Pichia pastoris a cell factory to produce recombinant lipases for enzymatic biodiesel production. Closing the circle.

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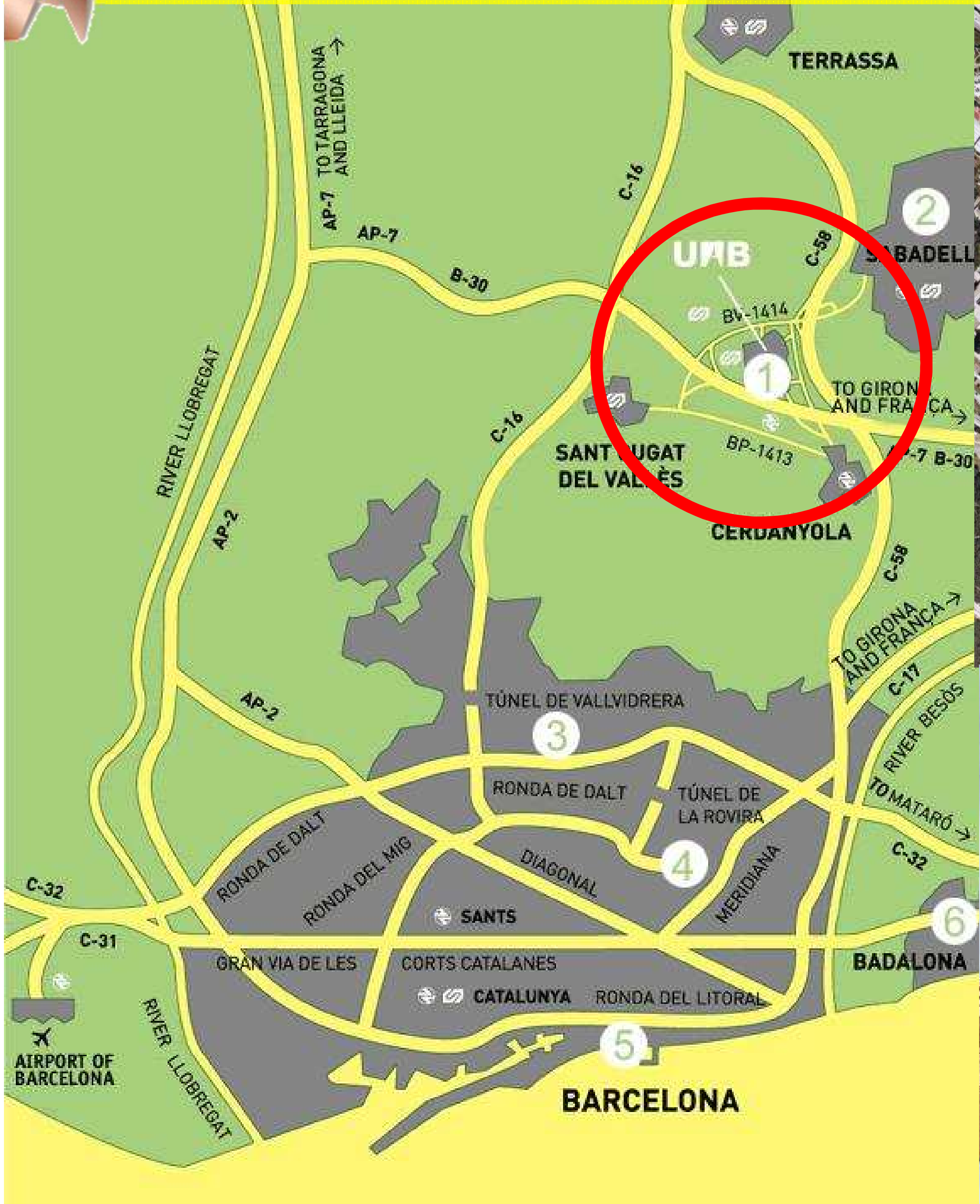
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BADALONA
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RONDA DEL LITORAL
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AIRPORT OF BARCELONA
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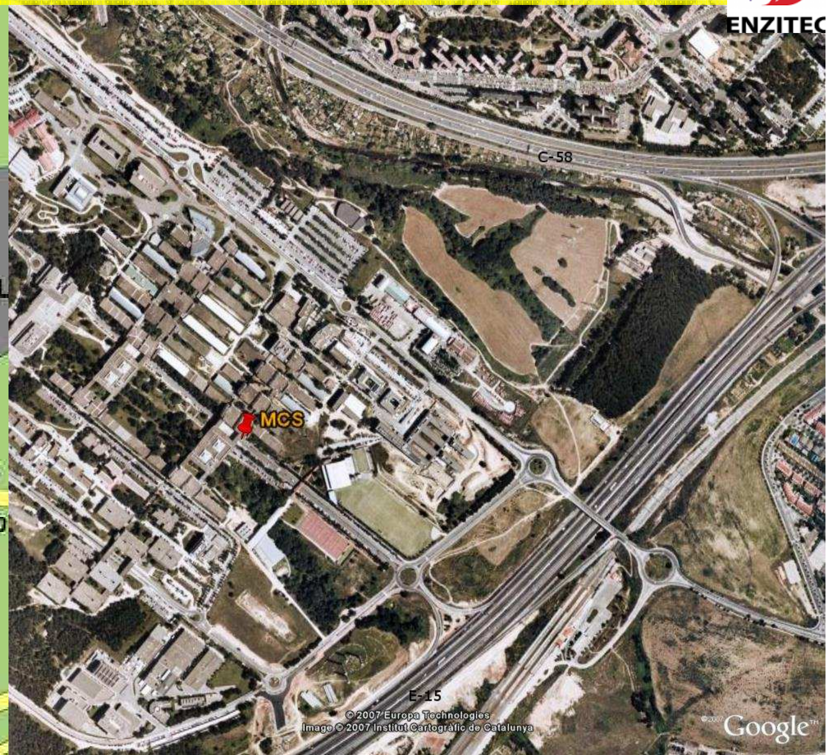
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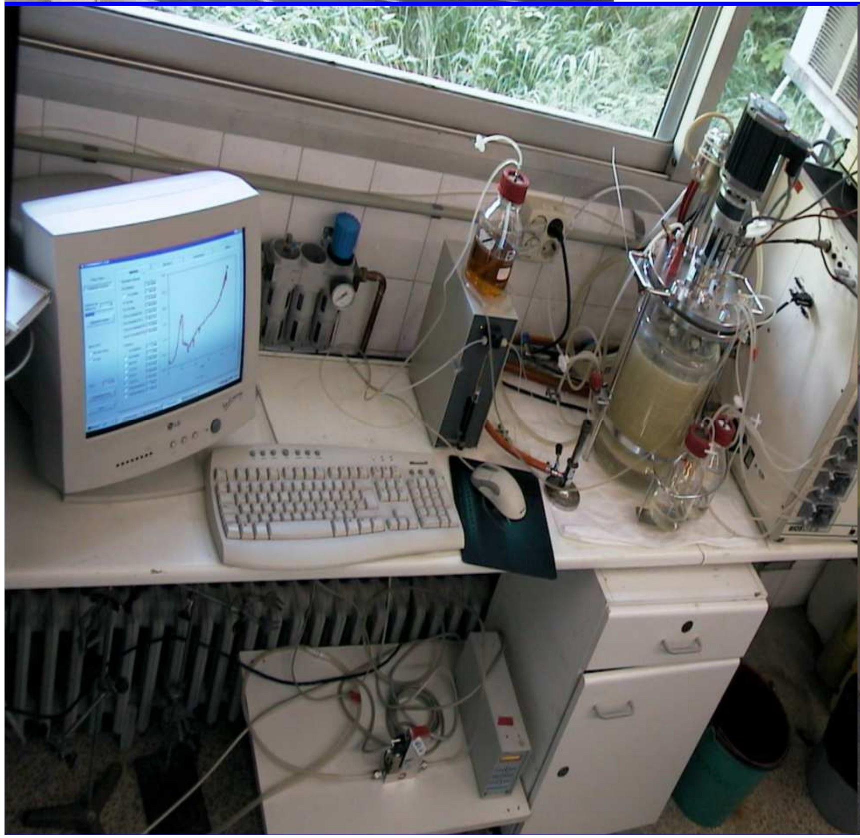
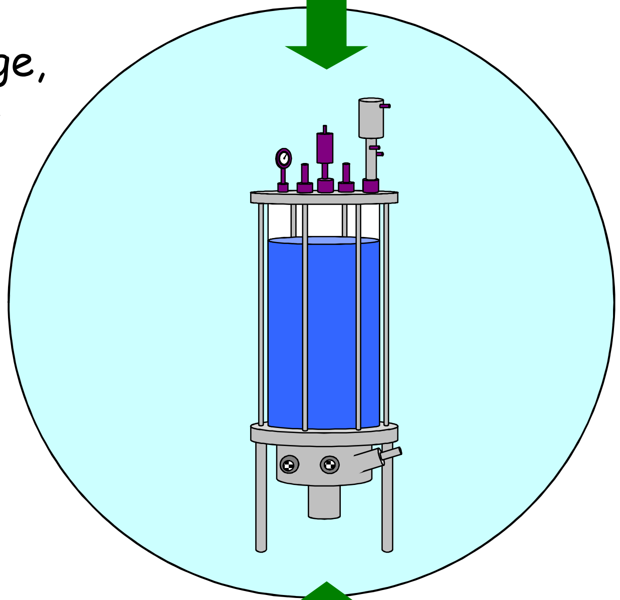



Development of recombinant protein production processes in *P. pastoris* using integrated strategies of:

Pichia pastoris



Genetic engineering: Combined use of genetic tools (codon usage, gene dosage, promoter, strains, etc)

➲ *integration with cultivation process development*

Quantitative physiology: analysis of physiological bottlenecks –metabolic flux analyses, transcriptomics, proteomics . Systems biology.

➲ *knowledge base for rational selection of cultivation conditions and metabolic engineering*

Monitoring, modelling and control of high cell density cultivation processes (software and hardware tools):

➲ *improved process performance and reproducibility, etc.*

Downstream processing

Scale-up

Applied Biocatalysis:

High quality product
Process robustness & reproducibility

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- Use of crude glycerol **with methanol** obtained from biodiesel industry to growth *Pichia pastoris* producing recombinant lipases from yeast

Glycerol

Lipases

Proof of concept

USE OF CRUDE GLYCEROL AS CARBON SOURCE FOR *Pichia pastoris* GROWTH. APPLICATION TO RECOMBINANT LIPASES PRODUCTION

GLYCEROL A

Soybean oil
Gly 61.2%
MeOH 0.3%
Bionet Europa

GLYCEROL B

Crude
Soybean oil
Gly 21.9%
MeOH 0.2%
Bionet Europa

GLYCEROL C

Commercial
99.6%
Panreac

GLYCEROL D

Crude
Animal fats
Gly 52.3%
MeOH 0.1%
Stocks del Vallés
S.A

GLYCEROL E

Loira
Soybean oil
Gly 84%
MeOH < 1000 ppm
NaCl 7 g/L
UFRJ

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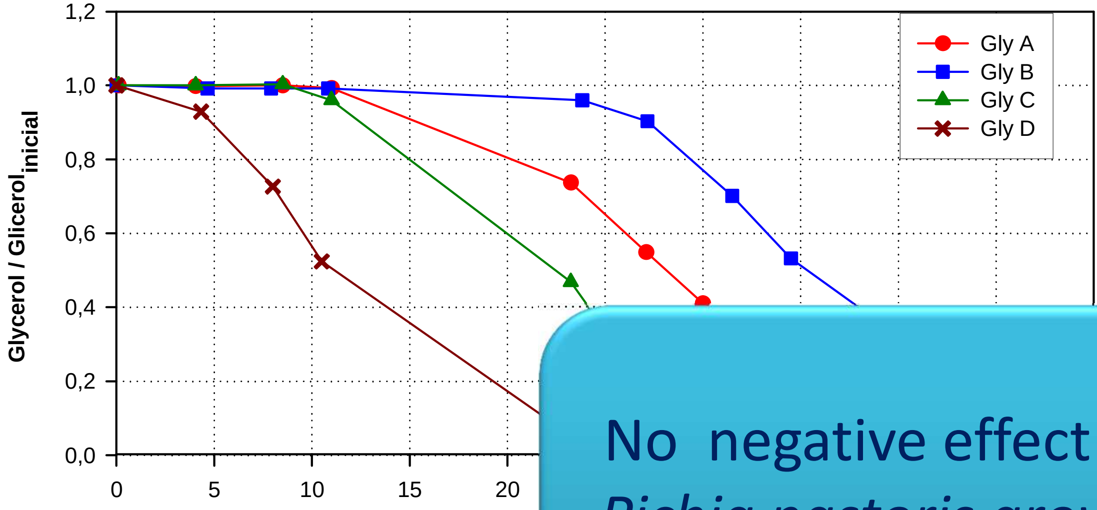
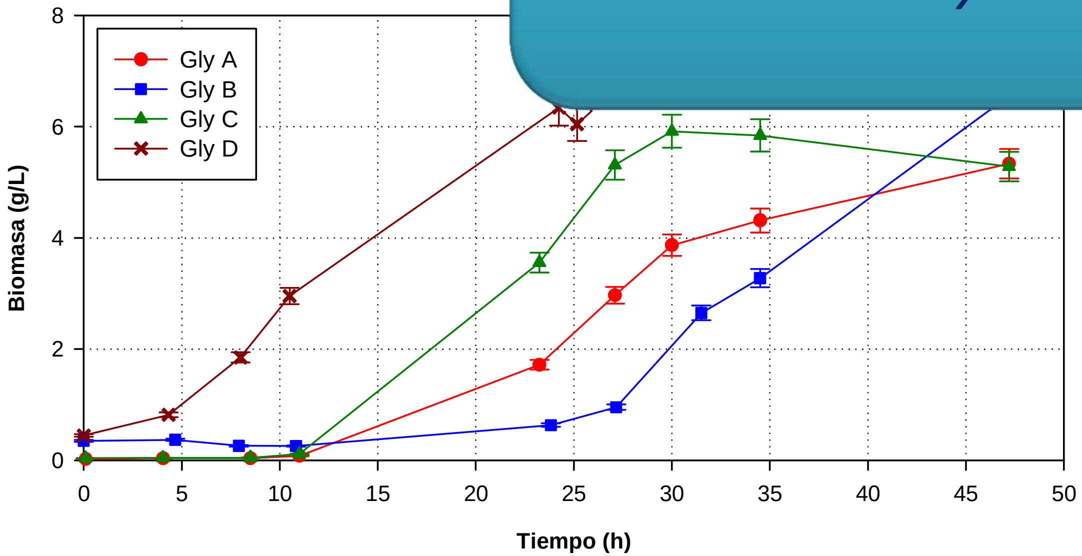
"Spanish" crude glycerol





$Y_{x/s}$ G_C = 0.45 g/g

$Y_{x/s}$ $G_{A,B,D}$ ≈ 0.5 g/g

No negative effect on *Pichia pastoris growth in erlenmeyer*

μ_C 0.18 h^{-1}

$\mu_{G\ A,B,D}$ 0.17 – 0.21 h^{-1}

RECOMBINANT EXPRESSION OF rLipB IN *Pichia pastoris*

LIPASE from *Candida antarctica B rLipB*:

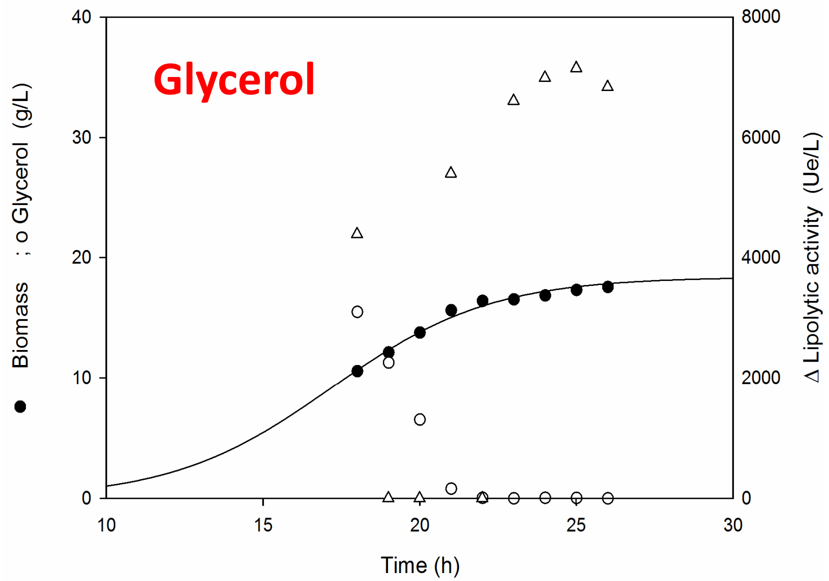
The most used lipase in applied biocatalysis. Industrial lipase supplied from Novo Nordisk

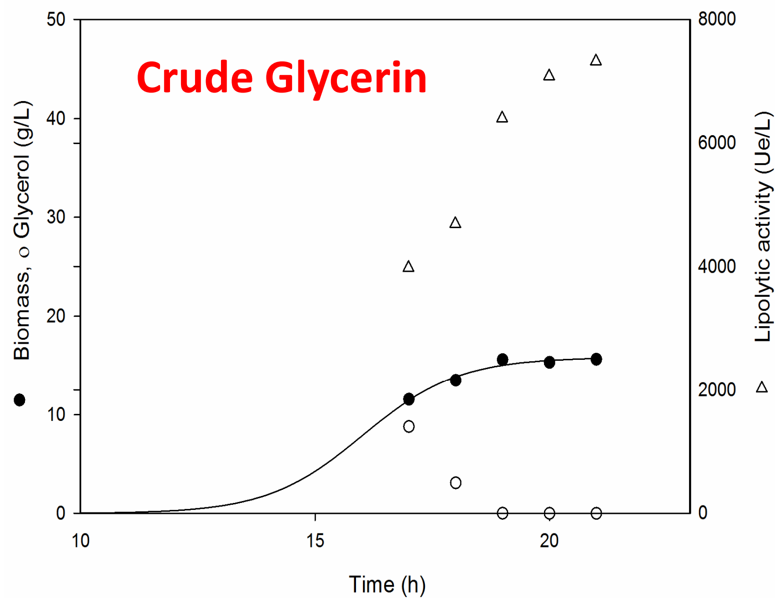
Institute of Chemistry UFRJ. J. Robert D. Freire

CALB CHARACTERISTICS:

33 kDa, pI $\geq$ 6

3 disulphide bond formation





Instituto de Química UFRJ

Batch fermentation producing rLipB

PGK constitutive promoter

	Crude glycerin	Glycerol
Yx/s (g/g)	0.46	0.46
Yp/x (U/g)	423,1	410.9
Yp/s (U/g)	192,6	189.4
Qp (U/g.h)	20.15	20.55
rLipB Activity (U/L)	6350	7108.6

No significant differences were observed

RECOMBINANT EXPRESSION OF ROL IN *Pichia pastoris*

LIPASE from *Rhizopus oryzae* (ROL):

Rhizopus oryzae (*R. arrhizus*) : filamentous fungi of high interest in Biotechnology and basic research.
Lipase for application in organic synthesis to obtain enantiomeric pure compounds, structured lipids, biodiesel...

