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Dossier

This paper is a part of the hereunder thematic dossier published in OGST Journal, Vol. 68, No. 5, pp. 789-946 and available online here

Cet article fait partie du dossier thématique ci-dessous publié dans la revue OGST, Vol. 68, n°5, pp. 789-946 et téléchargeable ici

DOSSIER Edited by/Sous la direction de : **A. Daudin et A. Quignard**

PART 2

Second and Third Generation Biofuels: Towards Sustainability and Competitiveness

Deuxième et troisième génération de biocarburants : développement durable et compétitivité

Oil & Gas Science and Technology – Rev. IFP Energies nouvelles, Vol. 68 (2013), No. 5, pp. 789-946

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Oil & Gas Science and Technology – Rev. IFP Energies nouvelles, Vol. 68 (2013), No. 5, pp. 899-911
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DOI: 10.2516/ogst/2013148

Dossier

Second and Third Generation Biofuels: Towards Sustainability and Competitiveness
Seconde et troisième génération de biocarburants : développement durable et compétitivité

Towards a Microbial Production of Fatty Acids as Precursors of Biokerosene from Glucose and Xylose

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Résumé — Vers une production microbienne d'acides gras en vue de l'application biokérosène à partir de glucose et xylose — Le développement de filières de production de molécules énergétiques en substitution au kérosène constitue un défi majeur pour l'industrie aéronautique afin de minimiser l'impact environnemental de son activité et de répondre à ses besoins en énergie, dont la demande est croissante. Le développement d'une nouvelle voie de production de lipides à partir de ressources renouvelables non alimentaires ouvre des perspectives prometteuses avec la certification ASTM des huiles hydrotraitées. Les travaux expérimentaux consistent en l'étude, d'une part, des potentialités de croissance de la levure oléagineuse *Rhodotorula glutinis* à partir de glucose et/ou xylose, substrats osidiques issus des ressources lignocellulosiques, et d'autre part, des potentialités d'accumulation de lipides à partir de glucose. Des cultures en mode *fed-batch* ont permis le contrôle des flux d'alimentation : en carbone, en condition de croissance, selon un ratio xylose/glucose variable pour la quantification du métabolisme, et en azote, en condition de production de lipides. Il a été montré que la levure *Rhodotorula glutinis* est capable de consommer simultanément le glucose et le xylose. Le taux de croissance et le rendement de conversion du carbone en biomasse diminuent en fonction de la composition du mélange xylose/glucose : à savoir 0,36 h^{-1} et 0,43 $C_{mol\ X^*}.C_{mol\ glu}^{-1}$ sur glucose pur, 0,15 h^{-1} et 0,56 $C_{mol}.C_{mol}^{-1}$ sur 10 % de xylose, 0,037 h^{-1} et 0,18 $C_{mol}.C_{mol}^{-1}$ sur xylose pur. Par ailleurs, lors d'expérimentation en condition d'accumulation lipidique, il a été mis en évidence la nécessité de maintenir une croissance résiduelle par le contrôle des flux d'azote et de carbone. Lors de la phase de production de lipides sur glucose, il a été ainsi obtenu une concentration finale en biomasse de 150 $g_{CDW}.L^{-1}$ contenant 72 % de lipides en masse; la productivité volumétrique maximale atteint 1,5 $g_{lip}.L^{-1}.h^{-1}$, avec un rendement de conversion du glucose en lipides égal à 95 % du rendement théorique limite. La température de culture se révèle un paramètre opératoire important pour la modulation du profil lipidique. Ces résultats sont originaux et ils ont permis l'obtention de très hautes performances, en culture intensive. Ils argumentent des potentialités de développement d'une stratégie de production de lipides par conversion de toutes les fractions osidiques des substrats lignocellulosiques pour des usages biokérosène après hydrotraitement.

Abstract — Towards a Microbial Production of Fatty Acids as Precursors of Biokerosene from Glucose and Xylose — *The aviation industry considers the development of sustainable biofuels as one of the biggest challenges of the next ten years. The aim is to lower the environmental impact of the steadily increasing use of fossil fuels on climate change, yielding greater energy independence and fuel security. Thus, the development of a new route for the production of lipids from renewable non-food resources is now being promoted with the recent ASTM certification of hydrotreated oils. Our study focuses on the potential of growth of the oleaginous yeast Rhodotorula glutinis using glucose and xylose which can come from renewable lignocellulosic substrates and of lipid accumulation using glucose as substrate. Experiments were carried out in fed-batch mode which allowed feed flux management. Carbon fluxes were controlled with modifying xylose/glucose ratios to quantify metabolism in optimal growth condition. Besides, the management of carbon and nitrogen fluxes allowed characterizing lipid accumulation. Thus, it has been shown that the yeast Rhodotorula glutinis can simultaneously consume glucose and xylose. When the ratio xylose/glucose increased, the growth rate and the carbon conversion yield into biomass decreased: it was of 0.36 h^{-1} and 0.64 $C_{mol\ X^*}.C_{mol\ glu}^{-1}$ for pure glucose, it was of 0.15 h^{-1} and 0.56 $C_{mol}.C_{mol}^{-1}$ for 10% xylose and it was of 0.037 h^{-1} and 0.18 $C_{mol}.C_{mol}^{-1}$ for pure xylose. The necessity to maintain residual growth and to manage carbon fluxes to optimize lipid accumulation performance was revealed. Lipid accumulation on glucose engendered a final biomass concentration of 150 $g_{CDW}.L^{-1}$, microbial production (72% of lipids) and maximal productivity over 1.48 $g_{lip}.L^{-1}.h^{-1}$. The culture temperature is an important parameter to modulate the lipid profile. The results were encouraging. Lipid accumulation using lignocellulosic feedstock was shown to be a highly promising route.*

INTRODUCTION

The environmental impact of the aviation sector represents 2.4% of global CO_2 gas emission [1] compared to the transport sector responsible for 13.5% of global CO_2 emission (*Fig. 1*). Every year 18 000 planes consume 200 million tons of jet fuel, corresponding to around 5% of world petroleum consumption [2]. The annual quantity of fuel consumed is growing by 1 to 2% per year and this could even reach 3% in 2050 [3]. Therefore, the International Air Transport Association (IATA) estimates oil demand to reach 300/350 million tons per year before 2030. This can be compared to the 190 million tons consumed in 2009 [3].

