing and transcriptional regulation in many organisms, including yeast. This review aims to provide a comprehensive overview of CRISPR application for different yeast species: from basic principles and genetic design to applications.

3.2 Fermentation and Downstream Processing - main S&T results/foreground

A 2-stage purification protocol has been developed and refined in order to increase both the yield and the purity of the fumaric acid. It was concluded that recycling the filtrate stream of the second filtration step, saturated in FA, improved the FA recovery yields. The selective precipitation of contaminants was attempted by testing a sequential pH decrease without apparent success. A polishing step based on ion exchange was introduced in order to improve the purity of the final product. Samples of different grades of FA were produced to assess whether less stringent, thus lest costly, purification protocols can be envisaged. The information required to develop a purification process for glutaric acid has been compiled and reported.

Biosci Biotechnol Biochem. 2017 Jun;81(6):1078-1080. doi: 10.1080/09168451.2017.1292839. Epub 2017 Feb 20.

A rapid method for analysis of fermentatively produced D-xylonate using ultra-high performance liquid chromatography and evaporative light scattering detection.

Almqvist H[1], Sandahl M[2], Lidén G[1].

Author information

Abstract

An ultra-high performance liquid chromatography (UHPLC) based method for the analysis of d-xylonate was developed using an amide column in combination with an evaporative light scattering (ELS) detector. Separation of d-xylonate from other components of the fermentation medium was achieved. The dynamic range of the method was 0.2-7.0 g/L.

KEYWORDS: ELS detector; UHPLC; amide column; d-xylonate

PMID: 28485215 DOI: 10.1080/09168451.2017.1292839

Fermentation **2017**, *3*(3), 46; doi:10.3390/fermentation3030046 Open Access Editorial

Carboxylic Acid Production

Gunnar Lidén

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(This article belongs to the Special Issue Carboxylic Acid Production)

Fermentation **2017**, *3*(2), 13; doi:10.3390/fermentation3020013 Open Access Feature Paper Article

Purification of Polymer-Grade Fumaric Acid from Fermented Spent Sulfite Liquor

Diogo Figueira, João Cavalheiro and Bruno Sommer Ferreira *

Biotrend-Inovação e Engenharia em Biotecnologia, S.A. Biocant Park, Núcleo 04 Lote 2, 3060-197 Cantanhede, Portugal

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Academic Editor: Gunnar Lidén

Received: 28 February 2017 / Revised: 29 March 2017 / Accepted: 30 March 2017 / Published: 1 April 2017

3.3 Novel biopolymers - main S&T results/foreground

Synthetic routes to obtain polyesters using glutaric acid have been identified. Presently, the most promising are diacids with small chain diols for production of medium and small polymers that can be polyurethanes precursors. The feasibility to transfer the synthesis to a continuous reactive extrusion process has been tested and mathematically simulated, and found to be scalable to a large scale. In order to determine the molecular weight of new biopolyesters a gel permeation chromatography method and a fast nmr method have been developedas well as and

an easy method to determine the polymer purity. The syntheses have been thoroughly optimized, and polymers were conveniently characterized (melting point, hydroxyl number and acid number, FTIR and DSC). The obtained polyesters were converted into commercially interesting products, such as thermoplastic polyurethane polymers,polyurethane dispersions and PLA copolymers. Polyurethane and PLA copolymers have been analysed according to their appearance, transparency, melting point, flexibility and brittleness. From novelty and commercial point of view some good results have been obtained. A market analysis has been conducted showing that it will be important to develop efficient processes for the production of the diacids.

Molecular weight prediction in polystyrene blends. Unprecedented use of a genetic algorithm in pulse field gradient spin echo (PGSE) NMR

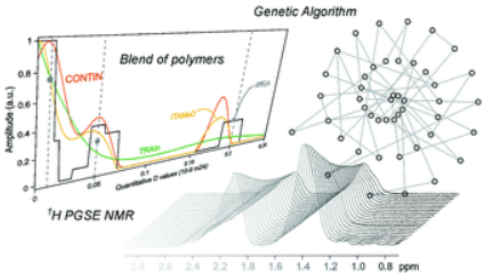
Check for updates

Francisco M. Arrabal-Campos,[a] José D. Álvarez,[b] Amador García-Sancho[c] and Ignacio Fernández*[a]

Author affiliations

Abstract

A genetic algorithm that uses boxcar functions (diffGA) has been applied for the first time in PGSE NMR. It reconstructs accurate diffusion coefficients for all the components of the mixture, and therefore predicts correct weight-average molecular weights for all of them. The results reported herein complement those obtained with established methods such as ITAMeD, CONTIN and TRAIn algorithms, and provide a detailed solution picture. Its robustness and limits have been stretched in order to ascertain the minimum separation within diffusion coefficients or relative proportion between components. In addition, the new genetic algorithm has been also applied to a mixture of small molecules, providing excellent results at very low computational times.