ROL CHARACTERISTICS:

30 kDa, pI ≥ 9.3

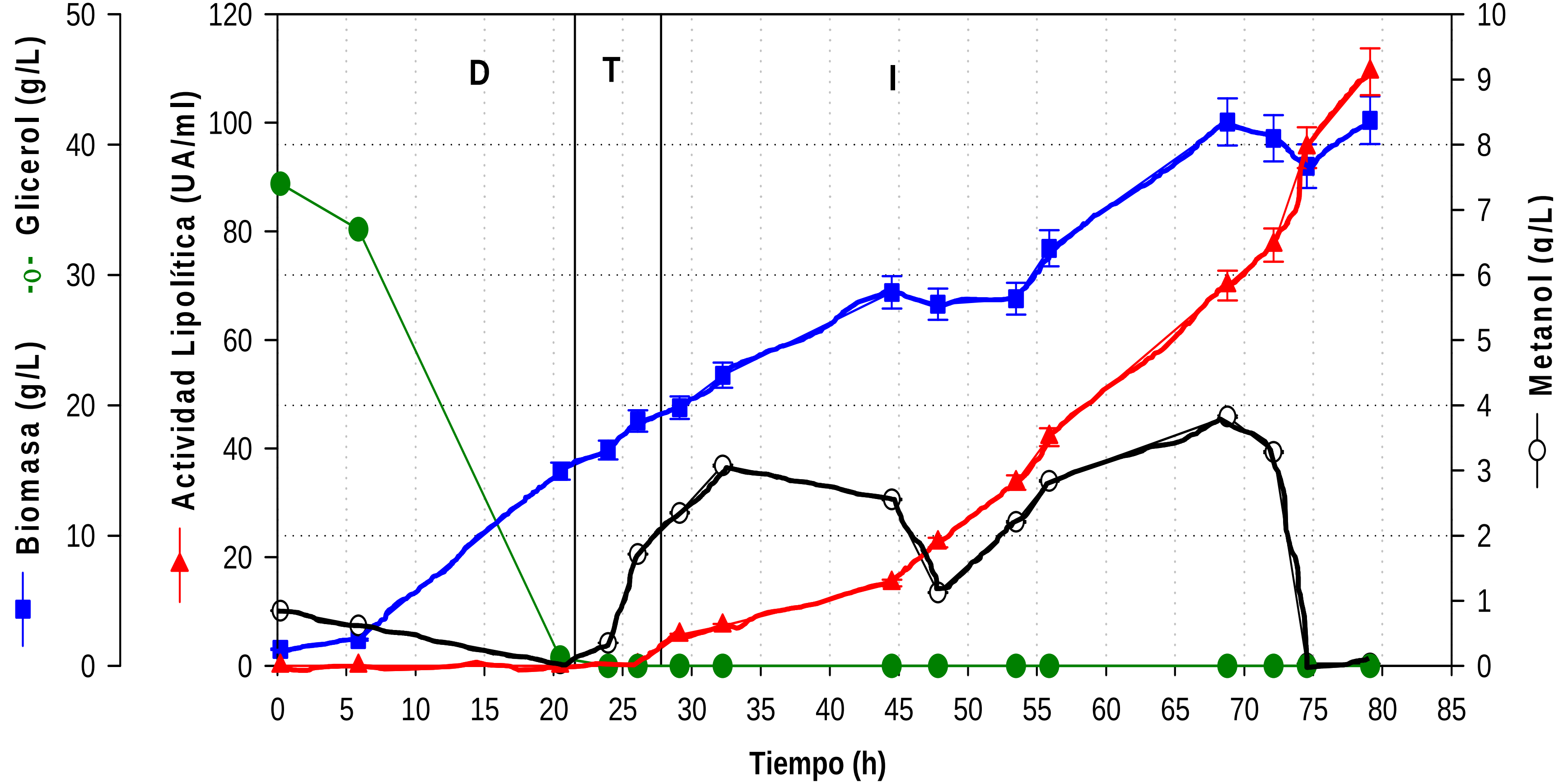
4 potential sites of N-glycosylation. 3 disulphide bond formation

Unfolding Protein response (UPR)

Fed batch strategy producing rROL under inducible AOX1 promoter

Act= 108 U/mL

μ_{med} = 0.024 h^{-1}



Fed batch strategy producing rROL

	Crude glycerol Methanol 2 g/L	Cos et al., 2005 Manual methanol control	Barrigón et al., 2013 Methanol 2 g/L
Actividad Máx. (U/mL)	108	150	103
$Y_{P/X}$ (U/g)	2567	2470	2004
Productividad (U/L·h)	1452	3000	2437
μ_{media} (h^{-1})	0.024	0.036	0.043
qs media (g/g·h)	0.11	0.14	0.19
qp media (U/g·h)	72	130	106

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BIOPROCESS ENGINEERING OF rROL PRODUCTION

ADVANTAGES of *Pichia pastoris* expression system

- Eukaryotic: post-translational modifications
- Secretion. easy downstream
- High cell cultures (> 100 g l^{-1})
- Non fermentative
- Easy genetic manipulation

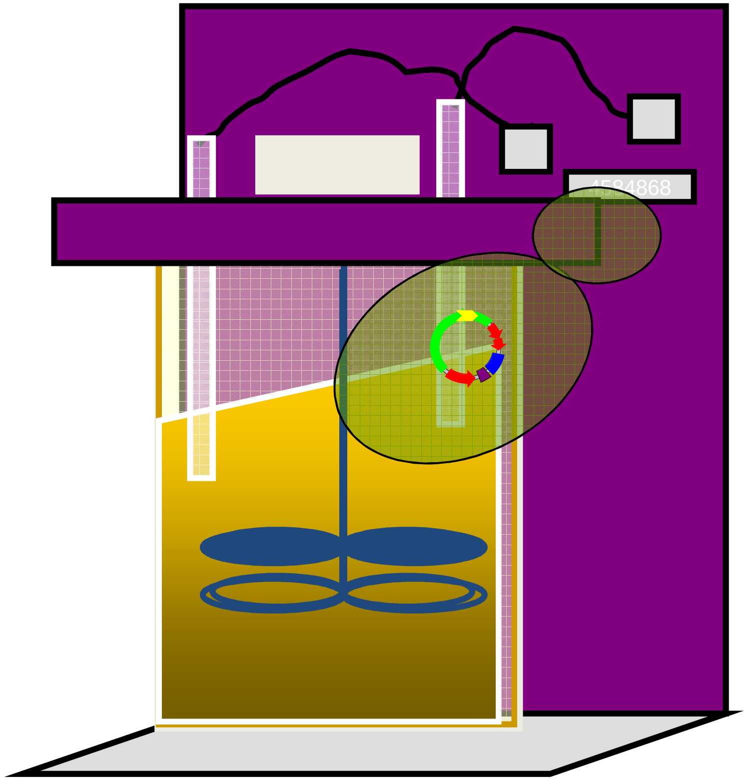
P_{AOX1}: STRONG AND REGULABLE

S. cerevisiae P. pastoris

1 OD_{600} 130 g dcw L^{-1} ~500 OD_{600}

Influent factors on expression

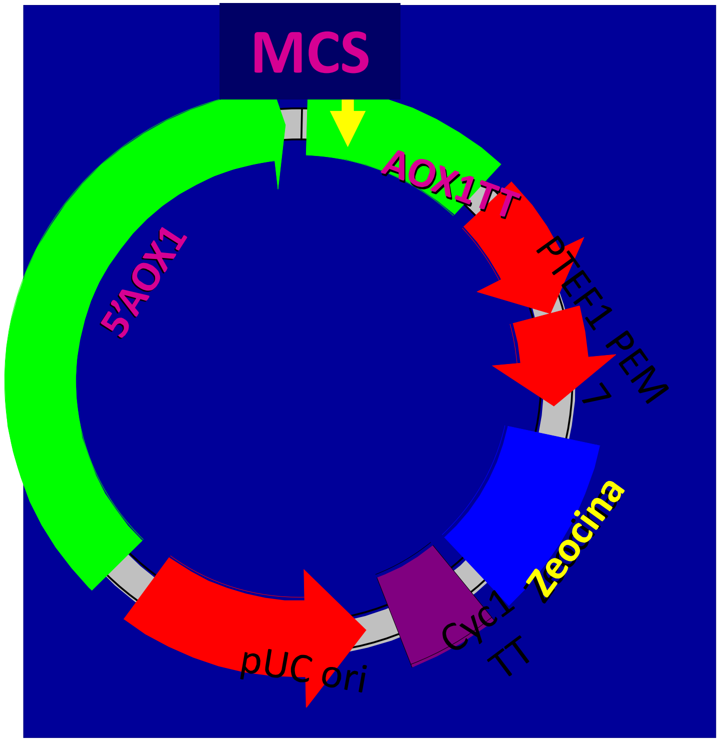
- Intrinsic characteristics of gen/protein.
- Phenotype Mut^{s}/Mut^{+}.
- Alternative promoters: GAP, PGK, FLD.
- Co-expression of other genes. Defective strains.
- Operational strategies.



Pichia pastoris: EXPRESSION SYSTEM

EXPRESSION SYSTEM based on P*AOX1*

2 GENES FOR THE SYNTHESIS OF AOX



AOX1	90%
AOX2	10%

PAOX1 | Gen AOX1

Functional AOX1 and *AOX2* → 100% AOX

Mut+ *(Methanol utilisation plus)*

PAOX1 | ~~Gen AOX1~~

Only functional *AOX2* → 10% AOX

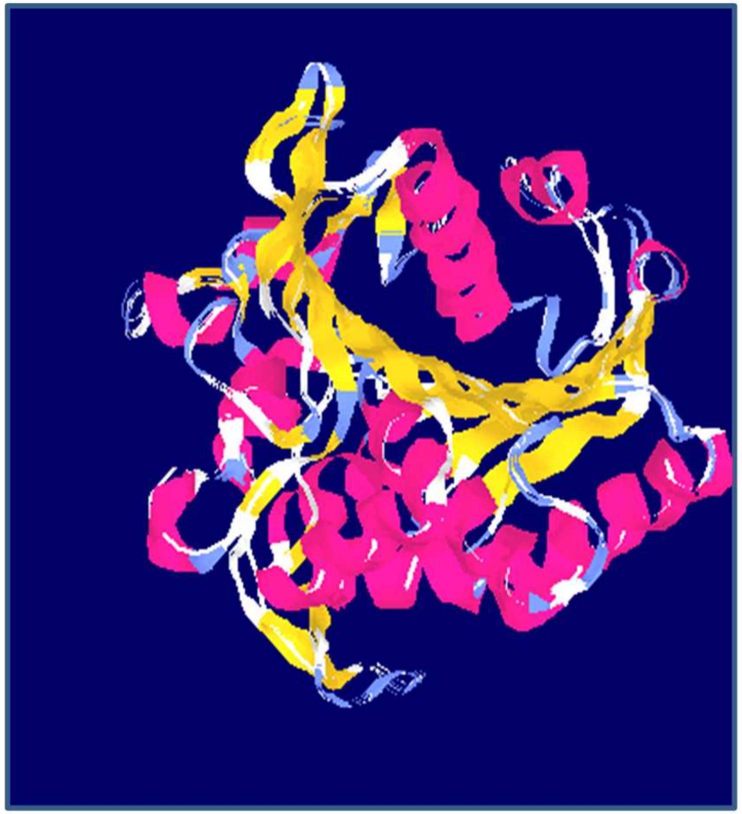
Mut^S *(Methanol utilisation slow)*

INFLUENCE OF ROL ON *Pichia pastoris* *SPECIFIC GROWTH RATE*

	μmax (h^{-1}) on Methanol	
	Mut^{+}	Mut^{s}
WILD STRAIN	0.15	0.05
ROL STRAIN	0.06	0.01

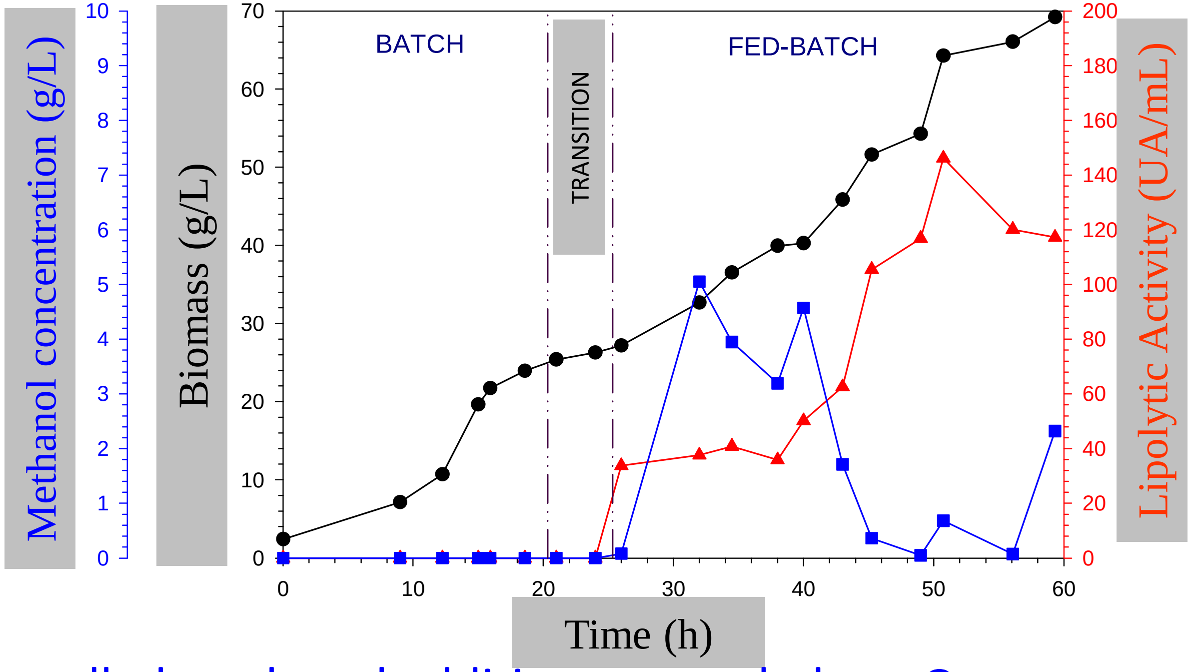
Important effect of heterologous production on *Pichia* growth

BIOPROCESS ENGINEERING OF RECOMBINANT EXPRESSION OF rROL IN *Pichia pastoris*

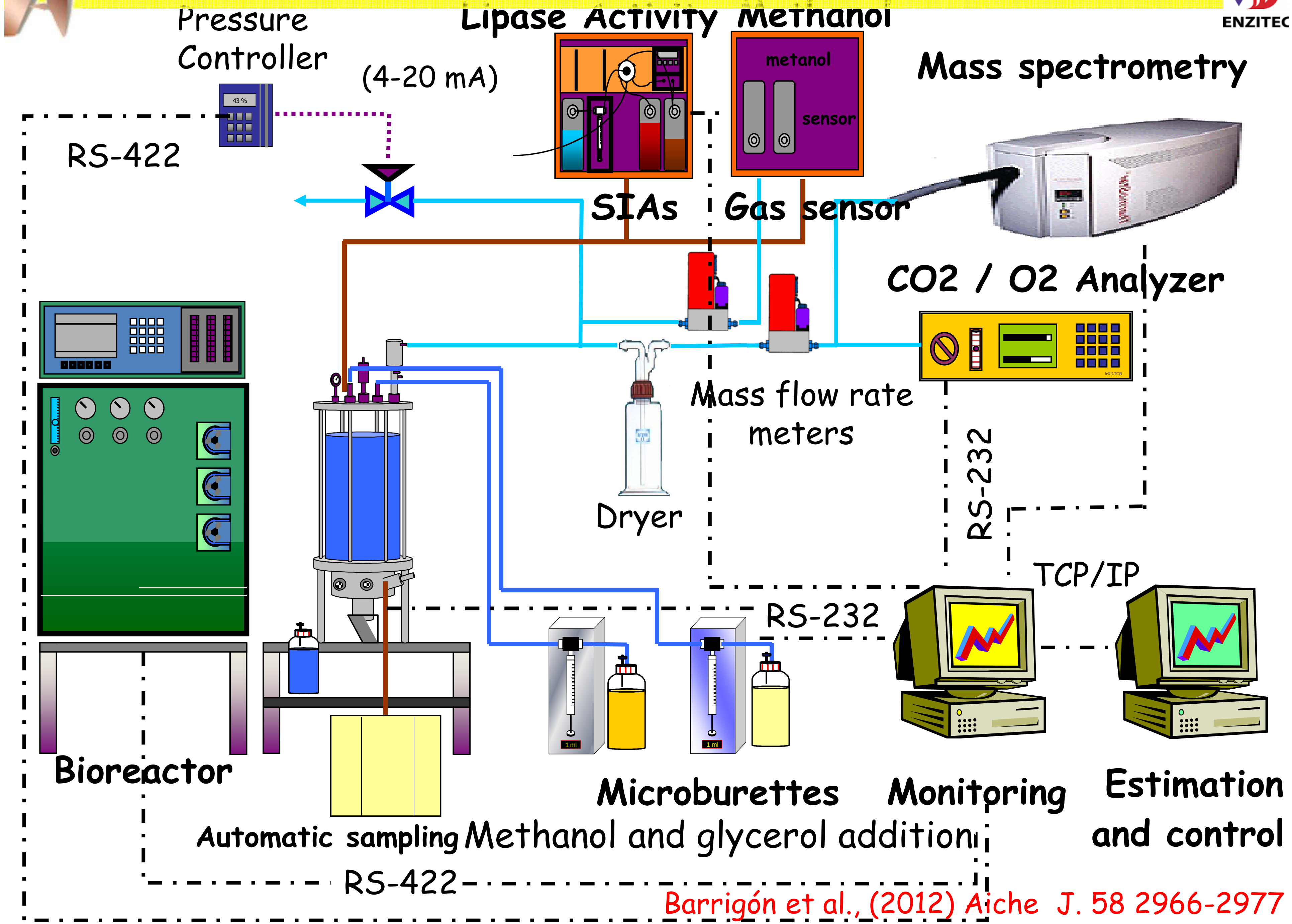


- Mut^{+} methanol as sole carbon source.
- Mut^{S} mixed substrates (sorbitol, glycerol).
- Fed-batch and continuous cultures.
- Different operational strategies. Methanol limited and non-limited, temperature limited cultures. Monitoring and control.
- *FLD* and *GAP* Promoter.
- Effect of co-expression of *HAC1* gene to minimize UPR phenomena.
- Knockout of the *P. pastoris* *GAS1* gene to increase cell porosity.