Facing petroleum resource rarefaction and crude oil price volatility (*Fig. 2*), the aim is to substitute jet fuel with bio jet fuel to both increase energy independence and reduce the environmental impact. This will

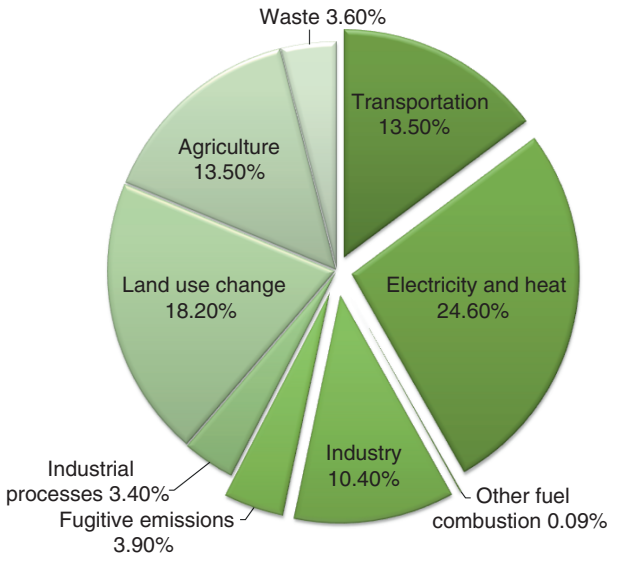

Figure 1

World CO_2 emissions by sector in 2009 [4].

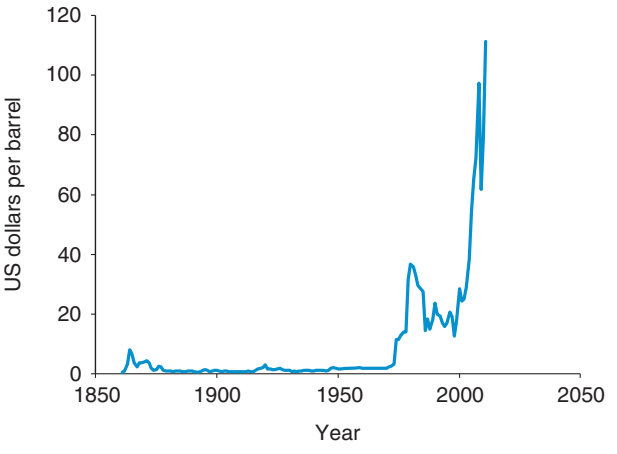

Figure 2

Crude oil prices since 1850, in money of the day [5].

promote the development of a new competitive economic sector which secures supplies and favours rural development [3].

In this context, the European aviation stakeholders are promoting researches into biojetfuel production in order to reach a target of 2 million tonnes of biofuel produced in Europe by 2020 [6]. Because of the extended lifetime of an aircraft (over 25 years), alternative fuels have to be compatible with current aircraft architecture and refueling infrastructures without any major changes in the fuel systems and with identical overall performance. This explains the needs to develop a "drop-in fuel", a fuel that will perfectly suit all aircraft functions without altering safety or performance. For this reason, biojetfuel must meet the specifications of jet A1 (*Tab. 1*) which is a mixture of a large number (5-16) of different hydrocarbons. The conditions of use of aircraft fuel imply stringent physical-chemical properties such as energy density (fuel tank volume is limited), low freezing point, high thermostability when temperature exceeds 40°C and is lower than −50°C, low viscosity, etc. [3]. All these parameters are precisely defined with international specifications [7] to ensure identical properties all around the world indispensable for intercontinental flights. Every specification is drastically tested with an ASTM methodology (*Tab. 1*).

Among the different alternatives recently studied, fully synthetic jet fuel (Synthetic coal to liquid from Sasol) [9], 50% blends of Fischer-Tropsch derived fuels and hydrotreated esters and fatty acid fuels have already been approved by ASTM [3, 10].

Considering the recent certification of hydrotreated oils, lipid-based biofuels seem to be a credible alternative to fossil jet fuel. Lipids are mainly extracted from oleaginous plants. However, to manage competition between food and non-food agriculture, the use of specific non-food crops such as camelina, moringa, or jatropha have been first investigated. Many recent flight tests have been successfully conducted with different blends, like the first-ever transatlantic biofuel flight on the 18th June 2011 with a blend of biokerosene from camelina and jatropha.

Lipids can also be produced by oleaginous microorganisms. Oleaginous microorganisms are able to accumulate lipid from 20% to 86% of its dry cell weight. There is a high biological diversity of oleaginous micro-organisms (*Tab. 2*).

But, considering yield, productivity, biomass concentration and lipid content, yeasts are the best candidate. Lipids accumulated by yeasts are mainly triacylglycerols [16]. *Rhodotorula glutinis* is a yeast of major interest as it is able to accumulate lipids to 70% of its dry cell weight

TABLE 1
Main specifications of Jet A1 and corresponding ASTM test method [8]

Specification	Composition	ASTM test method
Composition		
Acidity, total (mg KOH.g^{-1})	0.015	D3242
Aromatics (vol %)	25	D1319
Or total aromatics (vol %)	26.5	D6379
Sulfur total (wt %)	0.30	D1266, D2622
Sulfur Mercaptan (wt %)	0.0030	D3227
Volatility		
Distillation temperature		D86
10% recovery (°C) max.	205.0	
Final BP (°C) max.	300.0	
Distillation residue (vol %) max.	1.5	
Distillation loss (vol %) max.	1.5	
Flash point (°C) min.	38.0	D3828
Density 15°C (kg.m^{-3})	775.0-840.0	D1298 or D4052
Fluidity		
Freezing point (°C) max.	−47.0	D2386 or D5972
Viscosity −20°C (cSt) max.	8.000	D445
Combustion		
Specific energy, net. (MJ.kg^{-1}) min.	42.8	D4529
Smoke point (mm) min.	25.0	D1322
Or smoke point (mm) min.	19.0	D1322
Naphthalenes (vol %) max.	3.00	D1840
Corrosion		
Copper strip (2 h ± 5 min, 100°C ± 1°C) max.	1	D130
Thermal Stability		
JFTOT_P 260°C (mmHg) max.	25.0	D3241
Tube deposit rating (visual)	< 3	

TABLE 2
Main oleaginous micro-organisms with their maximal lipid accumulation [11-16]