3.4 Life Cycle Analysis (LCA) - main S&T results/foreground

For a first assessment of preliminary impacts of the diacid production, an environmental LCA has been performed in comparison with an established production process of succinic acid. Those results have been communicated with the project partners. The final life cycle sustainability assessment was performed building on the research of the project partners. Questions provided by the partners concerning sustainable options of the proposed processes have been considered through scenarios to inform on major hotspots and opportunities for more sustainable improvements. It was further condensed to provide a summary of the major findings for public audience. The table below shows a detailed view on the social LCA conducted for the Borregaard site.

Stakeholder	Subcategory	Country Performance	Site specific performance	Impact assessment
Local Community	Delocalization and migration	1	2	2
	Community engagement	1	1	1
	Cultural heritage	2	1	1
	Respect of indigenous rights	1	n. e.	n. e.
	Local employment	1	1	1
	Access to immaterial resources	1	1	1
	Access to material resources	1	2	2
	Safe and healthy living conditions	2	2	2
	Secure living conditions	2	1	1
	Total	**1**	**1**	**1**
Consumer	Health and Safety	1	1	1
	Feedback mechanism	1	1	1
	Privacy	1	n. e.	n. e.
	Transparency	1	n. e.	n. e.
	End-of-Life Responsibility	1	1	1
	Total	**1**	**1**	**1**
Workers	Freedom of Association and CB	1	1	1
	Child labour	2	1	1
	Fair salary	1	1	1
	Child poverty	1	n. e.	n. e.
	Working time	1	1	1
	Forced labour	1	n. e.	n. e.
	Discrimination	1	1	1
	Health and Safety	1	1	1
	Social benefits / social security	1	1	1
	Total	**1**	**1**	**1**
Society	Commitment to sustainable issues	1	1	1
	Prevention & mitigation of conflicts	1	1	1
	Contribution to economic dev.	1	1	1
	Corruption	1	1	1
				1
				1

Social Life Cycle Impact Assessment of the Foreground System (1=very low risk, 2=low risk)

3.5 Dissemination & Exploitation - main S&T results/foreground

The work during this project period resulted in the continuous up-date of the project website at www.biorefine2g.eu, the development of a project logo, common reporting templates and the organisation of two project workshops. The development of new marketing material (an up-dated 6-page flyer), the publication of the condensed summary report on Sustainability LCA, and the elaboration of the final dissemination and exploitation plan (PUDF) has also been part of the continuous dissemination work. Within the project, 14 peer reviewed publications and 4 PhD thesis have been completed or are in final stages of completion. More than 65 specific dissemination activities (e.g. presentations, posters, press releases) have been performed. Finally, AIMPLAS, DTU, Ecolpol Tech, IFU and ULund have developed valuable exploitable foreground material where future means of exploitation are investigated.

A professional, attractive and user-friendly project website was developed and launched in December 2013 (Month 2) using Joomla. The website can be accessed under www.biorefine2g.eu. The website provides information about the scope and objectives of the project as well as the activities implemented throughout the project duration.

Home page of the BioREFINE-2G website

In order to disseminate BioREFINE-2G project activities among important stakeholders, a project logo, common reporting templates as well as marketing material (two project flyers) have been developed. In October 2016 an up-dated 6-page flyer was elaborated presenting recent progress in strain engineering, process development, polymerization methods, scale-up and product development as well as contact details of all project partners. 2000 hardcopies of this flyer were printed in November 2016 and distributed by project partners on different occasions.

In order to disseminate project results, every partner prepared at least two publications and two presentations at national and international biorefinery events.