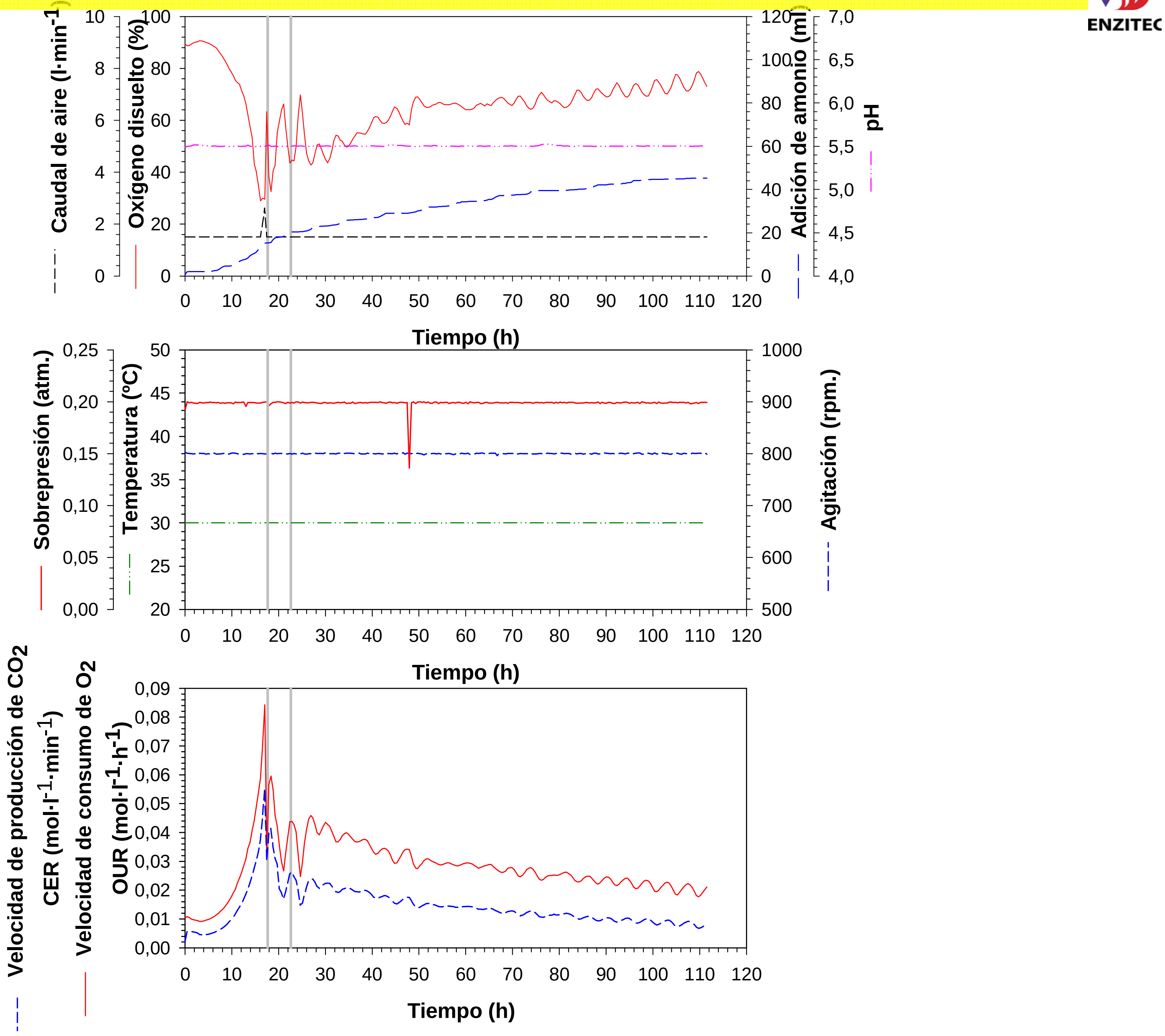
Pichia fermentation



- Non-controlled methanol addition or coupled to pO_2 measurement.
- Methanol feeding rate coupled to temperature.
- Methanol constant feeding rate.
- **Methanol open-loop control. Pre-programmed exponential feeding rate.**
- Methanol predictive control of oxygen or OTR.
- **Methanol predictive control. Constant methanol concentration.**



Barrigón et al., (2012) Aiche J. 58 2966-2977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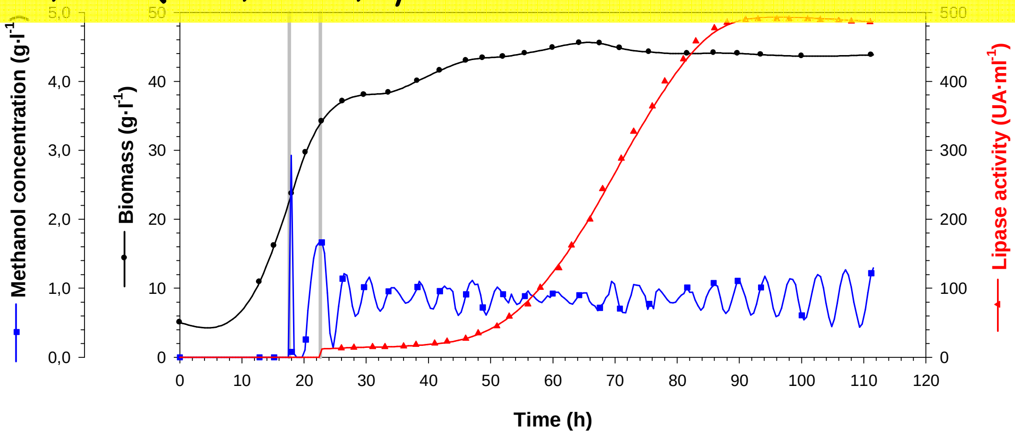
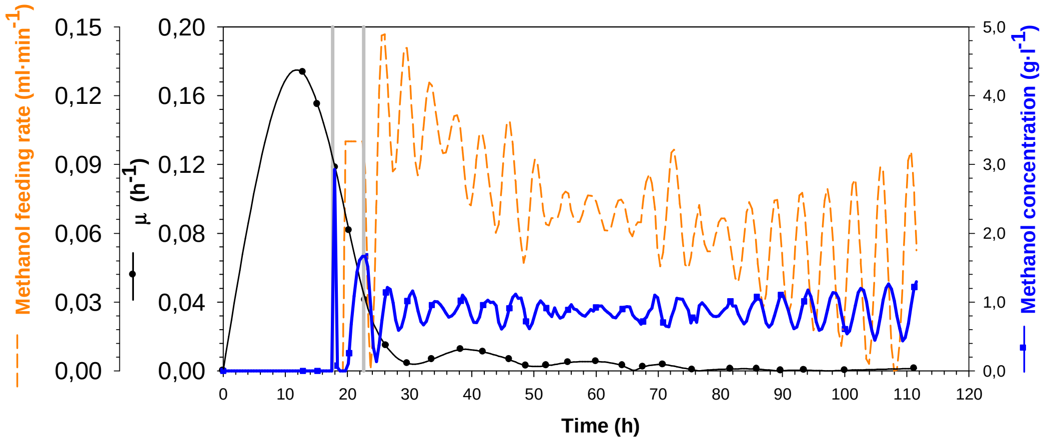
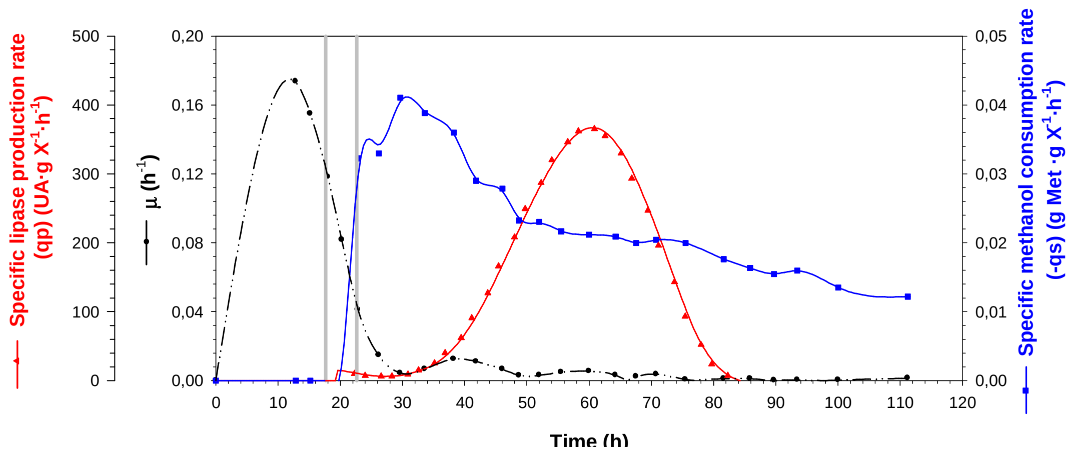
Caudal de aire ($l \cdot min^{-1}$)
Oxígeno disuelto (%)
Adición de amonio (ml)
pH
Tiempo (h)
Sobrepresión (atm.)
Temperatura (ºC)
Agitación (rpm.)
Tiempo (h)
Velocidad de producción de CO_2
CER ($mol \cdot l^{-1} \cdot min^{-1}$)
Velocidad de consumo de O_2
OUR ($mol \cdot l^{-1} \cdot h^{-1}$)
Tiempo (h)


Methanol concentration (g·l-1)
Biomass (g·l-1)
Lipase activity (UA·ml-1)
Time (h)


Methanol feeding rate (ml·min-1)
μ (h-1)
Methanol concentration (g·l-1)
Time (h)


Specific lipase production rate (qp) (UA·g X-1·h-1)
μ (h-1)
Specific methanol consumption rate (-qs) (g Met ·g X-1·h-1)
Time (h)

Mut+ phenotype

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Macrokinetic model of *Pichia pastoris* *Mut⁺ phenotype. Experimental*

Pre-programmed exponential feeding rate for methanol limited fed-batch cultures (MLFB).

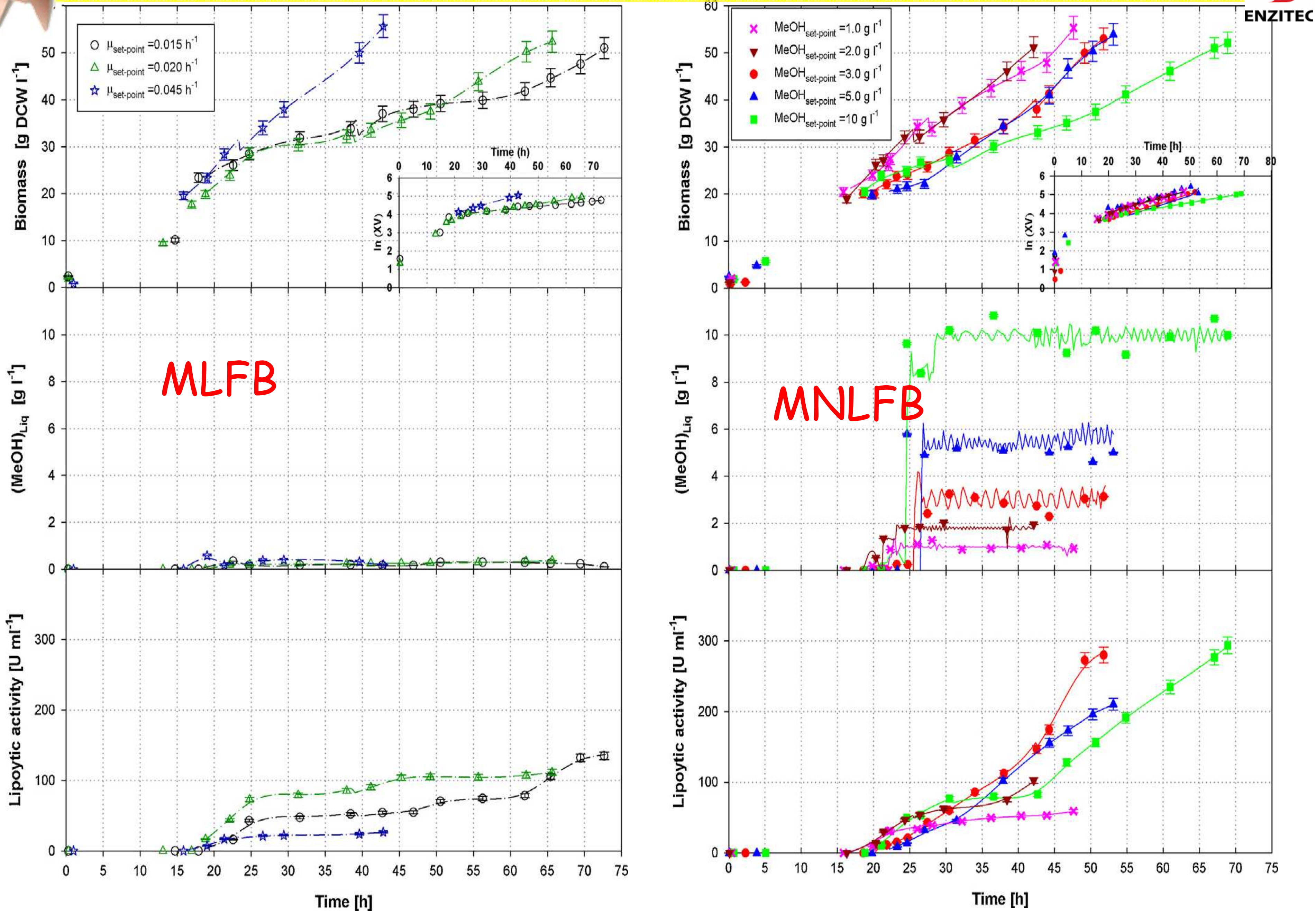
μ = 0.015 h^{-1} μ = 0.020 h^{-1} μ = 0.045 h^{-1}

Constant residual methanol control for methanol non limited cultures (MNLFB).

[MeOH] = 1 g L^{-1} [MeOH] = 2 g L^{-1} [MeOH] = 3 g L^{-1}

[MeOH] = 5 g L^{-1} [MeOH] = 10 g L^{-1}

Barrigón et al., (2013) Biochem. Eng. J. 75, 47-54



Barrigón et al., (2013) Biochem. Eng. J. 75, 47-54

Kinetic models of *Pichia pastoris Mut⁺ phenotype*. *Growth, consumption and production kinetics*

Monotonically increasing function. Monod model.

$$q_i = \frac{q_{max,i}\, S}{K_{s,i} + S}$$

q_i = i-specific rate.
$q_{max,i}$ = maximal value of i-specific rate.
$K_{S,i}$ = saturation constant.