Micro-organism	Lipid accumulation (% dry weight)
Bacteria	
Arthrobacter AK 19	78
Rhodococcus opacus	48
Fungi	
Aspergillus terreus	57
Cunninghamella japonica	60
Entomopthora coronata	43
Humicola lanuginosa	75
Mortiella isabellina	86
Micro-Algae	
Botryococcus braunii	25-75
Chlorella sp.	28-57
Cylindrotheca sp.	16-37
Dunaliella tertiolecta	71.4
Isochrysis sp.	25-33
Nannochloris sp.	20-63
Nannochloropsis sp.	31-68
Nitzchia sp.	45-47
Schizochytrium sp.	50-77
Yeasts	
Cryptococcus curvatus	58
Cryptococcus albidus	65
Lipomyces starkeyi	63
Rhodotorula glutinis	72
Trichosporon pullulans	65
Yarrowia lipolytica	36
Cunninghamella japonica	60
Rhizopus arrhizus	57
Penicillium spinulosum	64

under nitrogen limitation [17, 18] from glucose as carbon substrate. The accumulated lipids are mainly composed of the unsaturated fatty acid C18:1 [18].

In a perspective of industrial production, many research programs focus on the use of lignocellulosic feedstock hydrolysates as raw material, mainly composed of glucose and xylose. In the literature, catabolic repression of xylose metabolism by glucose when glucose is in excess in the culture medium has been reported [19-22]. In batch experiments using both glucose and xylose as substrates, it was observed with *R. glutinis* that xylose consumption occurred after a lag phase following the exhaustion of glucose [23]. No information on co-consumption of both substrates by *R. glutinis* has been previously reported.

In this context, the present paper reports the study of *R. glutinis* metabolism to optimize lipid accumulation using pentose and glucose as substrates. Controlling substrate feed with nutritional limitation or starvation, fed-batch cultures were carried out in bioreactors to investigate yeast growth using glucose and xylose as co-substrates and lipid accumulation using glucose as the sole substrate. The results presented here were used to quantify the dynamic behavior of the yeast during both growth and lipid accumulation cultures.

1 MATERIALS AND METHODS

1.1 Reagents

1.1.1 Microorganism

The yeast strain used was *Rhodotorula glutinis* wild type CECT 1137, supplied by the *Coleccion Espanola de Cultivo Tipo* (CECT), University of Valencia, Spain.

1.1.2 Culture Media

Before fermentor inoculation, three pre-culture of 12 h each were carried out in Erlenmeyer flasks containing successively 5 mL (yeast extract peptone dextose medium), which was then made up to 50 mL and finally 500 mL with mineral medium (see below) at 30°C on a rotary shaker (100 rpm).

For fed-batch culture, the glucose feed concentration was around 700 $g.L^{-1}$ and the xylose feed concentration was 545 $g.L^{-1}$.

Vitamin solution was prepared with the following composition:
- d-biotin: 0.05 $g.L^{-1}$,
- thiamine hydrochloride: 1 $g.L^{-1}$,
- pantothenic acid: 1 $g.L^{-1}$,
- pyridoxol hydrochloride: 1 $g.L^{-1}$,
- nicotinic acid: 1 $g.L^{-1}$,
- *p*-aminobenzoic acid: 0.2 $g.L^{-1}$,
- myo-inositol: 25 $g.L^{-1}$.

TABLE 3
Mineral medium composition for growth experiments ($g.L^{-1}$)

KH_2PO_4	4.5
$(NH_4)_2HPO_4$	0.83
$(NH_4)_2SO_4$	1 710
$MgSO_4.7H_2O$	1.7
$ZnSO_4.7H_2O$	0.016
$FeSO_4.7H_2O$	0.070
$MnSO_4.H_2O$	0.0029
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.0031
$Na_2MoSO_4.2H_2O$	0.0012
H_3BO_3	0.010
$CaCl_2.2H_2O$	0.018
NaCl	0.039
Glucose	20

TABLE 4
Concentrated salts feed composition for growth experiments ($g.L^{-1}$)

$MgSO_4.7H_2O$	36
$MnSO_4.H_2O$	0.103
$CaCl_2.2H_2O$	0.092
$Na_2MoO_4.2H_2O$	0.301
$FeSO_4.7H_2O$	3
H_3PO_4	90.733
$ZnSO_4.7H_2O$	1.143
H_2SO_4	212.565
$CuSO_4.5H_2O$	0.5
H_3BO_3	0.035
$CoCl_2.6H_2O$	1.49
NaCl	10
KCl	55.83

pH regulation was carried out with the addition of ammonia solution (16 $mol.L^{-1}$) or KOH solution (10 $mol.L^{-1}$).

The nitrogen feed was a 5 $mol.L^{-1}$ ammonia solution.

The mineral medium composition for growth experiments was detailed in Table 3. It was added with 10 mL vitamin solution (see above) and the pH was adjusted to 5.5 with phosphoric acid.

The concentrated salts feed for growth experiments had the composition detailed in Table 4.

Mineral medium composition for lipid accumulation experiments was presented in Table 5. It was added with 10 mL vitamin solution. The pH of this medium was adjusted to 5.5 with phosphoric acid.

The concentrated salts feed for lipid accumulation experiments had the composition listed in Table 6.

Vitamins were all purchased from *Sigma-Aldrich Chimie* (Lyon, France) with a purity of at least 99.9%. Glucose was provided by *Roquette* (France). All other chemicals were purchased from *VWR* (Fontenay-sous-Bois, France) with a purity of at least 99%.

TABLE 5
Mineral medium composition for lipid accumulation experiments ($g.L^{-1}$)

KH_2PO_4	4.54
$(NH_4)_2HPO_4$	0.83
$(NH_4)_2SO_4$	2.47
$MgSO_4.7H_2O$	1.7
$ZnSO_4.7H_2O$	0.016
$FeSO_4.7H_2O$	0.070
$MnSO_4.H_2O$	0.0029
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.0031
$Na_2MoSO_4.2H_2O$	0.0012
$CaCl_2.2H_2O$	0.018
NaCl	0.039
Glucose	20

1.2 Operating Conditions

1.2.1 Reactor

Fed-batch experiments were performed in a 20 L working volume fermentor using the *Braun Biostat E* fermenting system (*Braun*, *Biostat E*, *Braun Diessel*, *Biotech*, Melsungen, Germany) which was sterilizable *in situ*. Mixing was performed with three *Rushton* turbines.