Several peer reviewed publications were prepared in renowned journals including “CRISPR–Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains” and “EasyClone 2.0: Expanded toolkit of integrative vectors for stable gene expression in industrial *Saccharomyces cerevisiae* strains” by V. Stovicek, DTU, “Purification of Polymer-Grade Fumaric Acid from Fermented Spent Sulfite Liquor” by D. Figueira, Biotrend, and “A rapid method for analysis of fermentatively produced D-xylonate using ultra-high performance liquid chromatography and evaporative light scattering detection” by H. Almqvist, ULUND.

More than 65 other dissemination activities such as presentations at workshops and conferences, posters, and press releases have been implemented by all project partners.

With respect to outreach to the general public, "open door days" were organised by ULUND at the university premises on 8 April 2015 and by DTU in November 2015 and November 2016 permitting an open exchange as well as promoting public awareness among students about bio-refineries.

Synergies with other existing European initiatives were created by the exchange of information and results, cooperation concerning workshops and conferences and the mutual promotion of project activities.

Two workshops (in year 2 and year 4) were organised by the BioREFINE-2G consortium in order to bring leading experts in novel biorefinery processes for the production of biopolymers together and to discuss about the latest developments in this field.

The first BioREFINE-2G Workshop on Bioplastics from 2nd Generation Biorefineries was organized on the occasion of the 11th International Conference on Renewable Resources & Biorefineries (RRB-11) on 5 June 2015 in York, UK.

The Second BioREFINE-2G Workshop “Utilisation of Waste Streams for Bioproducts and Bioenergy” was organized on the occasion of the 2017 European Biomass Conference and Exhibition (EUBCE 2017) on 12 June 2017 in Stockholm, Sweden.

This Second BioREFINE-2G Workshop mobilized more than 50 international participants.

The BioREFINE-2G project includes training exchange opportunities for students among the involved research partners (DTU, ULUND, IFU), and between research partners and industry partners (Borregaard, Biotrend, Aimplas) in order to establish and strengthen close research cooperation links. Exchanges took place between DTU and AIMPLAS and training courses are offered by AIMPLAS in May 2016, by ULUND in August 2017.

Based upon the continuously up-dated draft dissemination and exploitation plan (deliverable report D7.3), a final dissemination and exploitation plan (PUDF) (deliverable report D7.9) was elaborated by WIP with contributions by all partners. It contains detailed information on all project dissemination activities such as project website, marketing material, publications, other dissemination actions, interactions with other projects, open door days, workshops, trainings and communication of LCA results as well as an overview of identified exploitable foreground.

The following exploitable results were identified and are explained in detail in the next session of this final report. Specific exploitation flyers are available under www.biorefine2g.eu/publications-reports.

1. DTU: Robust xylose-utilizing industrial yeast
2. DTU: Genetic engineering toolbox for manipulation of industrial yeast strains
3. ULUND: Yeast strain engineering for xylose oxidation
4. BIOTREND: Fumaric acid purification process from fermented lignocellulosic wastes
5. AIMPLAS: Novel polymerization methods by reactive extrusion to obtain new PLA-Copolymers with enhanced properties
6. ECOPOL: Polyester synthesis in batch and reactive extrusion
7. IFU: Integrated Life-Cycle-Sustainability-Assessment

4 Potential impacts and main dissemination activities and exploitation results

4.1 Main dissemination activities

All project partners contributed to the dissemination activities. For more information see Deliverable 7.9, the project Final Dissemination and Exploitation Plan (PUDF).

4.1.1 Peer reviewed publications

During the project period, 14 publications have been published, are in review or manuscripts are being finalized.

4.1.2 Presentations

During the project period, 32 presentations have been given reaching more than 9000 people in the audience.

4.1.3 Posters

During the project period, 14 posters were presented to a total audience of more than 3000 people.

4.1.4 Flyers

The BioREFINE-2G project flyer (Figure 3) was elaborated at the beginning of the project (D7.2, Task 7.2) to provide interested stakeholders of the specific target audiences in the scientific and industrial community with an overview of the aims and objectives of the BioREFINE-2G project, namely the development of commercially attractive processes for efficient conversion of pentose-rich side-streams from biorefineries into dicarboxylic acids, which can be used as precursors for bio-based polymers including biodegradable polymers.

