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LES MICRO-ORGANISMES PRODUCTEURS DE LIPIDES

DOSSIER

ProBio3 project: how to achieve scientific and technological challenges to boost the sustainable microbial production of lipids as biojet fuel and chemical compounds

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Abstract – The deal of ProBio3 project is to develop the microbial conversion on specific fatty acids of carbon substrates from renewable resources and industrial by-products. The main application fields are BiojetFuels and green chemistry. The objectives focus on the identification of renewable feedstock suitable for microbial nutritional requirements, the development of an intensive bioprocess, the proof of feasibility at pilot scale (m^3) with the evaluation of environmental, economic and societal impacts. During 8 years, 16 French partners (9 research units LISBP, MICALIS, IJPB, IGM, IMFT, SQPOV, TSE, TWB, IFPen, 4 industries Airbus, EADS, Sofiproteol, Tereos et 3 technical centres ITERG, CVG, CREOL) associate their interdisciplinary competences from Life Sciences to Process Engineering including Economic and Social Sciences. With Investissement d'Avenir financial support, the expected impacts are increases of fundamental knowledge of lipid metabolism in oleaginous microorganisms, development of high-throughput tools to fasten industrial engineering strains and fermentation strategies, new extraction processes involving green solvents and realistic scale up studies towards an industrial pilot; with undeniable innovative aspects, the deal is to get competitive assets for leading international positions on a new biological route.

Keywords: ProBio3 / renewable resources / BiojetFuel / green chemistry / oleaginous microorganisms / lipids / *Rhodotorula glutinis* / *Yarrowia lipolytica* / *Streptomyces lividans* / *Cupriavidus necator*

Résumé – Probio3 projet : relever les défis scientifiques et techniques pour accélérer la voie durable de production microbienne de lipides à usages biokérosène et chimie verte. Probio3 a pour ambition de développer la production microbienne d'acides gras spécifiques par conversion de substrats carbonés issus de ressources renouvelables et de co-produits industriels. Les champs d'application sont le biokérozène et la chimie verte. Les objectifs de Probio3 concernent l'identification de ressources renouvelables adaptées aux exigences nutritionnelles des microorganismes, le développement de bioprocédés intensifs, la preuve de faisabilité du concept à l'échelle d'un pilote (m^3) avec l'évaluation des impacts environnementaux, économiques et sociétaux. Durant 8 années, 16 partenaires français (9 publics LISBP, MICALIS, IJPB, IGM, IMFT, SQPOV, TSE, TWB, IFPen, 4 industriels Airbus, EADS, Sofiproteol, Tereos et 3 centres techniques ITERG, CVG, CREOL) associent leurs compétences interdisciplinaires associant les Sciences de la Vie au Génie des Procédés ainsi que les Sciences Economiques et Sociales. Avec le soutien financier des Investissements d'Avenir, les retombées attendues du projet sont des avancées dans la connaissance fondamentale du métabolisme lipidique chez les microorganismes oléagineux, l'accélération du développement de souches industrielles et de stratégies de fermentation par des technologies à haut-débit, des ruptures technologiques dans l'extraction des lipides et des études réalistes de changement d'échelle pour un pilote industriel ; avec des aspects innovants indéniables, il s'agit d'acquérir des atouts compétitifs pour une position de leader international en production microbienne de lipides.'etitifs pour une position de leader en production microbienne de lipides.

Mots clés : ProBio3 / ressources renouvelables / biokérozène / chimie verte / microorganismes oléagineux / lipides / *Rhodotorula glutinis* / *Yarrowia lipolytica* / *Streptomyces lividans* / *Cupriavidus necator*

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Introduction

Microorganisms, including yeasts and bacteria, have long been studied as alternative sources of oils and fats (Ratledge, 2004; Beopoulos *et al.*, 2011). Microorganisms synthesize lipids as a part of their metabolism, and as a source of energy. Some species have been reported to accumulate more than 20% of their dry cell mass in the form of lipids, and have been classified as "oleaginous" microorganisms (Thorpe, 1972; Ratledge *et al.*, 2002). Moreover, some oleaginous yeast species are particularly promising in this respect, as they can accumulate more than 70% of their dry cell weight as lipids (Ratledge *et al.*, 2002). In addition to this considerable capacity for lipid accumulation, oleaginous yeasts present various fatty acid profiles. In particular, they synthesize valuable polyunsaturated fatty acids, and are, therefore, a target of choice for potential applications as a renewable raw material for energetic and chemical production or as nutritional supplements.