Non-Monotonically increasing function. Haldane model. Substrate (methanol) inhibition.

$$q_i = \frac{q_{max,i}\, S}{K_{s,i} + S + \frac{S^2}{K_{I,i}}}$$

q_i = i-Specific rate.
$q_{max,i}$ = Maximal value of i-specific rate.
$K_{S,i}$ = Saturation constant.
$K_{I,i}$ = Inhibition constant

Barrigón et al., Biotechnol & Bioeng 12 (6), 1132–1145. (2015)

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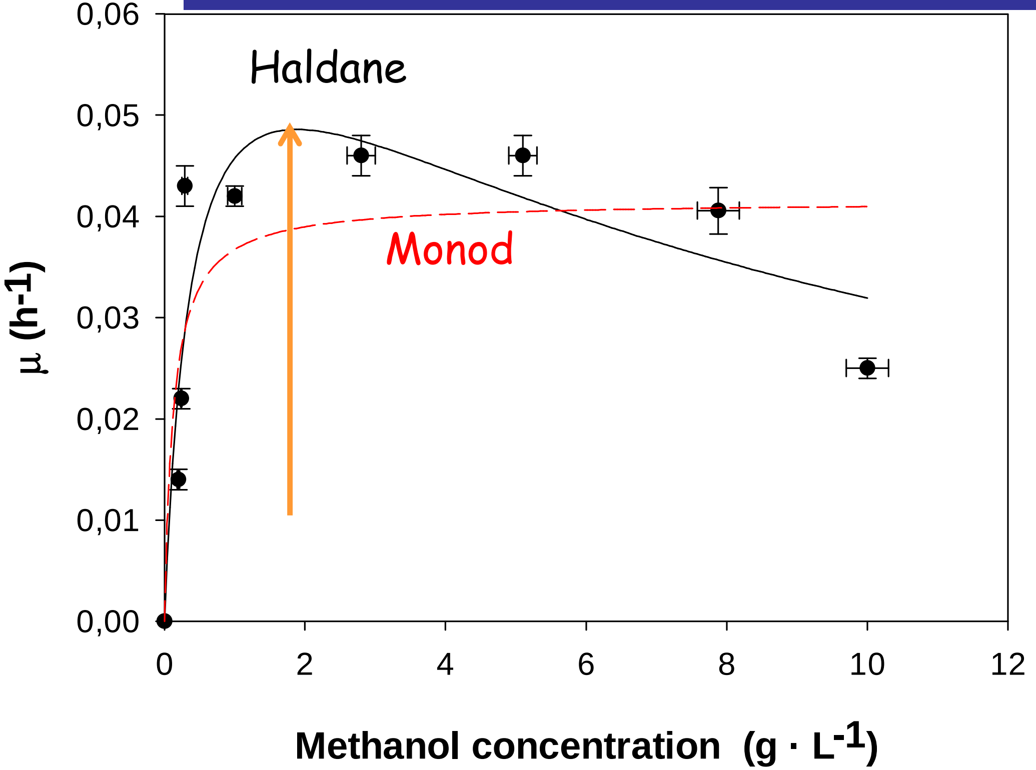
Kinetic models of *Pichia pastoris Mut+ phenotype.* *Cell growth kinetics*



Haldane
80% confidence level

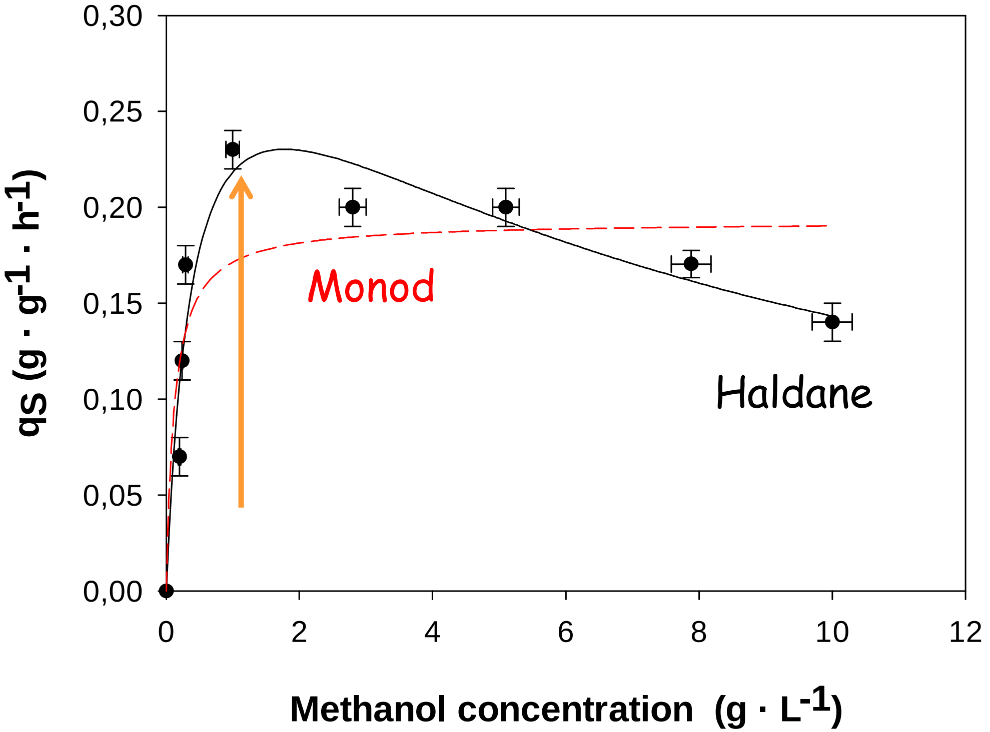
S_{crit} = maximal specific rate
1.9 g L^{-1}

Monod up to 2 g L^{-1}

S-non monotonically increasing function. Haldane

	Parameter	Units	Value	CV%	Statistics
q_X (μ) h^{-1}	μ_{max}	h^{-1}	0.069	33	R^2 = 0.81
	$K_{S,X}$	g L^{-1}	0.40	69.1	
	$K_{I,X}$	g L^{-1}	8.85	84.6	p = 0.006
	$S_{crit,X}$	g L^{-1}	1.9	---	

Kinetic models of *Pichia pastoris Mut⁺ phenotype.* *Substrate consumption kinetics*



Similar behaviour than growth kinetics, better statistics

Haldane
76% confidence level

Scrit = maximal specific rate
1.7 g L^{-1}

	S-non monotonically increasing function. Haldane				
	Parameter	Units	Value	CV%	Statistics
q_S g g^{-1} L^{-1}	$q_{max,S}$	g g^{-1} L^{-1}	0.34	21,6	R^2 = 0.76
	$K_{S,S}$	g L^{-1}	0.42	43.3	
	$K_{I,S}$	g L^{-1}	7.57	60.4	p = 0.003
	$S_{crit,S}$	g L^{-1}	1.7	---	

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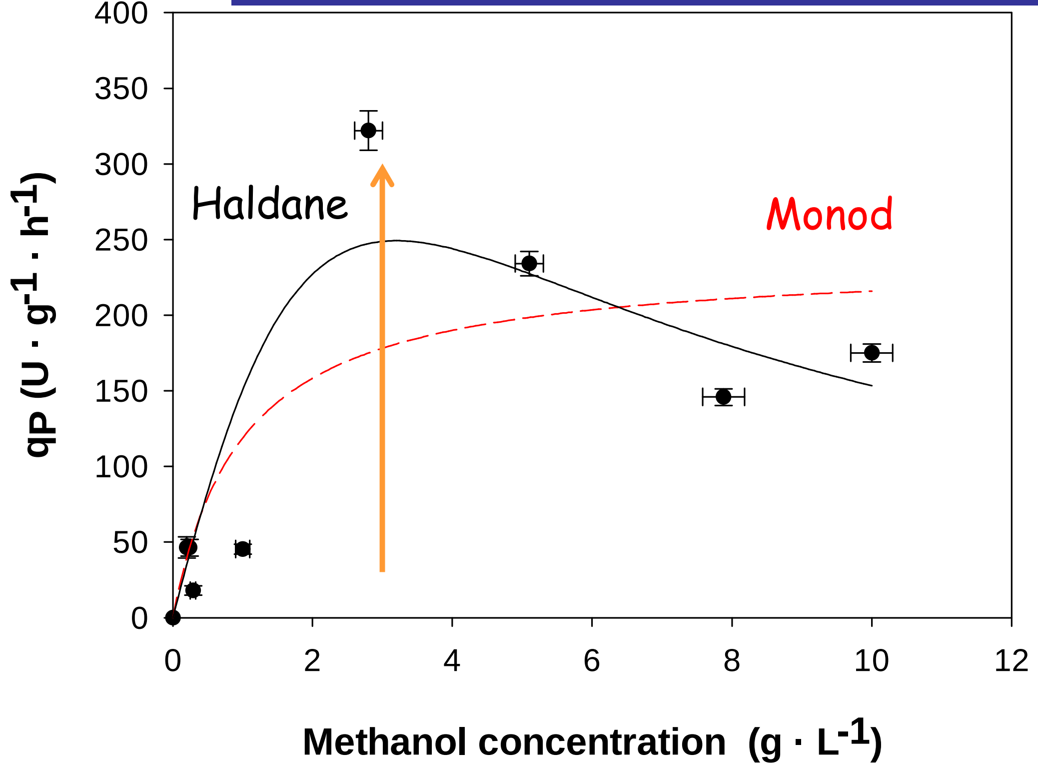
Kinetic models of *Pichia pastoris Mut⁺ phenotype.* *Product formation kinetics*



Similar behaviour but with the worst adjustment.

Haldane
64% confidence level

Scrit = maximal specific rate $3.2\ g\ L^{-1}$ higher than growth and substrate.

	S-non monotonically increasing function. Haldane				
	Parameter	Units	Value	CV%	Statistics
q_P $U\ g^{-1}\ L^{-1}$	$q_{max,P}$	$U\ g^{-1}\ L^{-1}$	1844	➢100	$R^2 = 0.64$
	$K_{S,P}$	$g\ L^{-1}$	10.2	➢100	
	$K_{I,P}$	$g\ L^{-1}$	1.0	➢100	$p = 0.010$
	$S_{crit,P}$	$g\ L^{-1}$	3.2	---	

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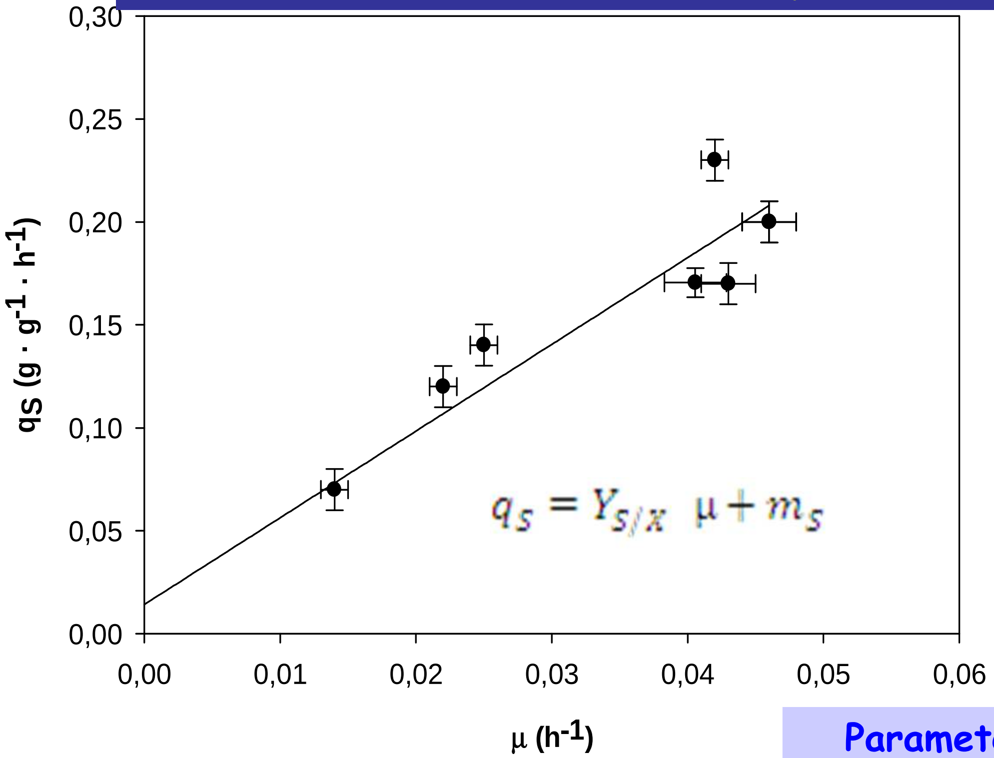
Specific substrate uptake model. Maintenance-energy Pirt's model

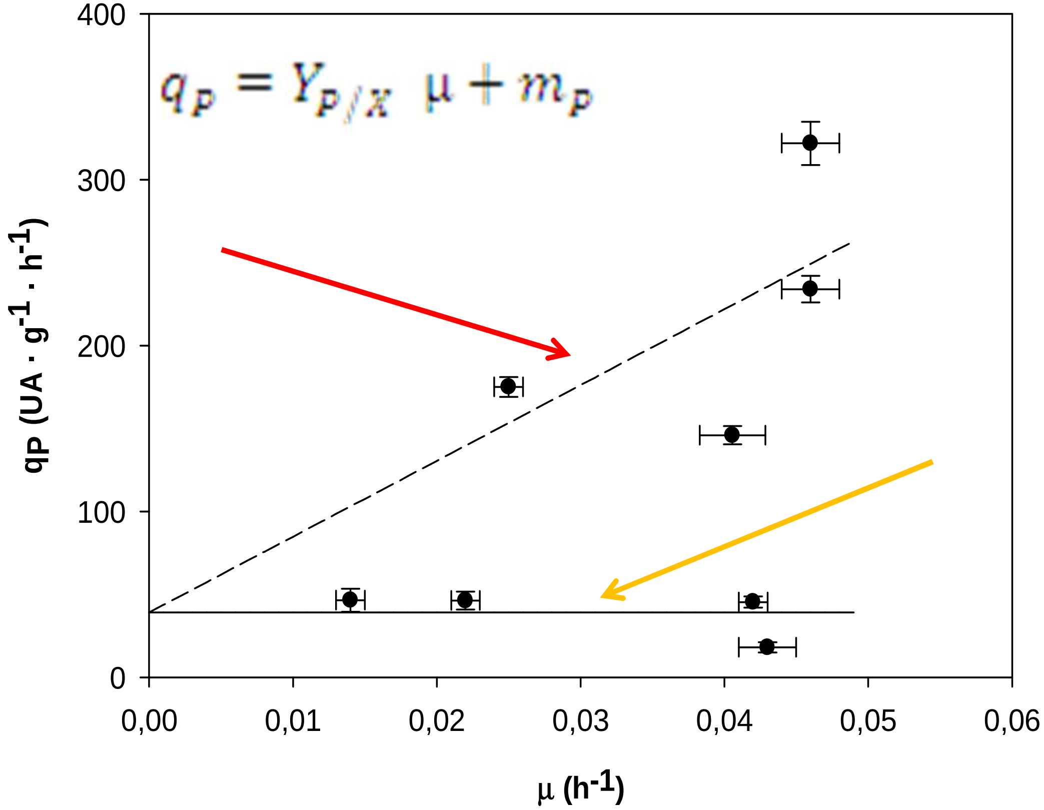


Pirt's model good correlation

Parameter	$q_s = g\ g^{-1}\ h^{-1}$		
	Units	Value	CV%
$Y_{S/X}$	$g\ g^{-1}$	4.21	11.6
m_S	$g\ g^{-1}\ h^{-1}$	0.0142	> 100
Statistical		R^2	p value
		0.92	< 0.001

Barrigón et al., Biotechnol & Bioeng 12 (6), 1132–1145. (2015)

Specific product model. Luedeking-Piret model



Luedeking-Piret 2 submodels:

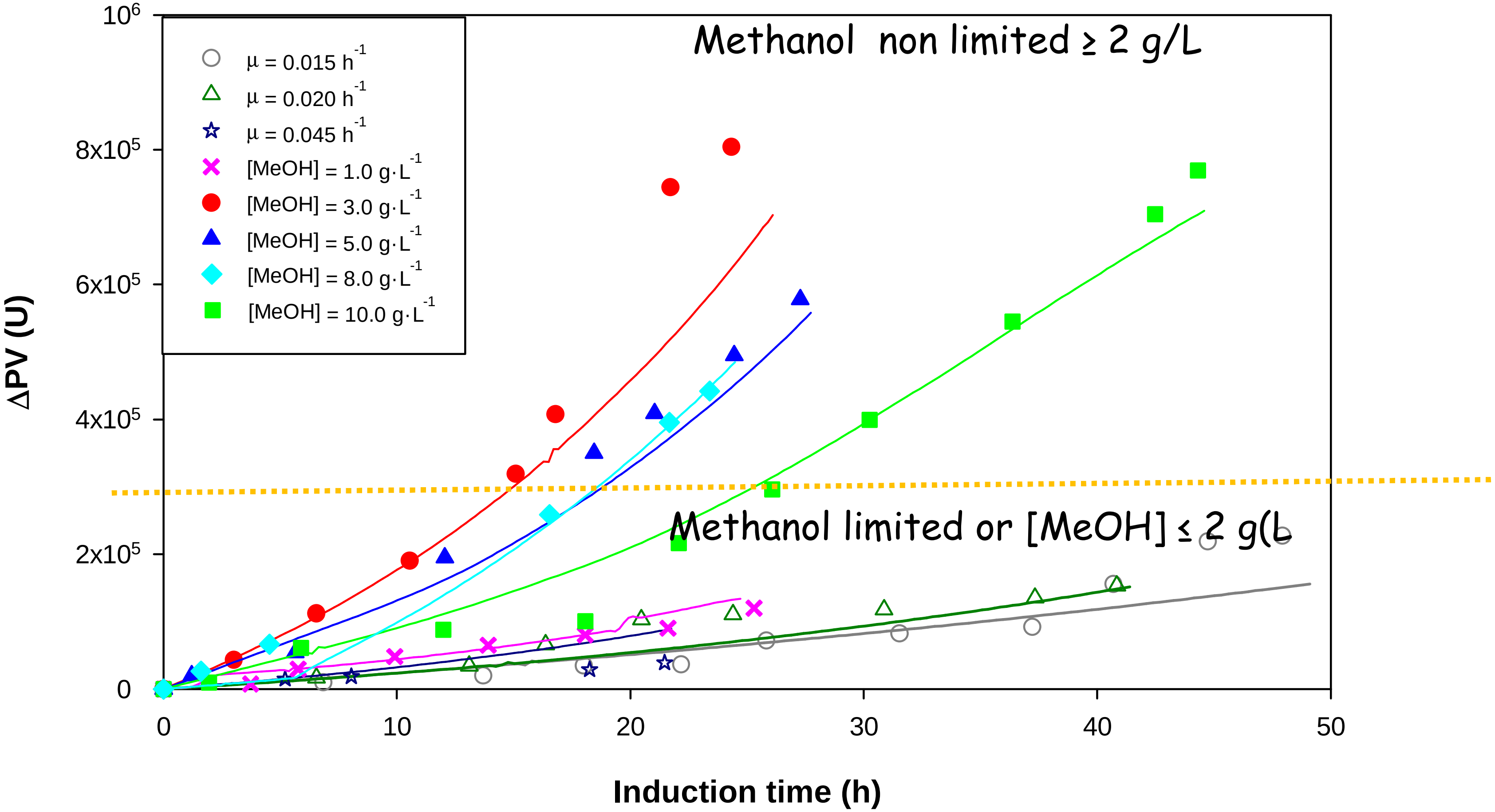
q_P constant for fed-batch cultures carried out at conditions lower than $S_{crit,X}$ (1.9 g L^{-1}) and $Y_{P/X} \approx 0$.