Up-dated BioREFINE-2G marketing material was developed during the course of the project (D7.4, D7.6). In October 2016 an up-dated 6-page flyer was elaborated presenting recent results of the BioREFINE-2G project (Figure 4). This flyer includes information on progress in strain engineering, process development, polymerization methods, scale-up and product development as well as contact details of all project partners. 2000 hardcopies of this flyer were printed in November 2016 and distributed by project partners on different occasions. Both flyers can be downloaded from the project website www.biorefine2g.eu.

4.1.5 Organisation of workshops

Two workshops (in year 2 and year 4) were organised by the BioREFINE-2G consortium in order to bring leading experts in novel biorefinery processes for the production of biopolymers together and to discuss about the latest developments in the field of biotechnology.

The first BioREFINE-2G Workshop on Bioplastics from 2nd Generation Biorefineries was organized on the occasion of the 11th International Conference on Renewable Resources & Biorefineries (RRB-11) on 5 June 2015 in York, UK.

The Second BioREFINE-2G Workshop “Utilisation of Waste Streams for Bioproducts and Bioenergy” was organized on the occasion of the 2017 European Biomass Conference and Exhibition (EUBCE 2017) on 12 June 2017 in Stockholm, Sweden.

A workshop summary report and all presentations of the second BioREFINE-2G workshop are available via the project website under:

http://www.biorefine2g.eu/newsand-events-biorefine2g/events

4.1.6 Press releases

During the project period, six press releases and magazine publications reaching an audience of more than 200.000 people.

4.1.7 Open door days

With respect to outreach to the general public, "open door days" were organized permitting an open exchange as well as promoting public awareness about biorefineries.

These project "open door days" were coordinated with similar activities already promoted by the partners (namely DTU or ULUND) for general outreach.

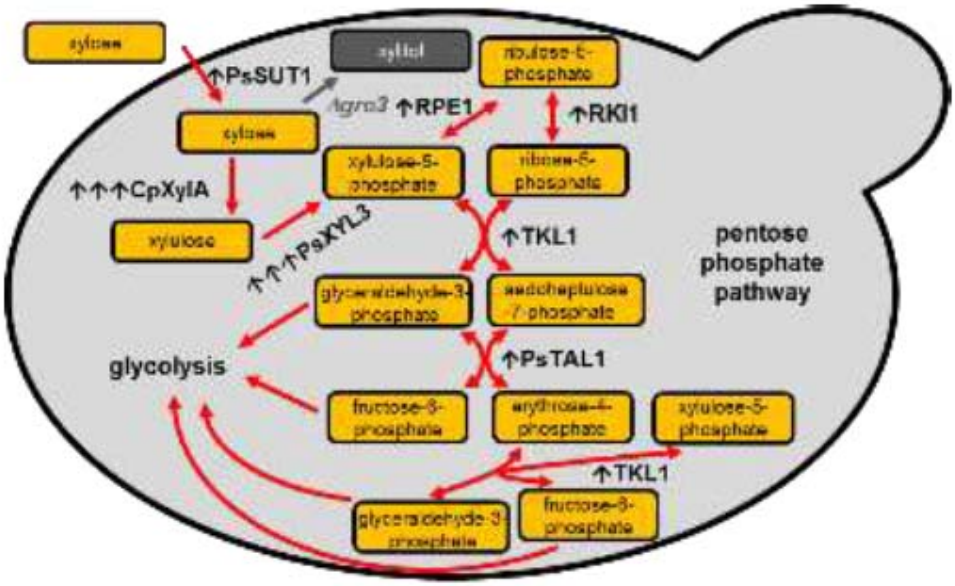
4.2 Exploitation of results and their potential impacts

a) Robust xylose-utilizing industrial yeast

Explanation and purpose

An industrial *Saccharomyces cerevisiae* strain Ethanol Red has been engineered for efficient utilization of xylose. The strain comprises overexpression of three native and four heterologous genes, including a xylose isomerase from *Clostridium phytofermentans*, and deletion of the native *GRE3* gene to prevent xylitol accumulation.

The strain was further adapted to hardwood spent sulfite liquor (SSL) by adaptive laboratory evolution. The strain is suitable for utilization of residual xylose in SSL streams.



Exploitation strategy

The strain is available for research and commercial use from Technical University of Denmark under standard terms. The strain will be employed as platform strain in other research projects.

IPR measures taken or intended

The isomerase gene is covered by a patent assigned to Lesaffre et Compagnie, hence a licence would be needed for commercial exploitation of the strain.