Therefore, ProBio3 project, selected for French financial support by "Investissements d'Avenir", investigates and develops the biocatalytic production of lipidic bioproducts from renewable resources and industrial by-products; the main application fields are the lipid production for alternative non petroleum compounds for aviation jet fuels and chemistry uses. The major objectives focus on scientific and technological bottlenecks of the microbial pathway related to:

- the identification of cheap and renewable feedstocks available with low dependence on fossil fuel price and suitable for microbial nutritional requirements;
- the development of an intensive bioprocess with the evaluation of its environmental, economic and technological impacts to reach the biofuel and green chemistry production targets;
- the demonstration of proof of feasibility at pilot scale (m^3) with the integration of all the unit operations with multi-stage scale-up approach from laboratory scale to commercial pre-production scale.

1 The challenges

The challenges of the development of valuable technological and economic process for sustainable microbial production of lipids, for energetic and chemical uses, are to lower environmental impact of fossil fuel use on climate change with increasing energy and chemistry demands and to greater petroleum independence. Two main application fields are of major interest: the biofuel for aviation and the green chemistry.

The aviation industry has identified the development of sustainable fuels dedicated to aviation as one of the biggest challenge. Using alternative sources to oil based kerosene is crucial for the European aeronautic industry competitiveness, economic growth and sustainable development. With an air traffic growth forecasted at a 5% rate annually and globally, the Jetfuel demand will be there in the future. Two targets were defined for aviation sector: a carbon-neutral growth at 2020 horizon and 50% CO_2 emission reduction compare to the 2005 level in 2050. Since 2005, aviation industry strongly intensifies researches and development in new routes for sustainable aviation fuel production all around the world with very stringent requirements: safety, "drop-in" to allow blends with traditional Jet Fuel, high-performances with international specifications (high energy density, high flash point, low freezing point . . .), benefit on full carbon life cycle, no competition with fresh water requirements and food production, no impact on biodiversity. As blends of up to 50% of hydrotreated ester and fatty acidst (HEFA) derived from any plant oils and fats, with conventional jet fuel have already been certified by ASTM for use in aircrafts operations, lipids from microbial production constitutes a very promising route involving oleaginous microorganisms that can be a valuable contributor to the aviation sustainable development.

Oleochemistry was also identified like an important field of interest for the 21st century. Products from lipids offer relevant and unique properties which can be used in several sectors: food, detergents, cleaning products, cosmetics, plastics and rubber, paints and coatings, crop protection, intermediate products or synthons. Microbial lipids offer then numerous industrial applications very similar to lipids extracted from oleaginous plants.

2 The targets

The analysis of the international state of the art revealed that oleaginous microorganisms have been studied over decades, but mechanisms underlying their metabolic specificities remain unclear. Some attempts have been made to describe potential set-ups for the large-scale production of lipids (Saenga *et al.*, 2010) but fermentation strategy needs also further investigations to develop process with very high performances according to industrial criteria. In this context, four microorganisms *Rhodotorula glutinis, Yarrowia lipolytica, Streptomyces lividans, Cupriavidus necator* were selected within the project according to their natural capacity to convert the selected substrates in lipids.

Yarrowia lipolytica is one of the most widely studied "nonconventional" oleaginous yeast species (Beopoulos *et al.*, 2009a; Beopoulos *et al.*, 2009b; Barth, 1997). It has been isolated from various food-related environments (*e.g.* cheese, sausage), but also from sewage, soils and oil fields (Barth *et al.*, 1997). Its classification by the American food and drug administration as "Generally Recognized As Safe" (GRAS) paved the way for the development of various biotechnological applications, including (i) heterologous protein production (Madzak *et al.*, 2004), (ii) organic acids production (Finogenova *et al.*, 2005), and (iii) single-cell oil productions from agroindustrial by-products or wastes (Papanikolaou *et al.*, 2003). Under specific growth conditions, *Y. lipolytica* accumulates large amounts of lipid, sometimes accounting for more than 50% of its dry cell weight (Ratledge, 2005). One of the major advantages of this yeast is its ability to use hydrophobic substrates (*e.g.* alkanes, oils, fatty acids . . .) efficiently as a sole carbon source (Barth *et al.*, 1997; Fickers *et al.*, 2005). *Y. lipolytica* cells accumulate large amounts of lipids on these substrates, using specialized protrusions formed on their cell surface to facilitate the uptake of hydrophobic

compounds (Mlickova *et al.*, 2004). These characteristics, together with the availability of the complete genome sequence, render *Y. lipolytica* a model of choice for investigations of lipid accumulation in oleaginous yeast species. Various studies have already made use of the genome sequence to decipher aspects of lipid metabolism in *Y. lipolytica*, and some of the genes involved in the bioconversion, synthesis and mobilization of lipids have been described (Beopoulos *et al.*, 2008).