The cultures were performed at a temperature maintained constant with a water-jacket and plate heat

TABLE 6
Concentrated salts feed composition for lipid accumulation experiments ($g.L^{-1}$)

$MgSO_4.7H_2O$	5.278
$MnSO_4.H_2O$	0.009
$CaCl_2.2H_2O$	0.051
$Na_2MoO_4.2H_2O$	0.0360
$FeSO_4.7H_2O$	0.215
H_3PO_4	28.620
$ZnSO_4.7H_2O$	0.078
H_2SO_4	16.370
$CuSO_4.5H_2O$	0.009
H_3BO_3	0.0025
$CoCl_2.6H_2O$	0.158
KCl	7.450

exchanger, or thermostatically-controlled water bath. This system was required to ensure accurate temperature control for high-density cell cultures.

The reactor was instrumented with the following probes: a pression sensor, a temperature sensor, an oxygen probe (*Ingold*, Urdorf, Switzerland) and a pH probe (*Fermprobe Broadley-James Co.*, Santa Ana, USA). The dissolved oxygen concentration was maintained in the non-limiting range (above 10% of saturation) by manual adjustment of stirring and aeration conditions. pH was automatically kept at 5.5. An anti-foaming agent (*Struktol*®J673) was added automatically as necessary through an antifoam probe inserted through the top plate of the reactor.

Three pumps were dedicated to the addition of substrates into the reactor: the first was used to make fast additions of substrate (maximum flow 15 $L.h^{-1}$) and two others provided a very precise flow (maximal flow of 0.5 $L.h^{-1}$). A fourth pump fed the concentrated salt solution. They were controlled through the software detailed in Section 1.2.2.

The amounts of substrates added were estimated from the variation of the weights of the substrate jars (balances model 12000 and I GP5202 *Sartorius*, Goettingen, Germany) using the software described in Section 1.2.2.

The composition of the inlet and outlet gases was analyzed by mass spectrometry (Dominated 600S, *VG gas*) or by a gas analyzer with an infrared spectrometry detector for carbon dioxide and a paramagnetic detector for oxygen (Egas 8, *Braun*, Germany).

1.2.2 Control Software

Homemade software enabled on-line acquisition, monitoring and regulation of operating parameters (stirring rate, pH, temperature, partial pressure of dissolved oxygen, base and antifoam additions). Oxygen consumption rate and carbon dioxide production rate were calculated from mass balances, taking into account the evolution of the gas volume in the reactor, inlet airflow (measured by a mass flowmeter, *Brooks*, USA), temperature, humidity and pressure. The residual substrate concentration in the fermentor was evaluated by homemade software based on carbon and redox balances taking into account on-line acquisition data.

1.2.3 Microbial Culture Parameters

The temperature was set at 30°C, and the pH at 5.5. An overpressure of 0.3 bar was maintained in the reactor. The bioreactor was supplied with a constant ratio 1:10 of mineral medium feed to substrate feed.

During the culture of *R. glutinis* in fed-batch mode, culture parameters such as pressure, pH and temperature were accurately controlled, in agreement with the values of the settings. Only stirring and aeration were variable parameters, manually adjusted during the experiment so as to maintain a dissolved oxygen concentration satisfying the needs of the microorganism: *i.e.* it was kept at over 10% of the saturation value.

1.3 Analytical Methods

1.3.1 Growth Characterization

Yeast concentration was quantified by spectrophotometric measurement at 600 nm (Spectrophotometer *Hitachi* U-1100, range 0.1-0.6 UOD) and by cell dry weight (catalytic biomass + lipids) measurements (filtration with 0.45 µm membrane, drying 60°C-200 mmHg for 48 h).

1.3.2 Analysis of Residual Substrates and Extracellular Metabolites

Alcohols, Organic Acids and Sugars

Determination of the alcohols, organic acids and sugar concentrations from supernatants was performed by HPLC using an *Aminex* HPX-87H^+ column (300 mm * 7.8 mm) under the following conditions: temperature 50°C, with 5 mM H_2SO_4 as eluent (flow rate of 0.5 $mL.min^{-1}$) and dual detection (refractometer and UV at 210 nm). Compounds were identified and quantified with reference to standards. During culture glucose concentration was determined in culture supernatants

with a glucose analyzer YSI model 27 A, *Yellow Springs Instruments*.

Ammonium Ion

An ammonium ion electrode (PH/ISE meter model 710A + Ammonia Gas Sensing Electrode Model 95-12, *Orion Research*) was used to quantify the residual nitrogen concentration in the culture medium.

1.3.3 Analysis of Intracellular Lipids

Lyophilisation

For biomass samples from yeast culture, cells were washed twice with saline solution (NaCl 9 $g.L^{-1}$) and then lyophilized (*Serail*, RP35).

Extraction

The PLE system, ASE 300, was provided by *Dionex*. The sample was placed in a stainless steel cell (11 mL) linked to electronic controllers which maintain extraction parameters (pressure, temperature, volume of extraction solvent and extraction time) at the programmed set points. The extraction solvent was pumped through the extraction cell, fitted with a cellulose filter and a stainless steel frit at the outlet. For each experiment, 0.4-0.7 g of lyophilized biomass was subjected to PLE extraction. Up to 1 g of Hydromatrix® (*Varian*) was used as a dispersant [24] in the extraction cell. Several cycles of extraction with different solvent mixtures were used. The method used was the optimized Bligh and Dyer method adapted for yeast lipid extraction with three extraction cycles using three different chloroform/methanol solvent mixtures: 1:2; 1:1 and 2:1 (v/v). For each solvent mixture, 2 static cycles were applied.

Washing

In order to remove the non-lipid components extracted, the organic phase was mixed with an acidified KCl solution (0.08 $g.L^{-1}$, pH = 1) for 15 min on a roller mixer; then centrifuged (5 000 × g, 10 min) to recover lipids as dry material after evaporation of the solvent with *Genevac* EZ-2 plus® (35°C, 200–5 mbar).

Analyses of Total Lipids

– *Gravimetry*
Total lipid content was quantified by weight after total drying (variation less than 0.2 mg).