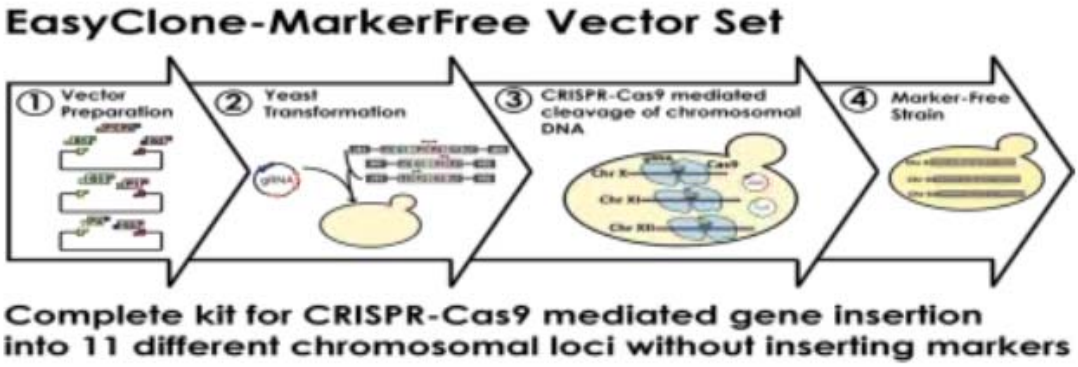
Further research necessary

The strain will be further engineered for production of various bio-based fuels and chemicals.

Potential / expected impact of exploitation
The yeast strain ferments glucose and xylose present in the hardwood spent sulfite liquor. The sugars can be converted to ethanol or, if the strain is further engineered, to other products.

b) Genetic engineering toolbox for manipulation of industrial yeast strains

Explanation and purpose
Polyploid industrial strains of *Saccharomyces cerevisiae* can be rapidly engineered using the provided genetic toolbox. The toolbox comprises a set of integrative vectors that target eleven specific well-characterized genomic locations. The vectors allow for selection in prototrophic yeast strains using six different dominant selection markers. The markers can subsequently be removed using loxP/creA recombination. Alternatively, the integration is ensured by CRISPR/Cas9 system. The vectors allow for efficient overexpression of multiple genes.
CRISPR/Cas vectors can also be employed for gene deletions and other genome edits, including combinations of several different genome edits in a single transformation event.



Exploitation strategy
The vector toolbox has been distributed to 30+ academic and industrial laboratories. The vectors are available for research use via public despository – Addgene, including user guidelines:

https://www.addgene.org/kits/borodina-easyclone-v2/
https://www.addgene.org/kits/borodina-easyclone-markerfree/
https://www.addgene.org/browse/article/22359/

For commercial use, the standard terms of Technical University of Denmark apply.

Detailed description of the toolbox is included in the following research papers:

1) Stovicek V., Borodina I., Forster J. (2015): CRISPR-Cas system enables fast and simple genome editing of industrial *Saccharomyces cerevisiae* strains. Metabolic Engineering Communications 2:13-22

2) Stovicek V., Borja G., Forster J., Borodina I. (2015): EasyClone 2.0: Expanded toolkit of integrative vectors for stable gene expression in industrial *Saccharomyces cerevisiae* strains. Journal of Industrial Microbiology and Biotechnology 42(11):1519-1531

3) Jessop-Fabre MM, Jakočiūnas T, Stovicek V, Dai Z, Jensen MK, Keasling J, Borodina I. (2016): EasyClone-MarkerFree: A vector toolkit for marker-less integration of genes into *Saccharomyces cerevisiae*. Biotechnol J 1(8):1110-1117.

IPR measures taken or intended
For commercial exploitation, refer to general CRISPR patents (if any) in the given country.

Further research necessary
Not applicable.

Potential / expected impact of exploitation
Industrial strains of *S. cerevisiae* can be engineered rapidly and efficiently using the developed genetic toolbox.

c) Yeast strain engineering for xylose oxidation

Explanation and purpose
A *Saccharomyces cerevisiae* strain for the conversion of xylose into dicarboxylic acids has been constructed by expression of genes from the bacterial Weimberg pathway.
In contrast to other xylose assimilation pathways expressed in *S. cerevisiae* that connect xylose to glycolysis, this 5-step oxidation pathway connects xylose to alpha-ketoglutarate, an intermediate of the tricarboxylic acid cycle.
Xylose oxidation should then occur without carbon loss, which opens the route for the generation of a whole new range of carboxylic acids.