Over $S_{crit,X}$ q_P depending lineraly on μ

Parameter	q_P = U g^{-1} h^{-1}		
	Units	Value	CV%
$Y_{P/X}$	U g^{-1}	4.56	17.3
m_P	U g^{-1} h^{-1}	39.3	20.7
Statistical		R^2	p value
		0.92	$<$ 0.001

Barriaón et al.. Biotechnol & Bioeng 12 (6). 1132–1145. (2015)

Simulation and experimental state variables PV



Overall protein productivities estimated from mean slope are between $3.2 \cdot 10^3$ and $2.7 \cdot 10^4$ U h^{-1}

Barrigón et al., Biotechnol & Bioeng 12 (6), 1132–1145. (2015)

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Comparison cell growth kinetics

Reference	Protein	Model	μ_{max} h^{-1}	S crit g L^{-1}	K_S g L^{-1}	K_I g L^{-1}
Kobayashi et al., 2000	HA2	Haldane	0.154	3.05	< 0.2	---
Schenk et al., 2007&2008	Avidin	Haldane	0.139	$1 < S_{crit} < 6$	< 0.6	---
Curvers et al., 2002	hCRTB	Monod	0.084	$S_{crit} < 4$	0.22	---
Zhang et al., 2000	BoNT/ A(Hc)	Haldane	0.08	3.65	1.5	8.86
Jacobs et al., 2010	GlycoSwi tch-Man5	Haldane	0.063	2	---	---
Zhou and Zhang 2002	HV2	Haldane	0.046	3.09	1.35	7.08
Our work	ROL	Haldane	0.069	1.9	0.4	8.85

μ_{max} range 0.154 – 0.046 h^{-1}

S_{crit} range < 6; around 2-3 g L^{-1}

Target protein affect significantly to growth kinetics

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Comparison substrate consumption kinetics

Reference	Protein	Model	$Y_{S/X}$ g g^{-1}	m_S g g^{-1} h^{-1}	$Y_{overallS/X}$ g g^{-1} μ= 0.02h^{-1}
Kobayashi et al., 2000	HA2	μ-qS linear	2.57	0.0226	3.7
Schenk et al., 2007&2008	Avidin	μ-qS linear		-----	2.85
Curvers et al., 2002	hCRTB	μ-qS linear	3.53	0.0298	5.02
Zhang et al., 2000	BoNT/ A(Hc)	μ-qS linear	3.05	0.0160	3.85
Jacobs et al., 2010	GlycoSwitch-Man5	μ-qS linear		-----	3.53
Zhou and Zhang 2002	HV2	μ-qS linear		----	2.0*
Khatri and Hofmann 2006	scFV	μ-qS linear	4.17*	0.042*	6.27*
Pais et al., 2003	MPI	μ-qS linear	2.05	0.016	2.85
Our work	ROL	μ-qS linear	4.21	0.0142	4.92

* Conversion factor WCW-DCW =4.2

Different values function of target protein

$Y_{overallS/X}$ range

2.85 – 5.02 g g^{-1}

Maintenance coef.range

0.042-0-016

Barrigón et al., Biotechnol & Bioeng 12 (6), 1132–1145. (2015)

• CONCLUSIONS

MNLFB strategy better than MLFB strategy for ROL production. For other target protein?

Target protein affects significantly on kinetics of the bioprocess.

Exploitation of model B including estimation, control and optimization applications to be developed in the future.

Is it possible to know the effect of a target protein previous to cloning and its production in a bioprocess?. Up to day is not clear.

- **Higher oxygen consumption rate.**

- **Higher heat production rate.**

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Mut^s phenotype

PROMOTOR AOX

Mixed substrates strategy (methanol + ?)

Mut^s

↓

Carbon source

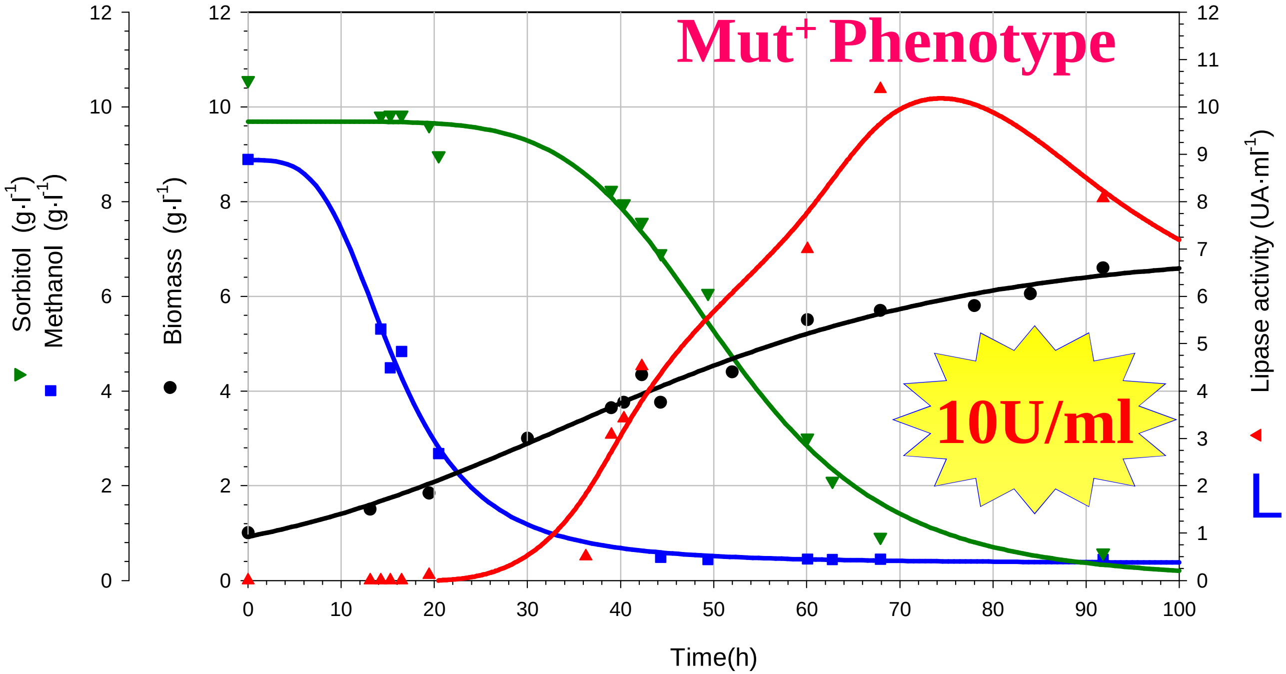
↙ ↘

Methanol → **Low growth**

Co-substrate

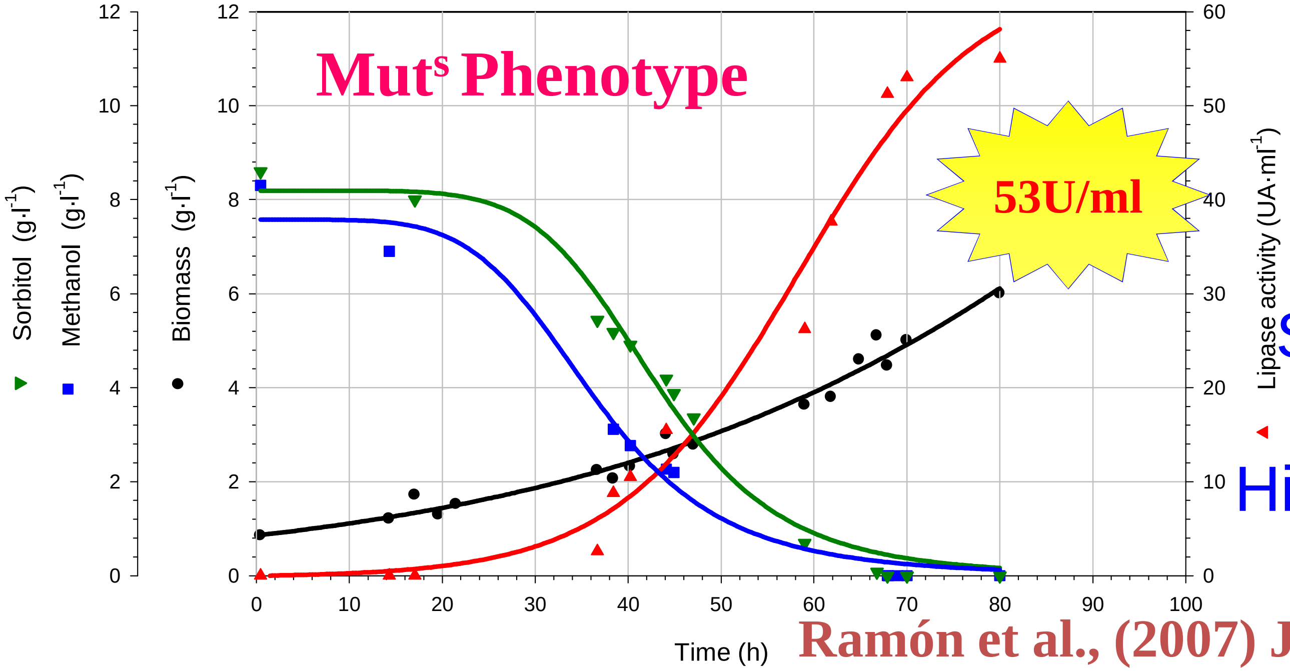
- **Glycerol** → **AOX repressor**
- **Sorbitol**

Mut+ versus Muts mixed substrates strategy



Mut+ phenotype
Sequential growth
First methanol

Low protein production



Muts phenotype
Simultaneous growth

High protein production

Ramón et al., (2007) J. Biotechnol. 130 (1), 39-46

Mut^+ versus Mut^s mixed substrates strategy

	Methanol Mut^+	Methanol + Sorbitol Mut^+	Methanol + Sorbitol Mut^s
Max. lipase activity* (U/ml)	6	10	53
$Y_{P/X*}$ (UA g^{-1} X)	1413	1825	10600
Productivity* (UA L^{-1} h^{-1})	201	153	757
Specific productivity (UA g^{-1}X h^{-1})	47	27	151

Ramón et al., (2007) J. Biotechnol. 130 (1), 39-46

Mixed substrates studies sorbitol

- ✓ Development of a new operational fed-batch strategy with mixed substrates.
- ✓ No on-line monitoring of sorbitol and glycerol.

Fed-batch operational strategy at constant μ by preprogrammed exponential feeding rate for sorbitol/glycerol and methanol concentration control

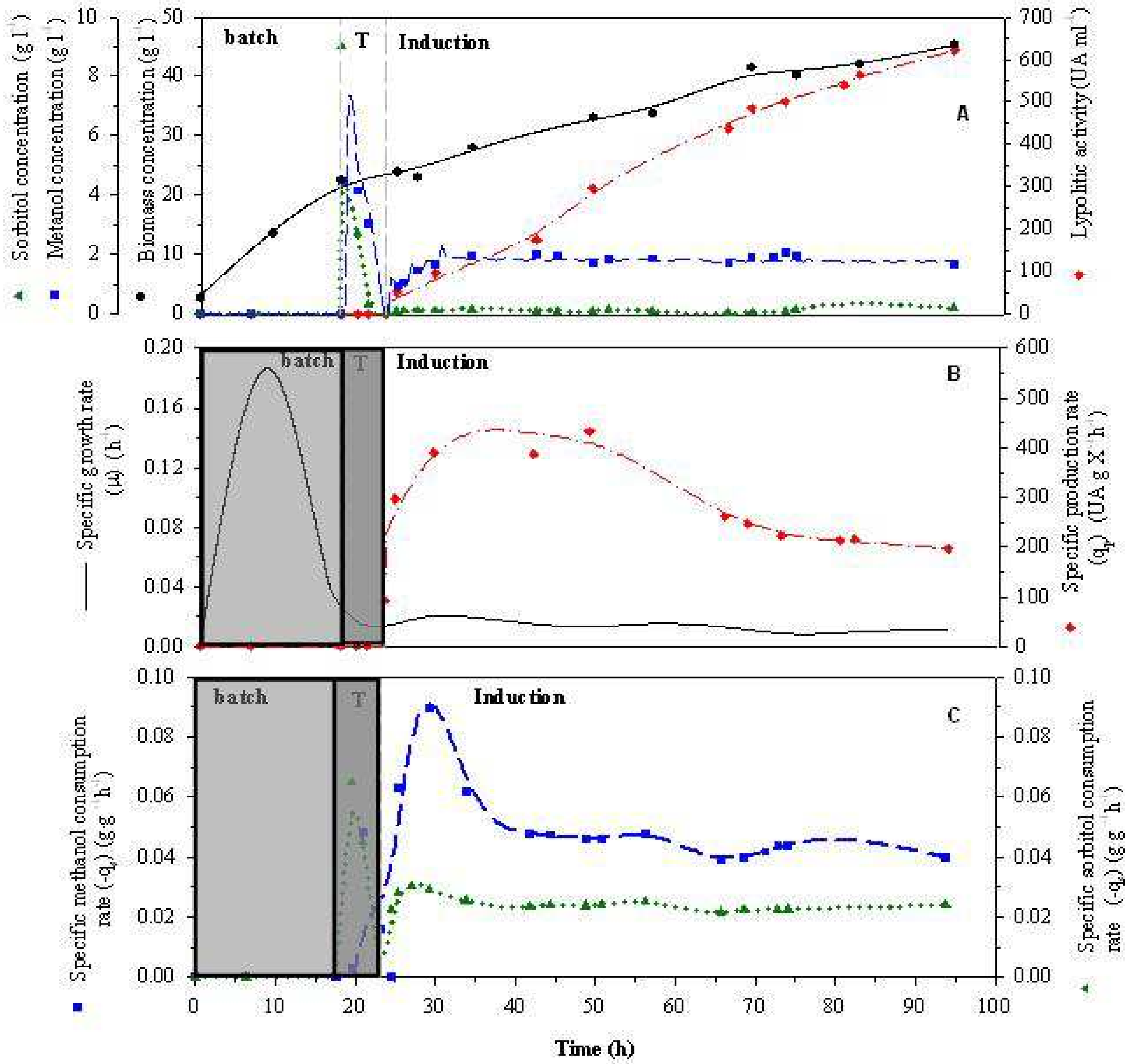
Mixed substrates studies sorbitol

Effect on growth, production yield and productivity of:

- ✓ **Methanol set-point concentration (0.5, 2 and 4 $g \cdot L^{-1}$)**
- ✓ **Specific growth rate (sorbitol) (0.01 and 0.02 h^{-1})**

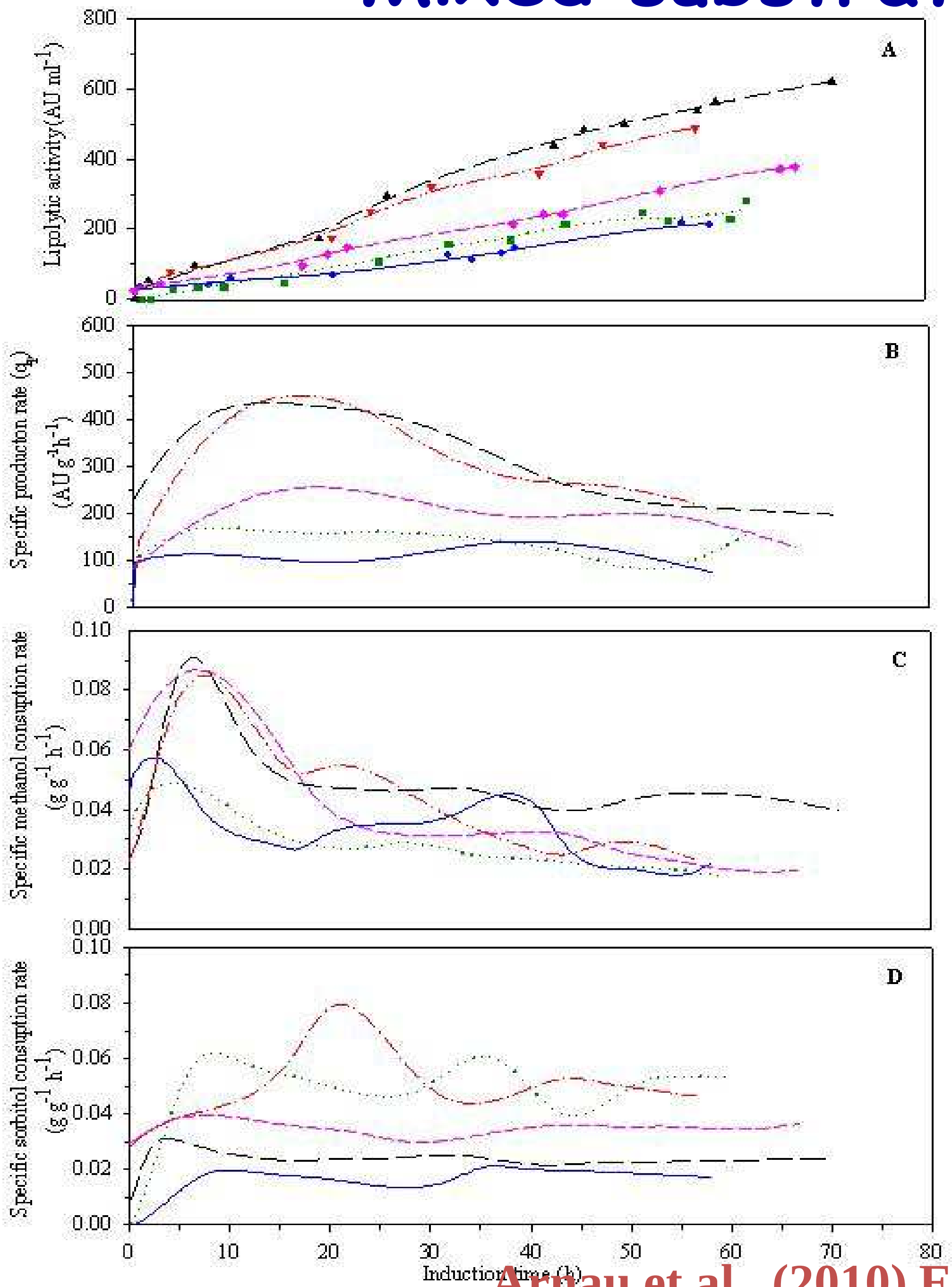
Mixed substrates studies sorbitol

Fed-batch fermentation
$\mu = 0.01\ h^{-1}$
$[MeOH] = 2\ g\ L^{-1}$



Arnau et al., (2010) Enzyme Microb. Technol. 46 (6), 494-500

Mixed substrates studies sorbitol



✓No influence of sorbitol growth rate.