– *Gas chromatography with an extraction step*
The free or linked fatty acids were methylated to yield Fatty Acid MethylEsters (FAME) using TriMethyl Sulfonium Hydroxide (TMSH, 0.2 M in methanol, *Macherey-Nagel*, Germany) [25]. GC analysis was carried out on a *Hewlett-Packard* gas chromatograph equipped with a 30 m × 0.25 mm × 0.25 μm WCOT fused silica column with the polar bonded phase CP-Select CB for FAME (*Varian*) and a flame ionization detector with Chromeleon® (*Dionex*) acquisition software, under the following conditions: carrier gas N_2 flow 1 $mL.min^{-1}$, column pressure 2.05 bar, oven temperature 50-75°C at 9°C min^{-1}, 75-140°C at 13°C min^{-1}, 140-180°C at 1.5°C min^{-1} and finally 180-240°C at 4.5°C min^{-1}, injector temperature 140°C, detector temperature 250°C with 40 $mL.min^{-1}$ H_2 flow and 450 $mL.min^{-1}$ air flow. Identification and quantification of methyl esters were based on the comparison of retention times and peak areas of serial dilutions of commercial standards. Internal standards were C9:0 for short-chain fatty acids and C19:0 for medium-chain fatty acids.

1.4 Culture Strategy

In order to investigate dynamic metabolic behavior of the yeast during growth fed-batch experiments were performed in two phases with glucose as sole substrate (phase I) and glucose and xylose as co-substrates (phase II). Lipid production was investigated with fed-batch culture carried out with 3 phases involving specific carbon and nitrogen feeding strategies.

1.4.1 Growth Conditions Using Co-Substrates

The two phases of fed-batch strategies for growth quantification were performed according to the following conditions:

– phase I: glucose feeding phase. In the first phase, only glucose solution was brought to the fermentor till the biomass concentration reached about 5 $g_{CDW}.L^{-1}$, this value is sufficient to quantify metabolic yeast behavior. Growth phase occurred at a maximal specific growth rate;
– phase II: co-substrate feeding phase. Following the first phase, xylose was incorporated. Different ratios of xylose/glucose (from 0 to 100% $C_{mol\ xylose}/C_{mol\ glucose}$) were gradually applied. Cultures were performed under carbon limiting conditions; to allow the co-consumption of both glucose and xylose, glucose uptake was controlled to maintain a residual glucose concentration near 0 g/L.

1.4.2 Lipid Accumulation

The 3 phases of fed-batch cultures for lipid accumulation were managed as following:

– phase I: growth phase using glucose as substrate. During the growth phase (I), an exponentially increasing glucose flow allowed a constant specific growth rate. Nitrogen flow was managed by pH regulation, kept constant at 5.5 with NH_4OH solution;
– phase II: transition phase to set up nitrogen limitation. The transition phase was the period during which nitrogen limitation with an excess of carbon source (glucose) occurred. It started when nitrogen medium depletion was initiated by shifting pH regulation from NH_4OH solution to KOH solution (10 $mol.L^{-1}$). Owing to nitrogen consumption by the yeast, without the NH_4OH solution feed, the ammonium ion concentration in the broth quickly decreased. As nitrogen is essential for protein and nucleic acid synthesis, growth was largely slowed down;
– phase III: nitrogen limitation phase. When nitrogen concentration in the broth was below 10 $mmol.L^{-1}$, nitrogen limitation was controlled by the NH_4OH solution inlet flow to maintain a specific growth rate at 0.05 h^{-1}. Glucose flow was adjusted to allow for both residual growth (according to ammonium feed rate) and lipid neo-synthesis demands. Phase III started when the NH_4OH flow reached a value limiting the specific growth rate to 0.05 h^{-1}.

2 RESULTS AND DISCUSSION

From *R. glutinis* fed-batch cultures on mineral medium with glucose and xylose/glucose mixture substrates, microbial kinetics were quantified as following to further understand the metabolic pathways involved during growth and lipid synthesis phases.

2.1 Growth Phase

2.1.1 Using Glucose as a Sole Carbon Source

With the appropriate exponential carbon source feed which permit to maintain the residual concentration of glucose lower than 0.01 $g.L^{-1}$, the specific growth rate was maintained at 0.28 h^{-1} first and then 0.36 h^{-1}. The carbon conversion yield into biomass was 0.64 $C_{mol\ X}.C_{mol\ glc}^{-1}$ and the catalytic specific glucose consumption rate was 0.43 $C_{mol\ glc}.C_{mol\ X*}^{-1}.h^{-1}$.

Moreover, carbon yield was stable; as a consequence, the macromolecular biomass composition was constant. In particular, during growth phase I, the lipid intracellular amount was constant and equal of 0.08 $g_{lip}.g_x^{-1}$ which is consistent with the literature values [26].

Thus, considering the experimental results and the bibliographic data about amino acid composition and macromolecular composition [27], the elementary biomass composition, corresponds to $CH_{1.605}O_{0.414}N_{0.186}P_{0.014}S_{0.009}$. Using these data, the energy yield was estimated at $Y_{ATP} = 12\ g_x.mol_{ATP}$.

2.1.2 Using Glucose and Xylose as Co-Substrates

Experimental results from fed-batch cultures under growth conditions using both glucose and xylose as substrates are reported in Figure 3.

During the initial phase from start to 10 hours, only glucose was consumed for the production of a biomass

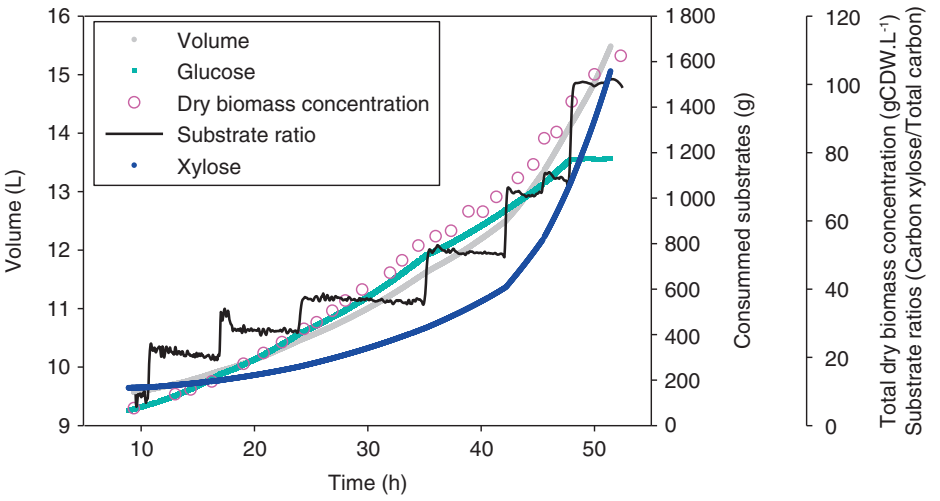

Figure 3

Total dry biomass concentration, substrates ratios, consumed substrates and reactor volume *versus* time during the culture of *Rhodotorula glutinis* in fed-batch mode, at 30°C, on mineral medium.