The basidiomycete yeast *Rhodotorula glutinis* is another relevant microorganism for lipid accumulation studies, as it is able to accumulate up to 70% lipid (w/w) of dry cell mass. The lipids accumulated are mainly TriAcylGlycerol (TAG) with fatty acids having aliphatic tails of 16–18 carbons (Ratledge, 1989), saturated and unsaturated (up to 2 unsaturations). *R. glutinis* is able to metabolize xylose, glucose and glycerol (Easterling *et al.*, 2009; Saenga *et al.*, 2010; Li *et al.*, 2010; Pan *et al.*, 1986). However, few experiments were done in bioreactors with co-substrates, under perfectly controlled conditions to quantify and manage yeast metabolism (Babau *et al.*, 2013).

Streptomyces lividans, a filamentous soil bacteria well-known for its ability to produce antibiotics, has the natural ability to degrade plant polymers, including lignocellulose, as well as to accumulate large reserves of TriAcylGlycerols (over 25% of its dry weight) when grown in a medium with a high C/N ratio and P limitation (Olukoshi *et al.*, 1994; Packter *et al.*, 1995). However, the genetic basis of these abilities remains to be established and very few works in the world have been published on these topics (Davis *et al.*, 2010; Arabolaza *et al.*, 2013; Kaddor *et al.*, 2009).

The facultatively autotrophic, Gram-negative β-proteobacterium, *Ralstonia eutropha* (recently renamed *Cupriavidus necator*), is best known for production of polyhydroxyalkanoates (PHAs), which are carbon and energy storage polymers synthesized from Acetyl-coenzyme A, the precursor for also fatty acids (Madison *et al.*, 1999). *C. necator*, in the presence of O_2, has the ability to utilize exogenously delivered H_2 (Schlegel *et al.*, 1971) for the reduction of CO_2 to cellular constituents and up to 75% (w/w) of intracellular PHA polymers (Schlegel *et al.*, 1971; Ishizaki *et al.*, 2001). This microorganism is of major interest to recycle CO_2, by-product of fermentation process, and produce lipidic molecules in an integrated process concept.

With the availability of the complete genome sequence, these four microorganisms are suitable candidates for genetic and metabolic engineering approaches aiming to develop optimized strains for the production and storage of large amounts of lipids with specific fatty acids composition.

Lipid accumulation is induced by nutrient limitation or deficiency (Cescut, 2009; Granger, 1992; Aggelis *et al.*, 1999, Ratledge *et al.*, 2002) with a carbon excess. The carbon to nitrogen ratio C/N is a key parameter to monitor fatty acid accumulation and profile with an optimum value depending on the strain. For higher values, nutritional deficiency becomes lethal. Fatty acid composition is also dependent on culture temperature, as the degree of saturation generally decreases with decreasing temperature (Granger, 1992) in order to maintain the cell membrane integrity. Most of the processes described in previous publications relate to batch (Rupcic *et al.*, 1996; Papanikalaou *et al.*, 2006; Kim *et al.*, 2000) and fed batch cultures. In batch cultures, minerals and carbon substrates are initially mixed in the bioreactor, with a high initial C/N ratio. As nutrients are consumed from the start of culture, C/N ratio continually increases and lipid production occurs (Granger *et al.*, 1993). Nevertheless, in batch mode, by-product production led to decreasing carbon conversion yield into lipids. In fed batch culture, nitrogen and carbon flows are monitored to monitor specific growth and lipid production rates with minimization of by-product production to perform the highest performances (Cescut, 2009). Fed-batch culture goes through three phases: (i) a growth phase, (ii) a transition phase and (iii) a lipid accumulation phase. In fed-batch cultures of yeast *R. glutinis*, on synthetic medium and glucose, at 30 °C, pH 5.5 without oxygen limitation (Cescut, 2009), a biomass concentration up to 132 dry cell weight g L^{-1} with 53% (w/w) of lipids was performed in 70 h with glucose to lipid conversion yield of 0.20 g_{Lip}^{-1} g_{Glu}. Fed-batch cultures of *Y. lipolytica* (at 28 °C, pH 5.5), on synthetic medium with glucose and glycerol substrates, were also recently reported (Cescut, 2009). The biomass concentration reached 103 dry cell weight g L^{-1} with a lipid accumulation quantity of 41% g_{lip} g_{yeast}^{-1} after 82 h of culture and a conversion yield of de 0.37 $Cmol_{lip}$ $Cmol_{glu}^{-1}$.