✓Key parameter [methanol].

✓ Needs to optimize methanol.

✓Inhibitory effect of high [MeOH].

✓ High fermentation time.

✓ $\mu = 0.01\text{-}0.02\ h^{-1}$

μmax = 0.02 h^{-1}; [MeOH] = 2 g L^{-1}

μmax = 0.01 h^{-1}; [MeOH] = 2 g L^{-1}

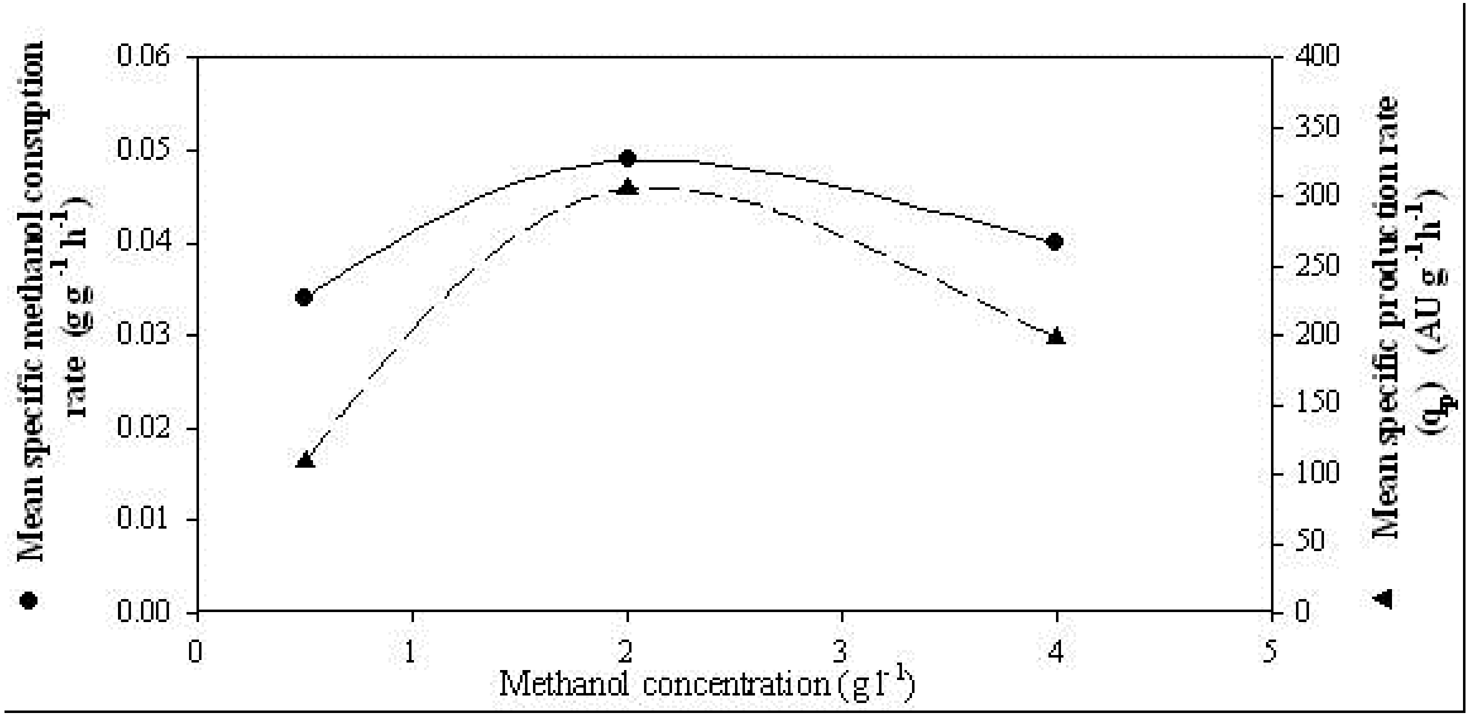
μmax = 0.02 h^{-1}; [MeOH] = 0.5 g L^{-1}

μmax = 0.01 h^{-1}; [MeOH] = 0.5 g L^{-1}

μmax = 0.01 h^{-1}; [MeOH] = 4 g L^{-1}

Arnau et al., (2010) Enzyme Microb. Technol. 46 (6), 494-500

Mixed substrates studies sorbitol



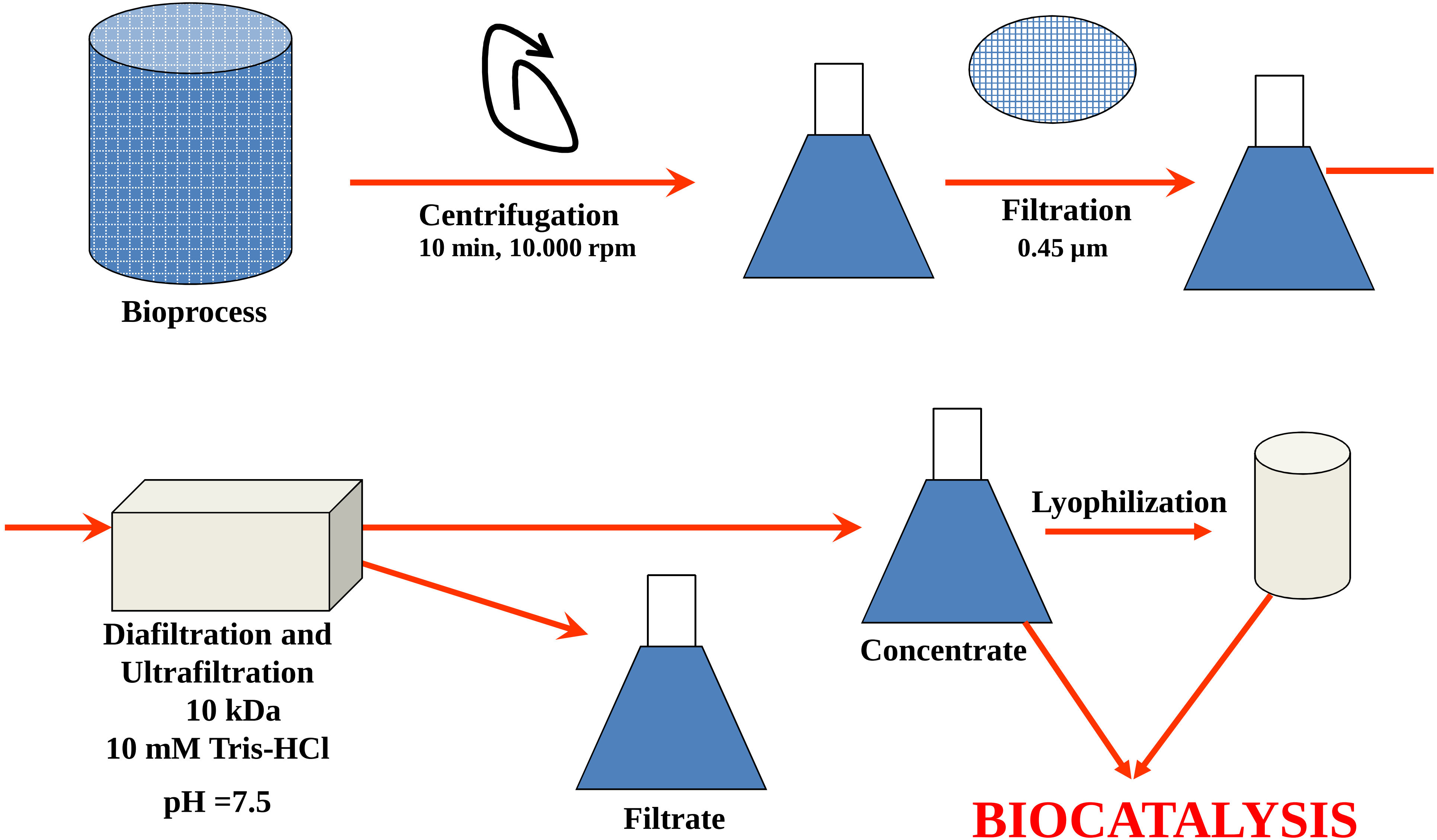
Optimized methanol concentration 2 g L^{-1}

Arnau et al., (2010) Enzyme Microb. Technol. 46 (6), 494-500

Comparison between strategies

	Metanol 1 g/L Mut^s	Metanol 2,5 g/L Mut^+	Met-Sor 2 g/l Mut+	New Strategy ?
Actividad máxima* (U/ml)	490	410	488	
$Y_{P/X*}$ (UA g^{-1} X)	11236	6120	10382	
Productividad* (UA L^{-1} h^{-1})	4901	5857	6421	

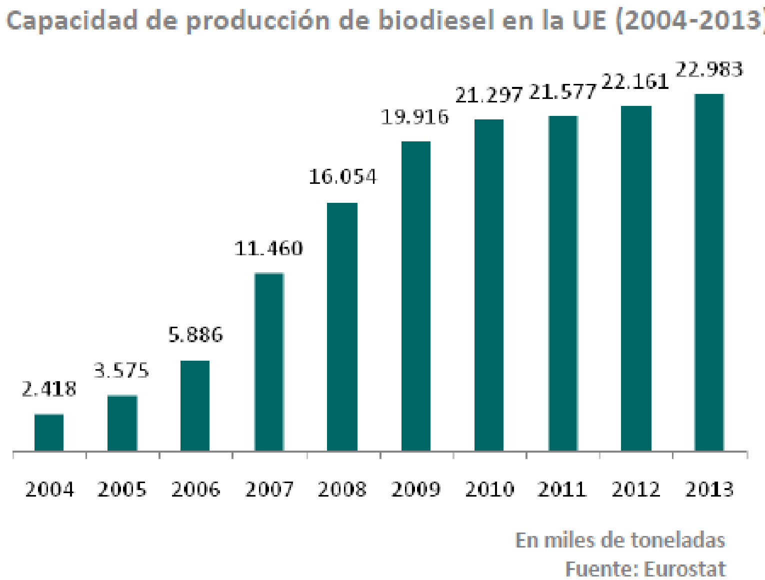
Downstream of ROL

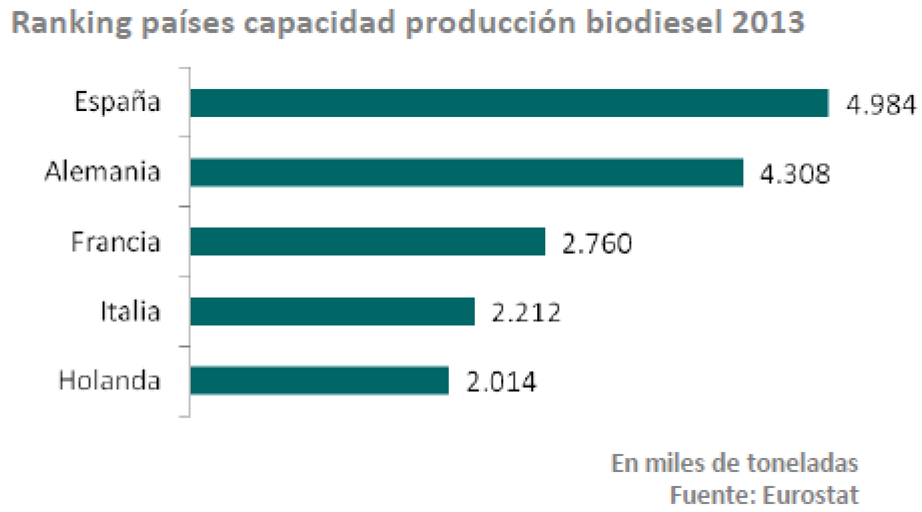


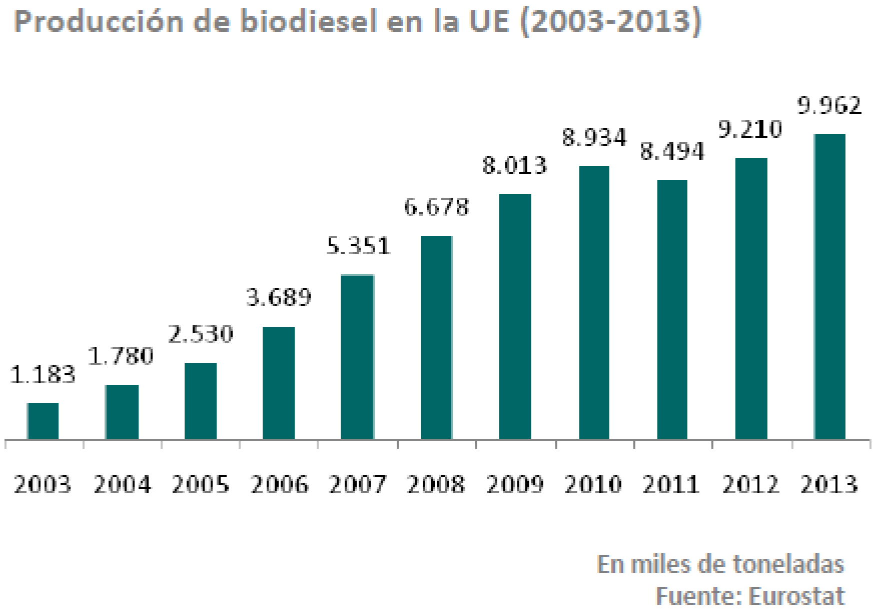
BIOCATALYSIS

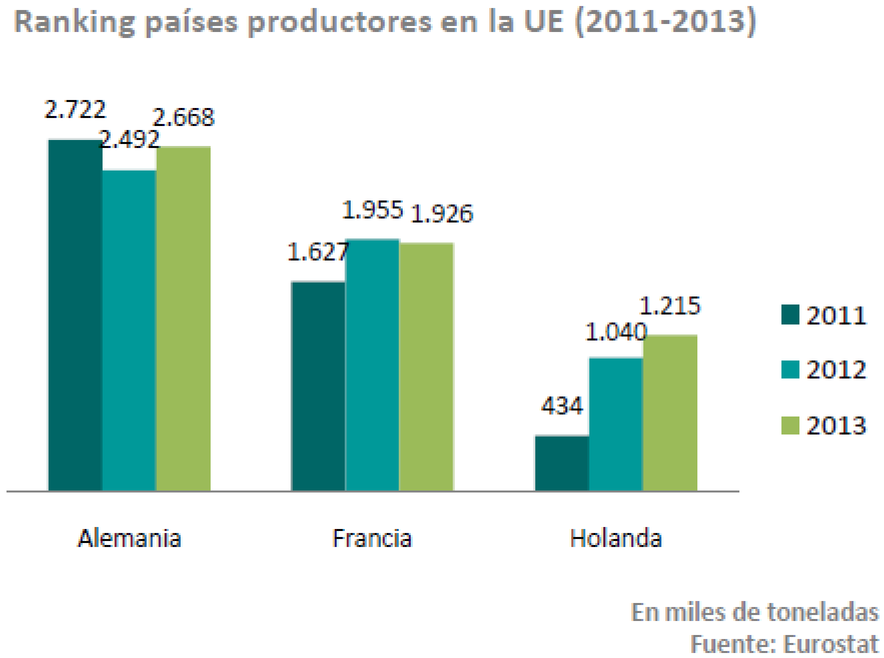
Storage (-20ºC) years without lost of activity