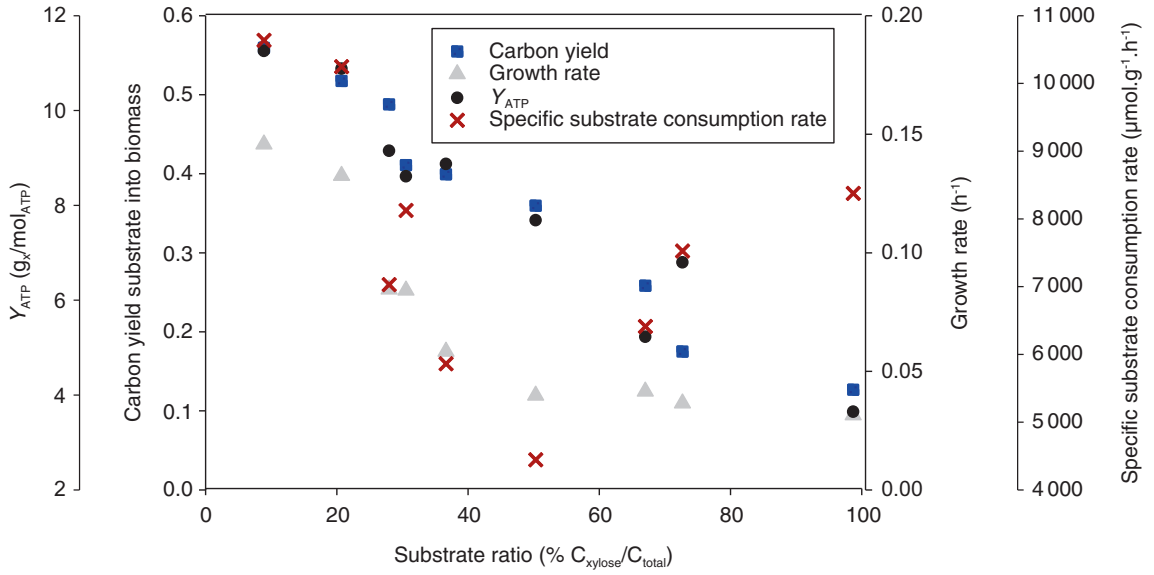

Figure 4

Growth yield, carbon yield, energetic yield and specific substrate consumption rate *versus* substrate ratio during the culture of *Rhodotorula glutinis* in fed-batch mode, at 30°C, on mineral medium.

concentration about 5 $g_{CDW}.L^{-1}$ suitable to study and quantify xylose metabolism phenomena.

During about 50 hours, 1 200 g glucose and 1 550 g xylose were consumed resulting in biomass production of 110 $g_{CDW}.L^{-1}$ (*Fig. 3*).

The duration of each step was long enough to ensure the total consumption of substrates, and to obtain stable metabolic behavior. Then, the growth rate, the carbon yield, the energetic yield (Y_{ATP}) and the total specific carbon flux were calculated for each substrate ratio (*Fig. 4*). These results showed the relevance of the culture conditions needed to ensure the simultaneous consumption of both substrates.

During this phase, the N/C ratio of the biomass composition decreased from 0.2 to 0.12, as the xylose ratio (xylose carbon/total carbon) increased from 0 to 100% which implies changes in macromolecular biomass composition. The elemental balances based on carbon, nitrogen, oxygen and hydrogen suggested intracellular accumulation of polysaccharides. Even if carbohydrate storage has been largely studied in *Saccharomyces cerevisiae* [28, 29], this metabolic behaviour has never been discussed previously in the literature in *R. glutinis*; therefore further investigations are needed and works are in progress.

Based on catalytic biomass – *i.e.* biomass composition without carbohydrate accumulation – growth rate decreased continuously from 0.015 h^{-1} to 0.0037 h^{-1} with an increasing fraction of xylose in the total carbon consumed. This tendency could be explained in terms of metabolism by a decrease in energy efficiency (Y_{ATP}).

When substrate ratio exceeded 50%, the specific substrate consumption rate increased while the growth rate decreased. As explained by the following equation:

$$q_s = \frac{\mu}{Y_{S,X}^{th}} + \frac{q_{polysacch}}{Y_{S,P}} + m \qquad (1)$$

with q_s: the specific substrate consumption rate, µ: the growth rate; $Y_{S,X}{}^{th}$: the theoretical substrate to biomass conversion yield; $q_{polysaccharide}$: the polysaccharide accumulation rate; $Y_{S,P}$: the substrate to product P conversion yield; µ: the specific growth rate of biomass without P, when the specific substrate consumption rate increased and the growth rate is maintained constant we can suppose a strong polysaccharide accumulation.

The coenzyme balance is essential for xylose assimilation [30]. Before entering the pentose phosphate pathway, xylose is assimilated *via* two reactions which correspond to a conversion of the reduced coenzymes NADPH, H^+ in NADH, H^+. The xylose catabolism leads to the production of glyceraldehyde-3-phosphate and fructose-6-phosphate. With glucose-6-phosphate isomerase fructose-6-phosphate can be reversibly