Further investigations are needed, based on quantitative physiology studies, to optimise the microbial capacity versus nutrient limitations to mimic industrial substrates within the wide range of substrates.

High importance is given in the substrate choice to ensure microbial and process requirements, economic and environmental criteria as cheap and sustainable; carbohydrate substrate resources from industrial by-products (from starch industry or sugar refinery, biodiesel production industry, food industries), lignocellulosic substrates, CO_2 and its derivatives within a recycling by-product strategy are under consideration. The four microorganisms selected have the best natural characteristics to convert a large range of renewable carbon substrates into lipids. Promising raw materials are lignocellulosic resources: lipids production from lignocellulose sources requires enzymatic hydrolysis of cellulose and hemicellulose (respectively by cellulases and hemicellulases) to release sugars (saccharification) that can subsequently be fermented by yeasts or bacteria to lipids. To be economically and environmentally viable on an industrial scale, this requires operating at high dry mass to achieve sufficiently high cellulose or hemicellulose levels. However, high substrate concentration in the form of fibrous, solid materials poses two principal problems that need to be investigated: (1) the increased concentrations of potential inhibitors hamper the performance of yeast and enzymes and (2) high viscosity results in more power consumption in the fermentor (Jorgensen *et al.*, 2007) and lowered mixing and heat transfer efficiency (Georgieva *et al.*, 2008; Jorgensen *et al.*, 2007; Rudolf *et al.*, 2005; Varga *et al.*, 2004).

Moreover, in order to reduce the cost of the conversion of lignocellulose to lipids, biomass-to-products conversion in one step (Lynd *et al.*, 2005; Kondo *et al.*, 2004; Steen *et al.* 2010; Van Zyl *et al.*, 2007) could be of major interest: this strategy called an integrated or consolidated bioprocess strategy is highly attractive and is studied in the project.

In addition, in a recycling strategy of fermentation by-products, glycerol and CO_2 as co substrate are carbon sources for the lipid production by, respectively, *R. glutinis* (Fakas *et al.*, 2009; Saenge *et al.*, 2010) and *Y. lipolytica* (Cescut, 2009), and *Ralstonia eutropha*. The management of the consumption of these substrates induces scientific biological and physical bottlenecks that need further investigations (Liang *et al.*, 2010).

Moreover, setting up an energetically efficient technique for oil extraction is among the most important targets of the project. Du to nutrient limitation or deficiency, cell wall is strengthened and become more resilient against disrupting techniques (Greenwell *et al.*, 2010). So, one bottleneck is the cell wall disrupting to free lipid bodies (Hanisch *et al.*, 2006; Chisti *et al.*, 1986; Ptasinski *et al.*, 2006; Töpfl, 2006).

Considering all these biological and physical targets, the system approach in ProBio3 allows:

- to identify the key genes involved in the biosynthesis of lipidic type molecules and its regulation through "omics" technologies;
- to elaborate new oleaginous microorganisms to control lipid composition and accumulation, including, in a highly attractive approach too, new strains producing both lignocellulotic cocktail and industrially-relevant molecules are constructed;
- to assess the performances of intensive fermentation strategies monitoring taking into account the specificities of the substrates, strains and products, with laboratory pilots (from 1 to 20 L) and small industrial pilot (m^3);
- to demonstrate proof of feasibility at pilot scale up to 1 m^3. The evaluation criteria involve energy consumption and process flexibility, yields, capital equipment costs, integration of process steps;
- to evaluate economic and environmental impacts of the lipid microbial production and the lipid compounds use to assess its competitiveness and its environmental relevance among other production pathways. In this way, it is highly required to analyse and quantify the market impact of developing this technology and to deal with competition for natural resources involved as production inputs. Such a competition is typically modelled through land-use decisions involving agricultural and forest resources, urban and other land uses. By combining several domains of economics (energy, natural resources agricultural and industrial economics), the modelling of an integrated framework, not yet available, allow assessing the economic impacts of this new generation of biofuels and chemical compounds. An important issue is to characterize these substitutions in order to determine the net impact that will indeed account for consequences of technological adaptation in the biofuel and chemistry sectors. On the environmental side, many aspects are evaluated: energy and greenhouse gas balance taking into account indirect aspects, soil use, biodiversity loss or threat, water or other resources use, pollutants consideration, land use competition as well as the evaluation of the production potential. The modelling of a specific LCA for heterotrophic microorganisms is required to properly envisage different scenarios.