APPIELD BIOCATALYSIS OF rROL. ENZYMATIC BIODIESEL PRODUCTION



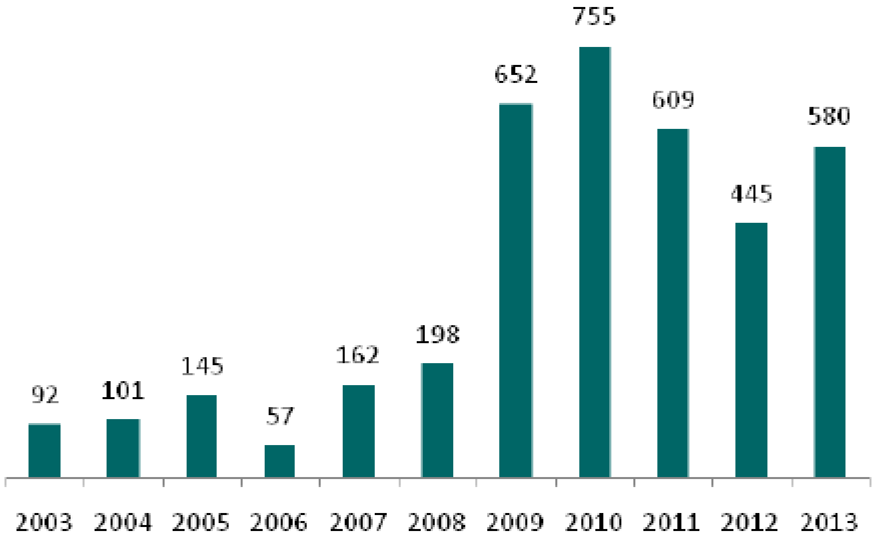






Informe sectorial infinita renovables 2015

Evolución producción biodiesel España (2003-2013)



En miles de toneladas
Fuente: Eurostat/Elaboración propia

Tabla. Consumo de biocombustibles en gasóleo 2012-2013. En toneladas

Ranking	País	2013	2012
1	Francia	2.293.324	2.268.977
2	Alemania	1.954.811	2.190.767
3	Italia	1.169.175	1.263.288
4	España	816.461	1.899.294
5	Reino Unido	603.755	497.349
	Total UE28	10.750.984	11.660.993

Fuente: Eurobserver

Capacity of production 4984 thousands of tones
Production 580 thousands of tones. Year 2013

Informe sectorial infinita renovables 2015

ORIGIN OF SUBSTRATES

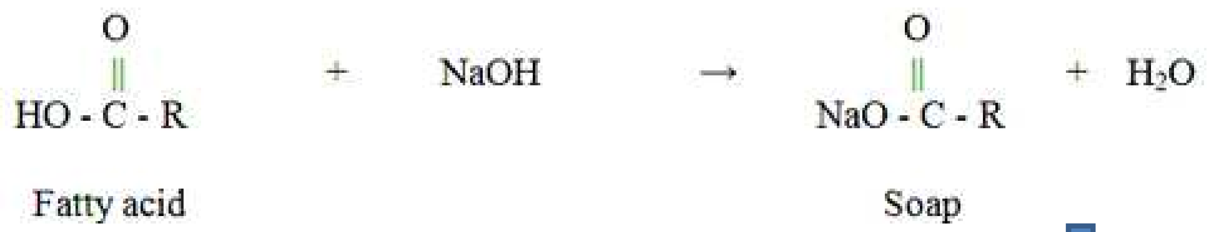
First generation biodiesel VS Second generation biodiesel

- Edible oils
(cottonseed, palm, rapeseed...)

- Non-edible oils
- Animal fats
- Waste oils

Biodiesel	First Generation	Second Generation
Advantages	· Already implanted · More environmentally friendly than fossil	· Easy available · Less GH emissions · Less-expensive substrates
Disadvantages	· Land Use · Higher food price · Net energy negative	· High FFA concentration · Neutralisation step

- **Problem**: **high concentration of FFAs** induces soap formation → neutralisation step is needed or acid catalysis.



Schematic representation of soap formation (Wen, 2012).

Biodiesel

Use of **lipases:**

WASTE OIL (FFAs) + LIPASE → COMPATIBLE

→ Alcohol inactivation?

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OUR SUBSTRATE

- Raw ***alperujo* olive oil (AOO)**: non-edible oil, by-product of the olive harvesting.
- Easily available in Spain.
- More than 20%wt of FFAs.

Raw *alperujo* oil

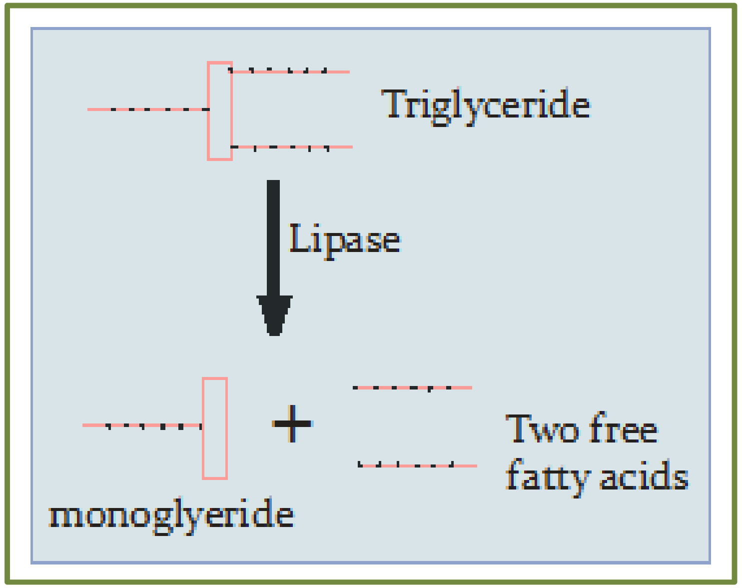
OBJECTIVE

The use of AOO for enzymatic biodiesel synthesis evaluating its FFA content as well as the methanol addition.

- Recombinant 1,3-positional selective *Rhizopus oryzae* lipase (rROL) produced using *Pichia pastoris* as a cell factory.

Maximum theorical yield → 66%

- Immobilised in a **glutharaldehyde-treated polymethacrylate amino-epoxide carrier** (Relizyme HFA-Glut) by COVALENT BOND.



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Effect of FFAs

Three types of susbtrate were used:

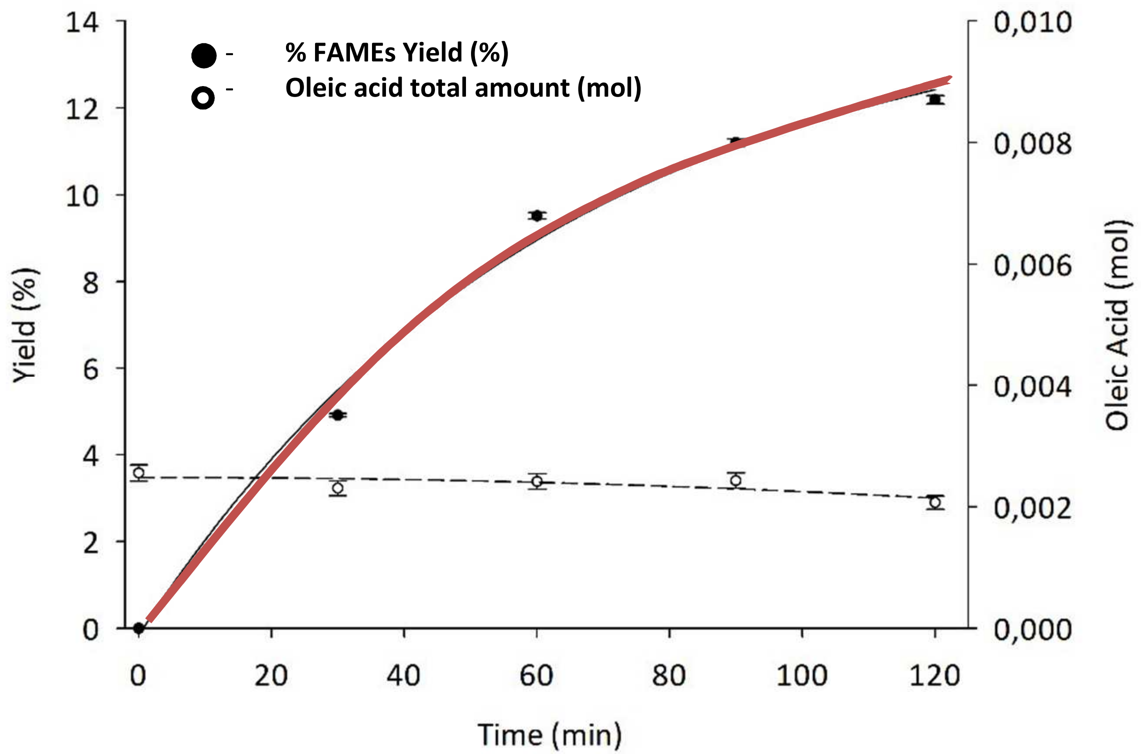
RAW ALPERUJO	NEUTR. ALPERUJO	SUPPL. ALPERUJO

INITIAL RATE

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Time evolution of FAME yield and oleic acid amount when raw alperujo is used.

1 pulse of methanol (14%) to evaluate initial rate

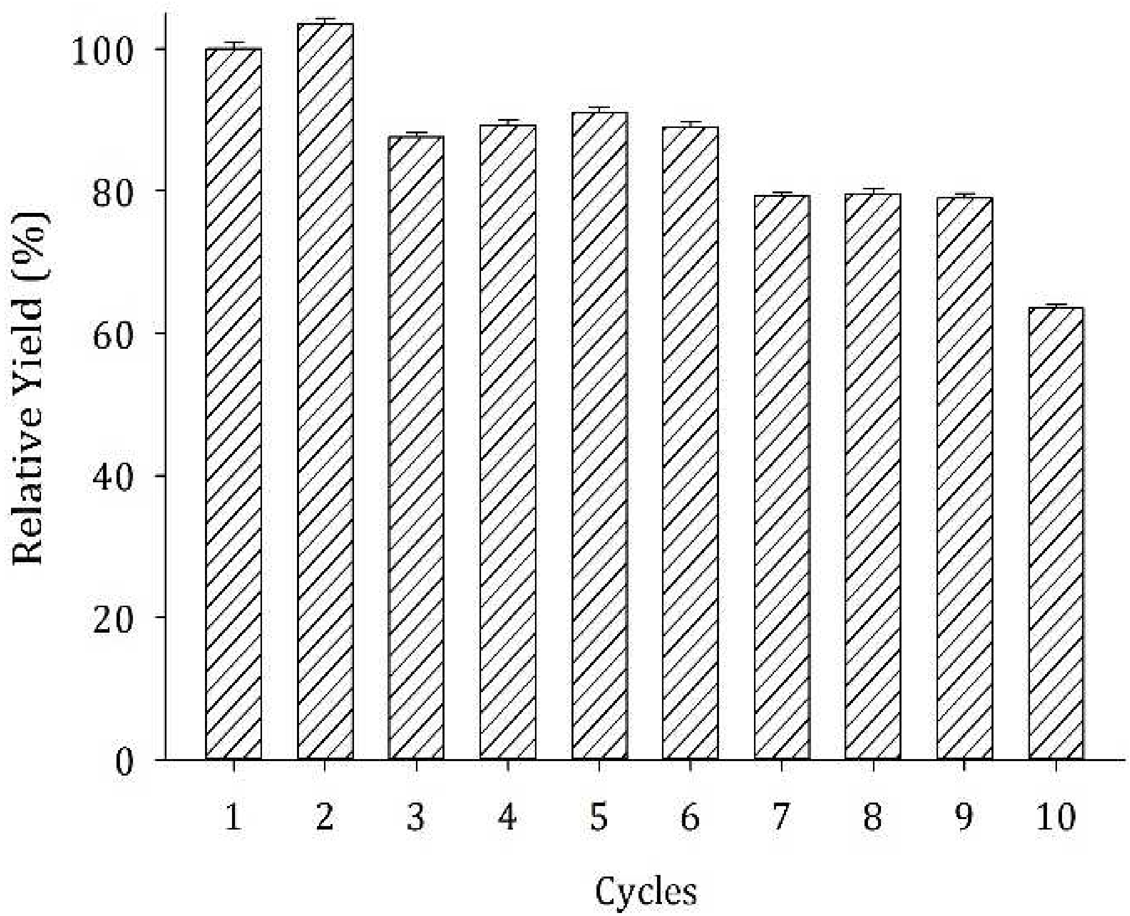
12% of yield in 2 hours.

Banet-Raqel et al., Fuel 161, 12-17. (2015)

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Re-utilisation cycles taking the first reaction yield as 100% of yield, when raw alperujo is used .

20-30% of activity loss in 20h

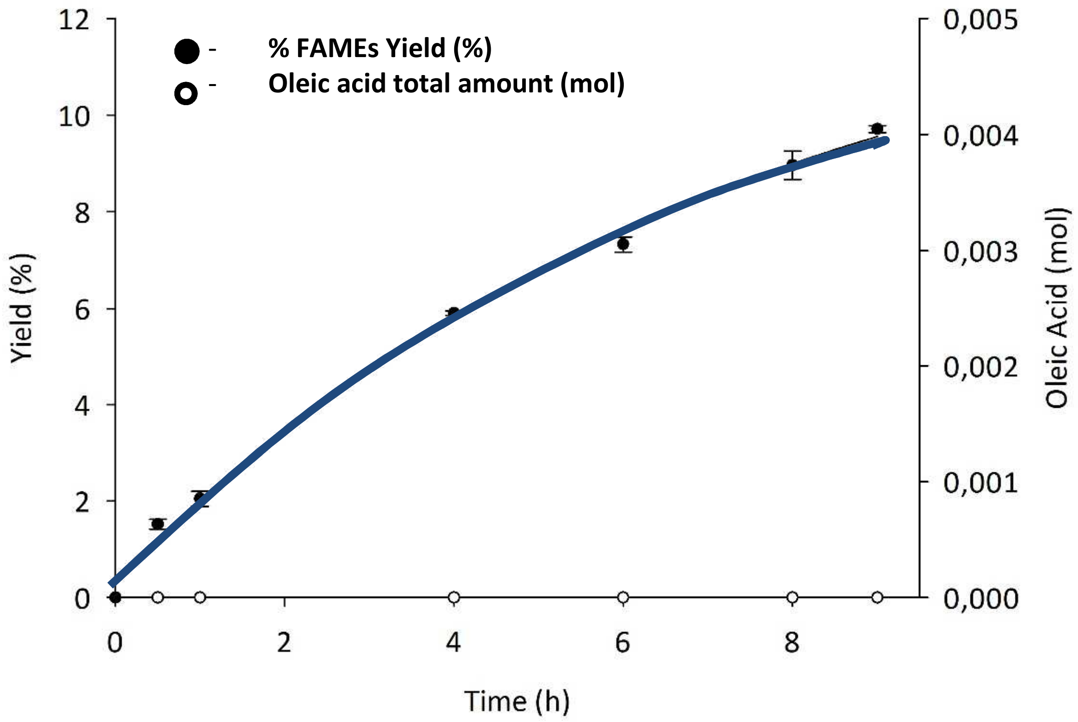
Banet-Ragel et al., Fuel 161, 12-17. (2015)

INITIAL RATE

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Time evolution of FAME yield and oleic acid amount when neutralized alperujo is used.

1 pulse of methanol (14%) to evaluate initial rate

10% of yield in 9 hours.

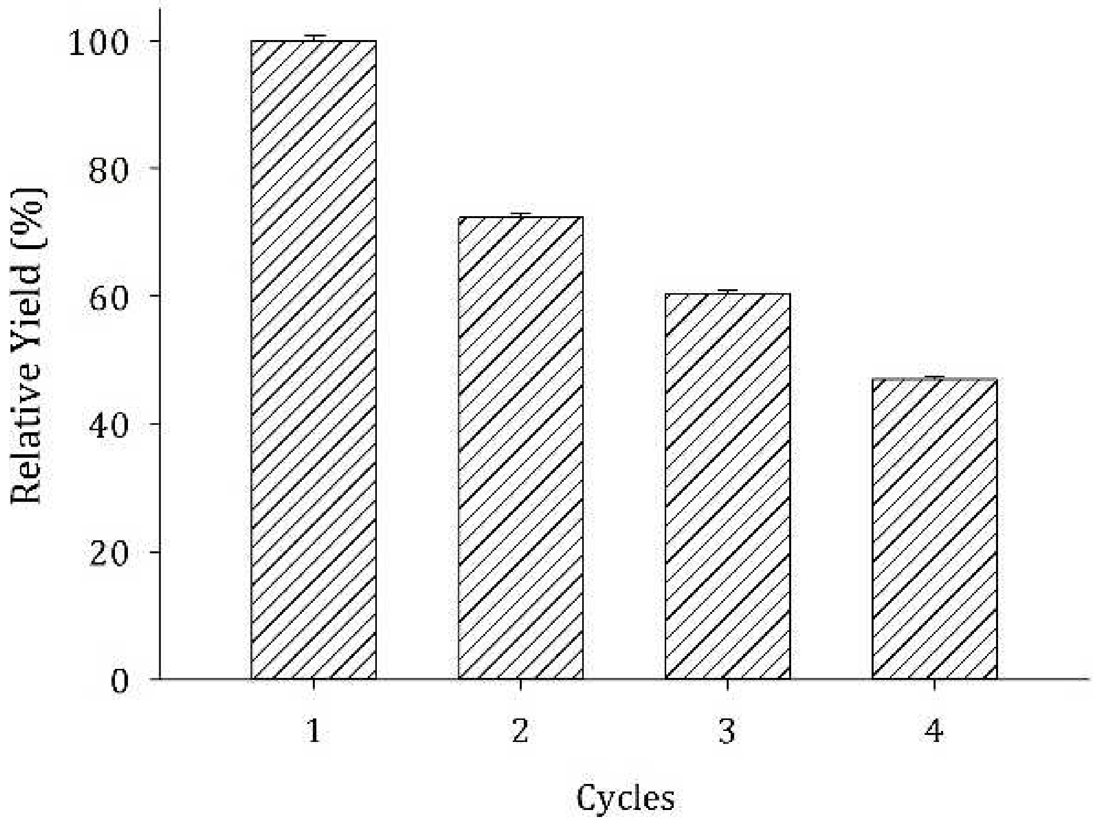
Banet-Ragel et al., Fuel 161, 12-17. (2015)

STABILITY

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Re-utilisation cycles taking the first reaction yield as 100% of yield, when neutralized alperujo is used .