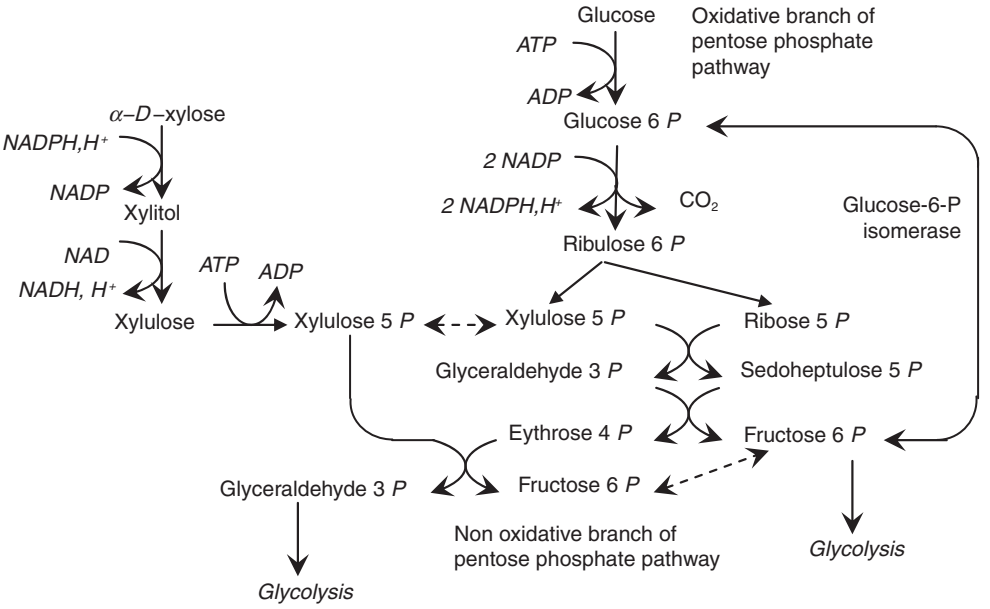

Figure 5

Cofactors and xylose metabolism in *Rhodotorula glutinis*.

converted into glucose-6-phosphate and thus join the glycolysis pathway. The oxidative branch of the pentose phosphate pathway allows NADPH, H^+ generation (*Fig. 5*).

When carbon excess occurred at the end of the culture (data not shown), xylitol was produced and accumulated in the fermentation broth with a maximum concentration of 14 $g.L^{-1}$. This phenomenon, never reported in the literature for *Rhodotorula glutinis*, is well known in *Candida sp*. [31]. Xylitol was also consumed when sugar supply was reduced. The xylitol production revealed that xylose assimilation was not limited by $NADPH_2$ generation but by its distribution into catabolism and anabolism. This strong demand of $NADPH_2$ implied significant recycling of the carbon from the non-oxidative branch of the pentose phosphate cycle. As a consequence, this phenomenon increased the pool of hexose phosphates, which could explain the accumulation of polysaccharides.

2.2 Lipid Accumulation

The transition phase (phase II) allowed to decrease nitrogen concentration in the broth; lipid accumulation started when the extracellular nitrogen concentration reached 10 mM (*Fig. 6*). The maximal glucose concentration was about 9 $g_{glc}.L^{-1}$ during phase II.

Growth rate decreased in a short time: specific growth rate was divided by two in two hours to match the set point. This trend could correspond to global intracellular nitrogen relocation (reduction of protein and nucleic acid pools). During Phase III, an exponential nitrogen flow corresponding to a growth rate of 0.05 h^{-1} was applied.

During phase III, the N/C ratio decreased from 0.2 to 0.03 $N_{mol}.C_{mol}{}^{-1}$.

Throughout the lipid synthesis phase, the glucose flux was successfully adjusted so that the glucose concentration in the broth did not exceed 6 $g.L^{-1}$ allowing the maximum conversion of carbon into lipid to be assessed.

These results combined a biomass concentration of 144 $g_{cdwx}.L^{-1}$ and a lipid content of 0.72 $g_{lip}.g_x^{-1}$. The global carbon yield of lipid accumulation using glucose as substrate was of 0.4 $C_{mol}.C_{mol}{}^{-1}$, which corresponds to 95% of the theoretical conversion yield of glucose into lipids calculated from stoichiometric reaction equations.

The lipid productivity of the culture depended on the duration of the different phases with a maximal value of 1.5 $g.L^{-1}.h^{-1}$. The best balance between substrate-to-lipid conversion yield and productivity during culture was obtained by triggering nitrogen limitation 20 h after the beginning of culture with an average specific growth rate of 0.28 h^{-1} during phase I and 0.05 h^{-1} during phase III.

Besides, in order to quantify limiting mechanism during lipid accumulation metabolism, the Relative Nitrogen Ratio (RNR) defined as the ratio between the specific consumption rates of nitrogen during the

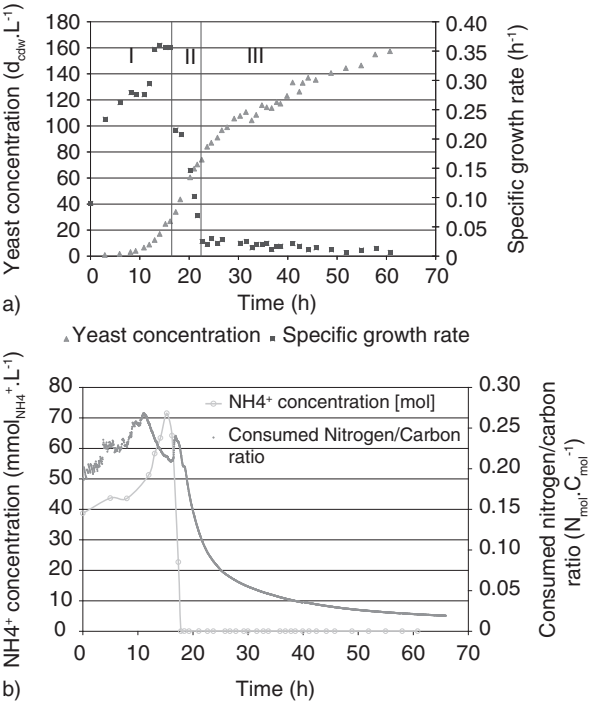

Figure 6

a) Biomass concentration [$g_X.L^{-1}$] and specific growth rate *versus* time during the culture of *Rhodotorula glutinis* in fed-batch mode, on glucose, at 30°C, on mineral medium. b) Ammonium ion concentration and N/C *versus* time during the culture of *Rhodotorula glutinis* in fed-batch mode, on glucose, at 30°C, on mineral medium.

production phase $q^N(t)$ relative to that measured in exponential growth phase ($q^N{}_{\max}$) was calculated according to the following equation:

$$RRN = q^N_{(t)}/q^N_{\max} \quad (2)$$

The evolution of the specific lipid production rate *versus* RNR was reported in Figure 7.

A maximum value of the specific lipid production rate of 0.035 $g.g^{-1}.h^{-1}$ was reached when RNR was equal to 0.018; it corresponded to a specific growth rate of 0.05 h^{-1}.