3 ProBio3 actions and partners

ProBio3 is an 8-years long research project combining fundamental and applied researches with 16 partners that associate their interdisciplinary competences from life sciences to process engineering including economic, social and human sciences. These partners are international industrial stakeholders as Airbus, EADS, Sofiproteol, Tereos, IFPen, international leading academic laboratories as Laboratoire d'Ingénierie des Systèmes Biologiques et Procédés, Microbiologie de l'Alimentation au Service de la Santé Humaine, Institut Jean-Pierre Bourgin, Institut de Génétique et Microbiologie, Institut de Mécanique des Fluides de Toulouse, Sécurité et Qualité des Produits d'Origine Végétale, Toulouse School of Economics, Toulouse White Biotechnology labelled by the two major scientific French research Agencies CNRS and INRA, also including three national centres of experts, ITERG, CVG and CREOL. The total cost is 24.6 M€.

Seven WP combine research and development works with high interactions between multidisciplinary approaches (genetic engineering, metabolism, microbial engineering, bioprocess, economy, environmental, social and ethic sciences).

- WP1 evaluates the fermentescibility of industrial pretreated substrates with a screening of industrial effluents or by-products and high dry matter content lignocellulosic substrates, CO_2 and its derivatives within a recycling by-product strategy;
- WP2 develops new and robust industrial microorganisms to modulate intracellular lipid profile and to optimise lipid accumulation; from physiology study, intensive bioprocess strategies are deduced according to realistic industrial criteria;
- WP3 transposes oilseed extraction methods to oil microbial cells and/or to develop new methods of pretreatment of microbial cells and extractive methods involving green solvents;
- WP4 quantifies the performances for each microorganism/substrate within the strategies of optimized fermentation taking into account the specificities of the substrates, strains and products, with laboratory pilot (from 1 to 20 L) and small industrial pilot (m^3). The valorisation of co-products, as CO_2, glycerol, proteins and various lipids, are studied to decrease the overall biofuel production costs. A multi-stage scale-up approach is employed with mass and energy balances. The deal is to demonstrate the proof of feasibility at pilot scale;
- WP5 evaluates all over the project, environmental, economic, technological and ethic impacts including life cycle assessment, to ensure an efficient and sustainable process for microbial lipids production;
- WP6 transfers research outputs and customises information and knowledge so that it is ready for uptake by different target end-users;
- WP7 provides a strong management component that allows ProBio3 to reach its ambitions.

4 ProBio3 innovation and scientific, technological and product outputs

Biocatalytic route has many advantages as a short and middle term solution:

- the ability of tuning lipid composition to modulate fatty acid profile by using different wild strains, genetically modified micro-organisms and culture strategies. It is of major interest to optimise overall process performances including post treatment processes (hydrotreatment, chemical reactions . . .);
- the wide range of potential substrates (glucose, pentose, lipidic residues and even recycled CO_2 and glycerol, main co-products of fermentation);
- the valorisation of co-products as proteins for animal food, specific lipids for alimentary or health according to the biorefinery principle;
- the quantitative and qualitative reproducibility of the microbial processes without geographic and climate dependence;
- the maturity of fermentation technology involved in the process, from laboratory pilot up to industrial scale: oleaginous yeasts and bacteria fermentation technology is already involved in very large industrial bioprocesses all around the world for many other applications.

Strong indications that the environmental gain of biofuel produced by microorganisms is greater than any other pathways of biofuels production have been reported in preliminary results coming from former projects CALIN, Alfa-Bird and SWAFEA.

All the actions of ProBio3 projects allow to develop methodologies and approaches for fasten up strain engineering program under industrial performance constraints. Technological breakups will be needed in the development of synthetic biology tools (such as enzyme engineering, promoter engineering, regulatory networks) to precisely redirect carbon flow within the desired metabolic pathways. Many data are produced which increase the fundamental knowledge of the regulatory network of lipid metabolism in the organisms studied; it allows gaining new fundamental knowledge, identification of bottlenecks and improvement strategies in fermentation and microbial oil extraction by multidisciplinary approach combining genetic engineering, metabolism, microbial engineering and bioprocess. Technological breakups are needed too in the development of industrial fermentation strategy and industrial oil extraction as microbial lipid production has been investigated at the scale proposed in ProBio3.

The ambition of ProBio3 project partners is to contribute to the development of French valuable technological and economic process for sustainable microbial production of biojet fuel and chemical compounds. It would then contribute to maintain French and European industry competitiveness, economic growth and sustainable development with reducing national petroleum energetic dependence.

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