Exploitation strategy
Not applicable.

IPR measures taken or intended
Once the pathway is fully functional in *S. cerevisiae*, the strain will be available for non-commercial purposes under MTA.

Further research necessary
Alternative enzymes with improved affinity and/or activity will be investigated.

Potential / expected impact of exploitation

This new strategy enables the conversion of xylose to carboxylic acids which opens the route for the generation of a whole new range of products.

d) Fumaric acid purification process from fermented lignocellulosic wastes

Explanation and purpose

A process for the recovery and purification of fumaric acid from a complex fermentation medium containing spent sulfite liquor (SSL) as a carbon source was developed. A simple procedure, involving separation unit operations, pH and temperature manipulation and polishing, allowed for the recovery of fumaric acid with high recovery yield and with specifications meeting the requirements of the polymer industry.

Exploitation strategy

Biotrend is a research-based company providing advanced bioprocess development services. The technology will be available for testing and implementation in different biotechnological processes producing fumaric acid or other dicarboxyic acids from complex raw materials.

IPR measures taken or intended

No patent application is planned, but the technology has potential application in the purification of other dicarboxylic acids from similar complex media. The technology will be kept as internal know-how.

Further research necessary

Upon the availability of robust fumaric acid producing strains at relevant scale, the purification process can be scaled-up and fully integrated, according to the existing integration proposal for swift transition to industrial application.

Potential / expected impact of exploitation

The application of this purification process will validate the possibility to produce fumaric acid using renewable resources as raw material while meeting the stringent standards of the polymer industry. It will provide a success story of production of a

biobased building block meeting the highest standards albeit being produced from complex industrial residual or side-streams.

e) Novel polymerization methods by reactive extrusion to obtain new PLA-Copolymers with enhanced properties

Explanation and purpose

Biopolymer markets are experiencing huge growth expected to continue in the near future, mainly driven by growing European and North American markets and intense focus on industrial expansions.

One example of this competitive advantage is the use of new bio-adhesives and biomaterials for use in Tetra Pak or in laminated complexes of PLA (Polylactic Acid) with other bio-polymers ensuring fully biodegradable products. In this context development of PLA derivatives with improved properties (in line with the bio-polymer concept) will guarantee access to the PLA market.

As European consumers are demanding more environmentally friendly products in the packaging sector, an increase in demand is expected. Finding new bio-adhesives derived from bio-glutaric and/or bio-fumaric acids will be a value proposition for these new polymers enabling companies to access a large market in which until today bioadhesives and enhanced biomaterials are poorly developed, thus offering a direct competitive advantage.

Exploitation strategy

Technical data sheets for new materials will be prepared and distributed at fairs and congresses.

Specific roadmap for PLA producers will be developed to offer them the new materials.

Furthermore, the concept of the integrated synthesis production process of reactive extrusion will be communicated to the relevant scientific community via conferences.

IPR measures taken or intended

Trademark application is planned and a patent will be submitted to protect the new copolymers as well as the fabrication process by AIMPLAS and EcopolTech together with interested companies.

Further research necessary

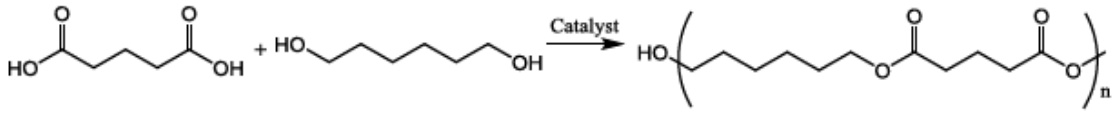
The polymerization by reactive extrusion is currently under optimization. Molecular weight and material properties will be adjusted to fulfil the requirements of the packaging industries.

Potential / expected impact of exploitation

The new PLA copolymers show improved properties and similar cost compared to fossil-based PLA.

f) Polyester synthesis in batch and reactive extrusion

Explanation and purpose
Our main goal is to synthesize polyesters from glutaric and fumaric acids obtained from renewable sources. Different conditions have been studied in order to prepare high quality polyesters with special properties and added value. Among them, a wide variety of diols, catalysts, temperature ranges, reaction times and characterization methods have been applied. Special attention has been focused on lowering the energy and waste generated during the processes. To this aim, lipases have been applied to our syntheses as reusable catalysts.