When the system is the cell, the mass balance equation of lipid accumulated is

$$\frac{dP^{in}}{dt} = \left(1 - P^{in}\right)\left(q_p * \left(1 - P^{in}\right) - \mu.P^{in}\right) \quad (3)$$

So, this equation suggests an asymptotic value of intracellular lipid accumulation of:

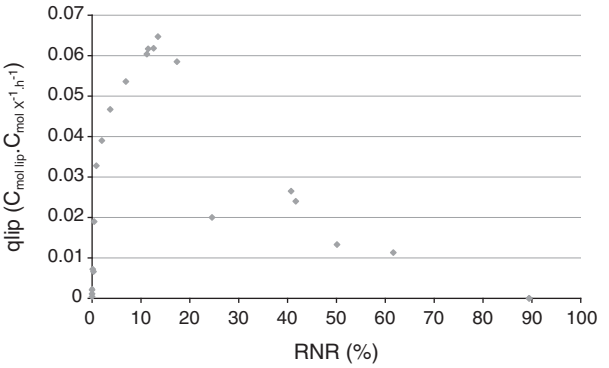

Figure 7

Lipid specific accumulation rate evolution *versus* Relative Nitrogen Ratio (RNR) during the culture of *Rhodotorula glutinis* in fed-batch mode, on glucose, at 30°C, on mineral medium.

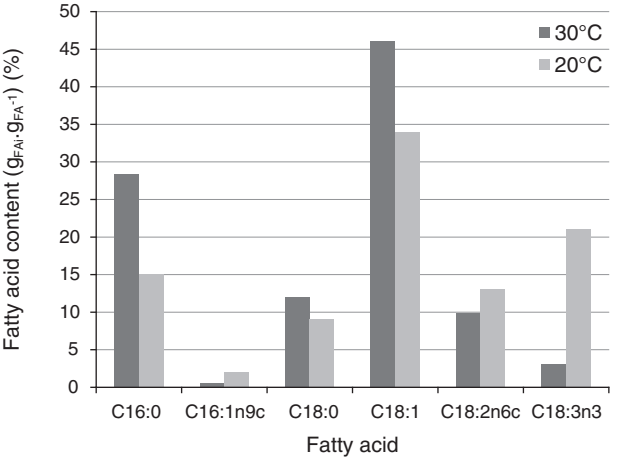

Figure 8

Accumulated fatty acid profile at the end of the lipid accumulation phase under two temperature conditions during the culture of *Rhodotorula glutinis* in fed-batch mode, on glucose, at 20°C and 30°C, on mineral medium.

$$\frac{dP^{in}}{dt} = \frac{q_S \cdot Y_{S,P}}{q_S \cdot Y_{S,P} + \mu} - \mu.P^{in} \quad (4)$$

Symbols are explained as detailed below:

- P^{in} corresponds to intracellular product P accumulation, for lipid, it was equal to $\%_{lip}$;
- q_s: specific substrate consumption rate;
- $Y_{S,P}$: substrate to product P conversion yield;
- μ: specific growth rate of biomass without P.

It obviously demonstrates that a residual growth rate has to be maintained to get this asymptotic value.

Fatty acids accumulated was mainly composed of oleic acid (C18:1; 0.46 $C_{mol\ C18:1}.C_{mol\ FA}{}^{-1}$) and palmitic acid (C16:0; 0.28 $C_{mol\ C16:0}.C_{mol\ FA}{}^{-1}$).

The impact of temperature on the accumulated fatty acid profile was also investigated and results relative to the end of the lipid accumulation phase are reported in Figure 8. When the culture temperature decreased from 30°C to 20°C, a decrease in the synthesis of palmitic acid (C16: 0) in favor of linoleic and linolenic acids (C18:2 and C18:3) was quantified. The specific rate of lipid production (data not shown) was significantly reduced by a factor 1.9 (0.25 $g.g^{-1}.h^{-1}$).

CONCLUSION

Fed-batch culture combined with stoichiometric model allowed to quantify co-consumption metabolism of *Rhodotorula glutinis* as it enabled substrate flow management. Thus, under limited carbon flux, co-consumption of both glucose and xylose without xylitol production was achieved for the first time in *R. glutinis*. The study of the dynamic behavior of the yeast highlighted:

– on glucose: a maximal growth rate of 0.36 h^{-1} and carbon-to-biomass conversion yield 0.64 $C_{mol\ X^*}.C_{mol\ glu}{}^{-1}$ for pure glucose;
– on glucose and xylose a decrease of growth rate from 0.15 h^{-1} for a 10% xylose ratio (xylose carbon/total feed carbon) to 0.037 h^{-1} for 100% xylose. A decrease of carbon-to-biomass conversion yield from 0.56 $C_{mol}.C_{mol}{}^{-1}$ for a 10% xylose ratio to 0.18 $C_{mol}.C_{mol}{}^{-1}$ for 100% xylose was quantified simultaneously.

In addition, from carbon mass balances, intracellular polysaccharides accumulation was observed under carbon excess and nitrogen limitation, probably due to enhanced $NADPH_2$ generation.

Finally, high lipid production performances were reached on glucose: 72% w/w of lipid with a volumetric productivity greater than 1.48 $g_{lip}.L^{-1}.h^{-1}$ and a biomass concentration close to 150 $g_{CDW}.L^{-1}$. The global lipid accumulation yield was 0.23 $g_{lip}.g_X{}^{-1}$.

The performance obtained here reached 95% of the theoretical conversion yield of glucose into lipids. This performance is the best reported until now in the literature. The lipids accumulated were mainly oleic acid and palmitic acid but the fatty acids accumulated can be modified especially by decreasing the culture temperature.

This work contributes to propose a bioprocess strategy combining biomass production using glucose and pentose and lipid production from glucose. The results are encouraging for the development of a biotechnological route for lipid production using renewable lignocellulosic resources, knowing that the lignocellulose feedstock is composed of 62 to 91% glucose, and 9 to 38% xylose, depending on the plant source [26]. As mentioned above, this lipid production route opens important opportunities for biojetfuel production when lipids are hydrotreated.

ACKNOWLEDGMENTS

Thanks for the financial support of the *CNRS*, *Airbus* and *EADS Industry* and thanks to Peter Winterton from the *University of Toulouse III* who helped to correct the English version of the manuscript.

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Manuscript accepted in April 2013
Published online in September 2013

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