Our next purpose has been the preparation of other commercially interesting products, such as water-borne polyurethane dispersions, which are in line with the philosophy of our company.

Exploitation strategy
Our idea is to produce high added value polyurethane polymers from bio-based polyesters. Achieving sophisticated products with special properties will allow us to manufacture in-house and exploit the research made within BioREFINE-2G. Another approach is to establish license agreements with big companies interested in our products.

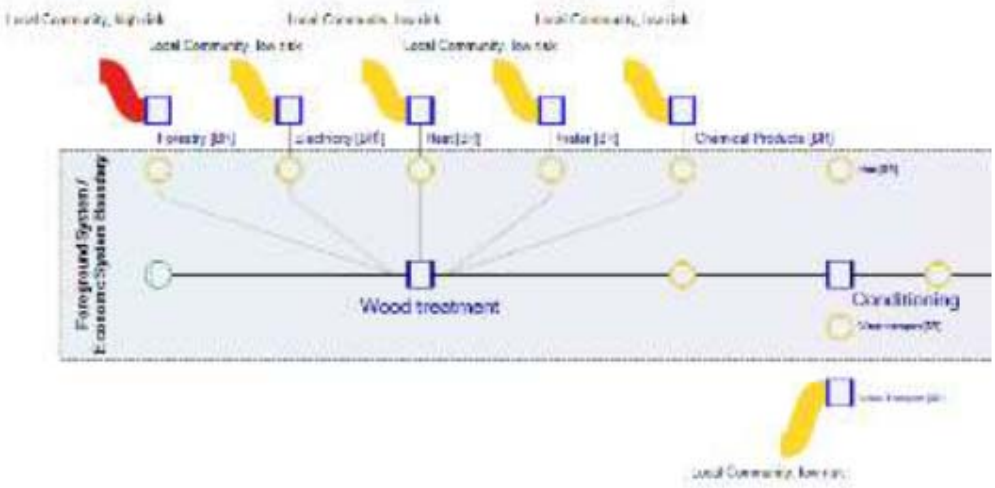
IPR measures taken or intended
The most promising polyurethane products and copolymers obtained from the starting polyesters will be protected through one or more patents. Then, Ecopol Tech and AIMPLAS will consider the possibility to publish the results in peer-reviewed journals.

Further research necessary are divided into two approaches: firstly, the methods involving organometallic catalysts will be studied so that the reaction temperature is lowered below 150 ºC. This is important to ensure that the products can be synthesized at Ecopol Tech's facilities. Secondly, the use of lipases will also be optimized in order to make the process more environmentally friendly and reduce energy and resource waste.

Potential / expected impact of exploitation can be very large given the applicability of polyurethane and PLA copolymers in various sectors of the market.

g) Integrated Life-Cycle-Sustainability-Assessment

Explanation and purpose
Life-Cycle-Sustainability-Assessment (LCSA) of the product system developed is an integrated model. Two calculations are necessary to produce the results for environmental and economic perspective. This is due to the different system boundaries that are applied. During the calculation of the environmental LCA, also the results of the social LCA are calculated. The results are qualitative, they are shown as 'flags' connected to processes (as shown in the figure below) in the model showing colours on a range from green to red indicating risk. : derive the LCSA with only one calculation step. The integration of social data and the display of social results was the biggest methodological challenge for the approach. It has been realised using the social hot spot database.



Exploitation strategy
The integrated approach will be tested on further case samples and it will be aimed at enabling the calculation of results for multiple system boundaries simultaneously. Furthermore, the social hot spot database, necessary for the SLCA, will be integrated into the standard software. The concept of the integrated model will be communicated to the relevant scientific community via conferences.

IPR measures taken or intended
No trademark application is planned, but the software feature will be commercialised by ifu Hamburg GmbH together with interested companies.

Further research necessary
The current approach is on a conceptual level and not implemented into the standard software. The social hot spot database needs to be harmonised with the existing data format of the Umberto software.

Potential / expected impact of exploitation
Combination of three Sustainability Assessments types in one Model increases the efficiency of analyses compared to parallel implementation of the assessments.

5 Address of project public website and relevant contact details

www.biorefine2g.eu

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