30-35% of activity loss in 20h

Banet-Ragel et al., Fuel 161, 12-17. (2015)

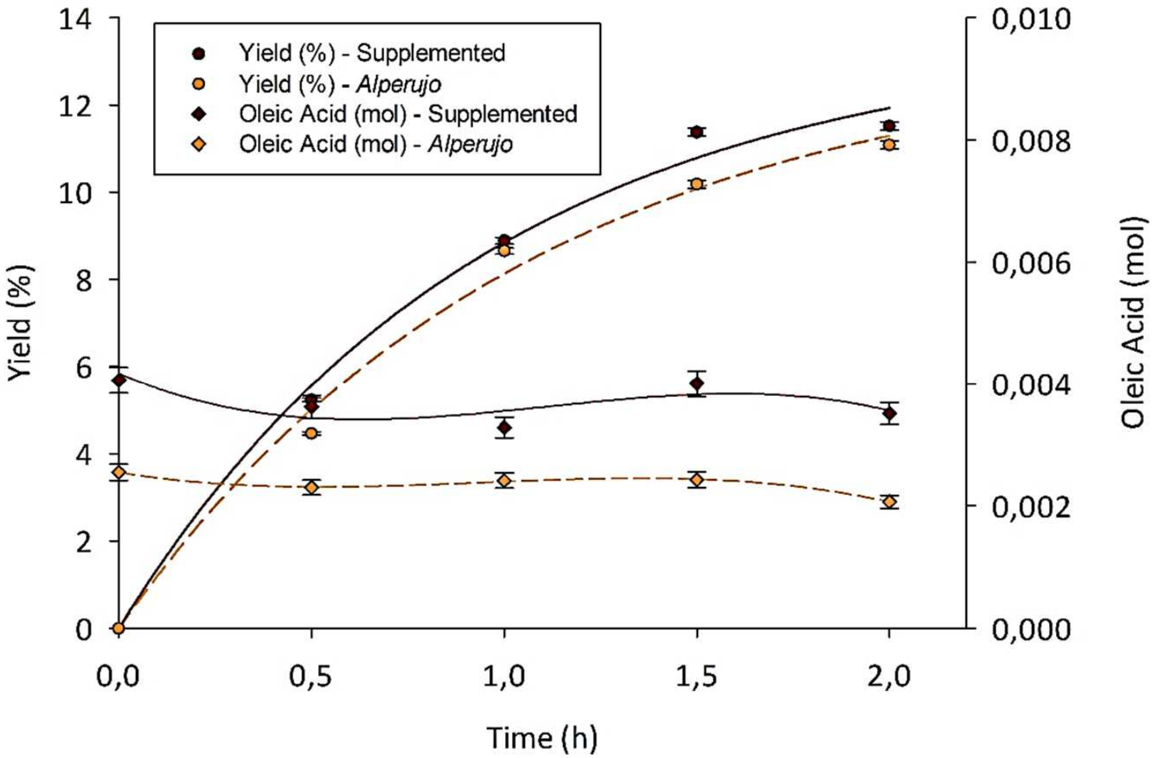
ENZITEC

INITIAL RATE

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Time evolution of FAME yield and oleic acid amount when supplemented alperujo is used (—) in comparison with raw oil (- -) .

1 pulse of methanol (14%) to evaluate initial rate

Same behaviour than raw *alperujo*

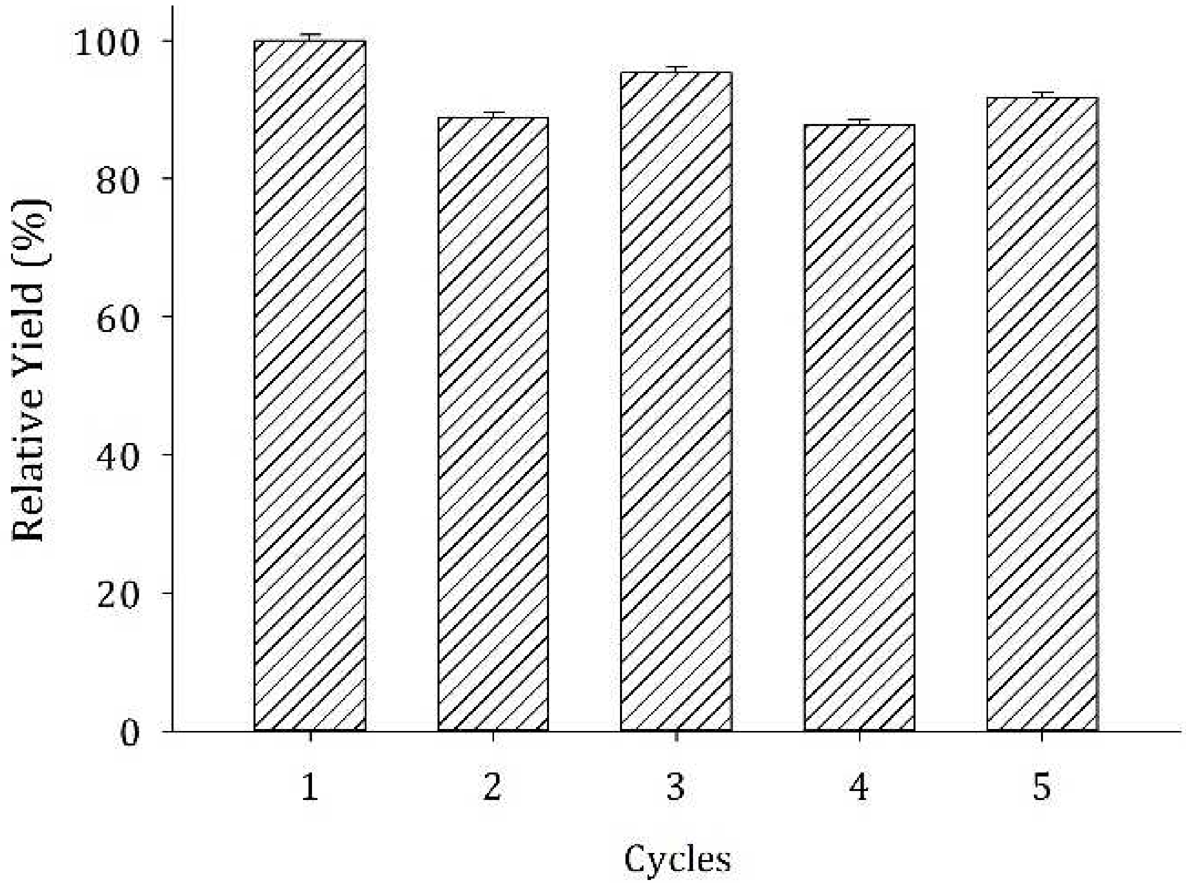
Banet-Raqel et al., Fuel 161, 12-17. (2015)

STABILITY

1 RAW ALPERUJO

2 NEUTR. ALPERUJO

3 SUPPL. ALPERUJO



Re-utilisation cycles taking the first reaction yield as 100% of yield, when supplemented alperujo is used .

Same retained activity as raw *alperujo*

Banet-Raqel et al., Fuel 161, 12-17. (2015)

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Comparison of initial rate values

Substrate used	Initial rate (μmol FAME·mL^{-1}·min^{-1})
Raw *alperujo*	6,48
Neutralised *alperujo*	0,64
Supplemented *alperujo*	7,28

Banet-Raqel et al., Fuel 161, 12-17. (2015)

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Effect of metanol addition

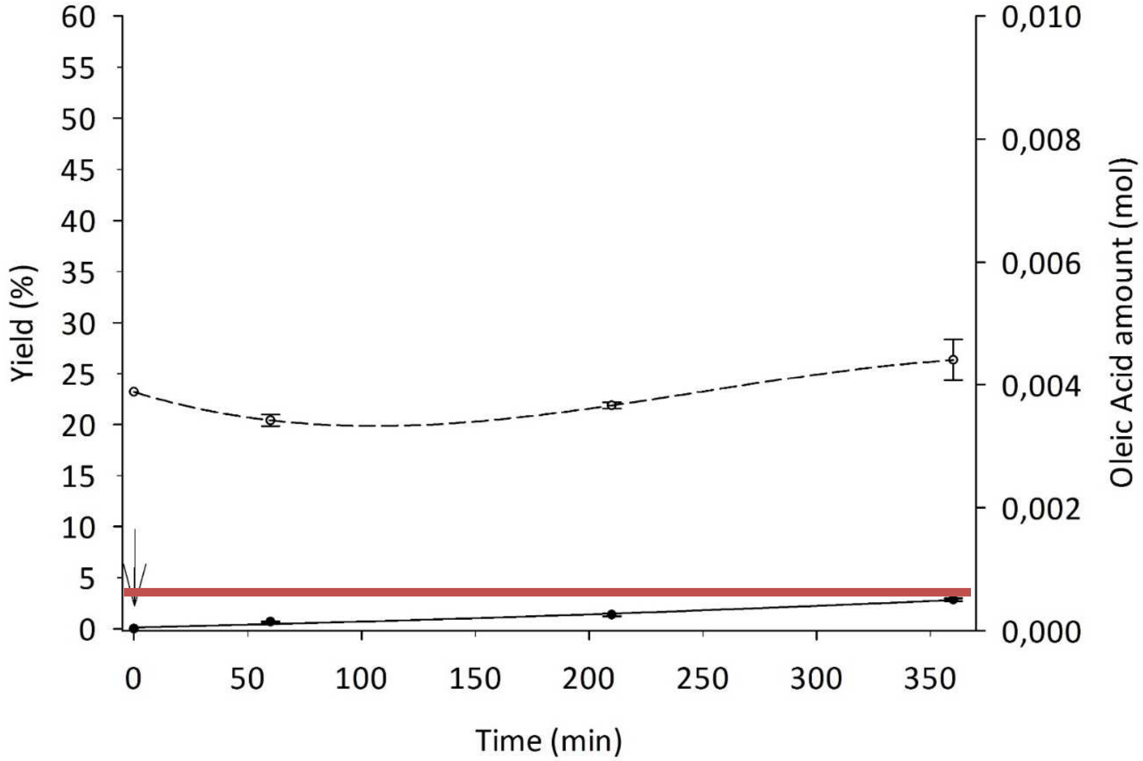
1 pulse

5 pulses

10 pulses

Banet-Ragel et al., Fuel 161, 12-17. (2015)

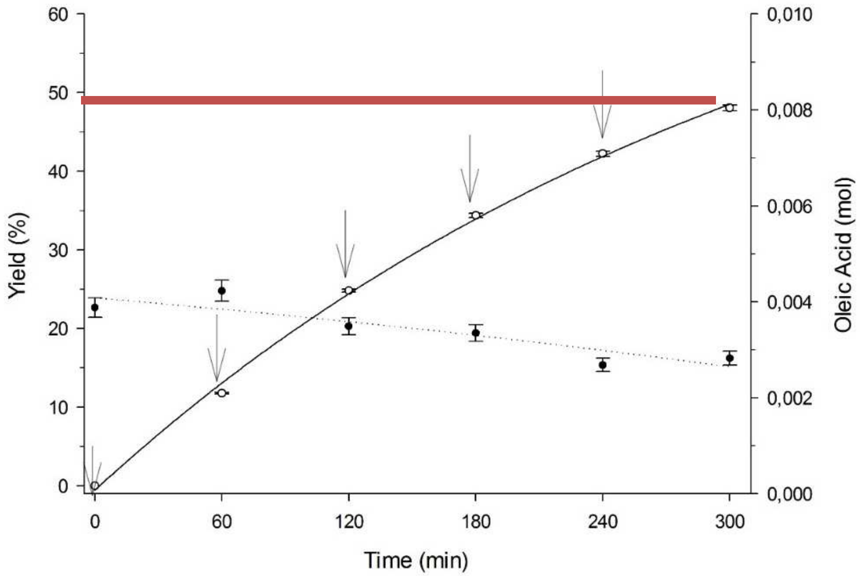
1 pulse of metanol, 66%



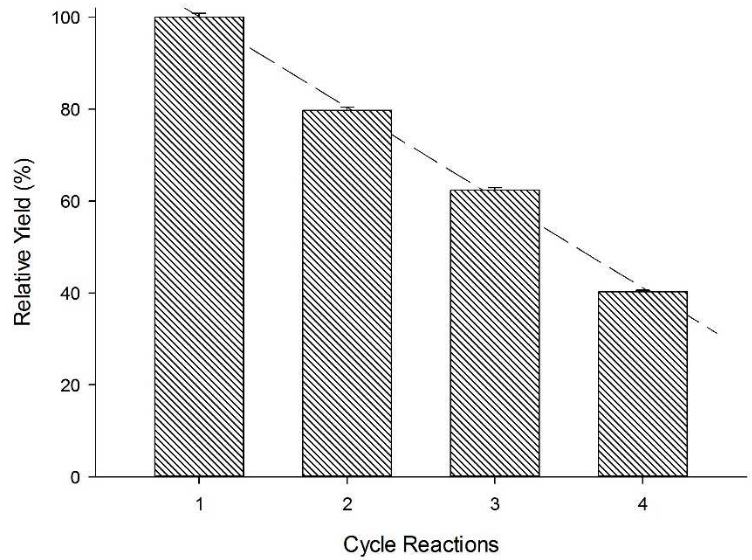
Time evolution of FAME yield and oleic acid amount when one pulse of methanol was added.

1 pulse → 5 pulses → 10 pulses

Banet-Ragel et al., Fuel 161, 12-17. (2015)



Time evolution of FAME yield and oleic acid amount when five pulses of methanol were added.



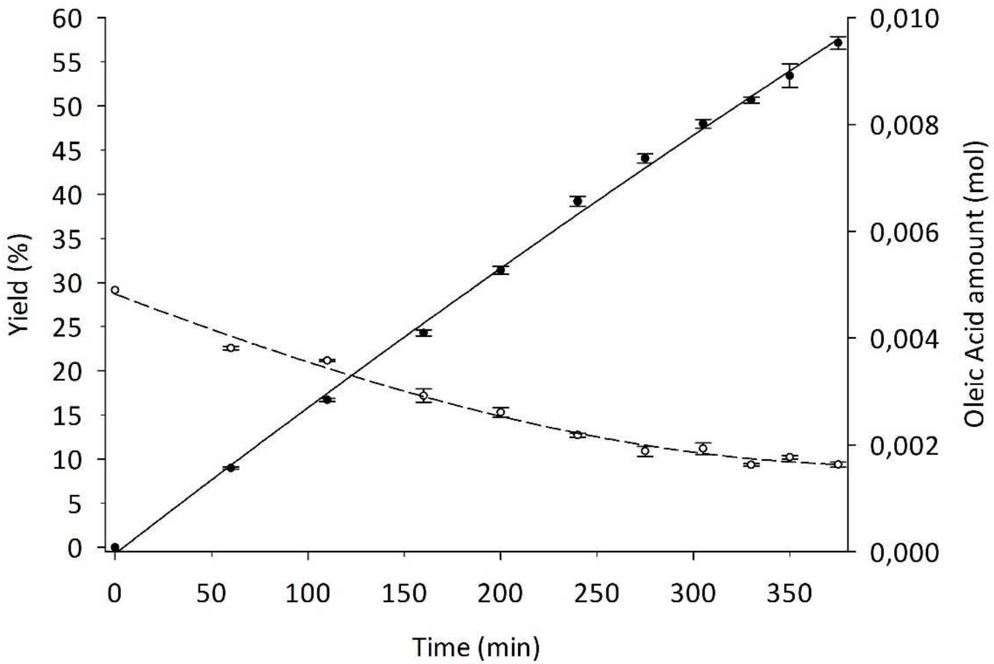
Re-utilisation cycles taking the first reaction yield as 100% of yield, when five pulses of methanol were added.

5 pulses of metanol, 66%

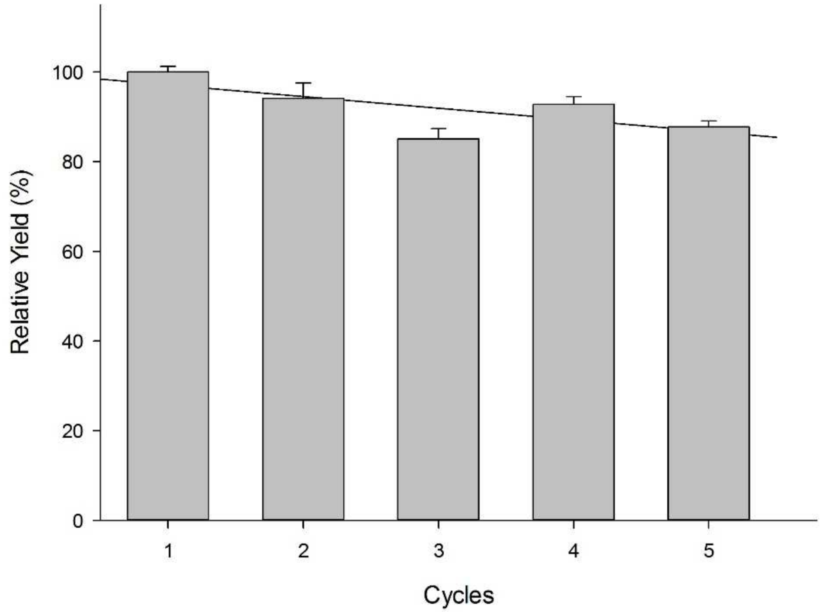
60% activity loss in 20 hours.

1 pulse → 5 pulses → 10 pulses

Banet-Ragel et al., Fuel 161, 12-17. (2015)



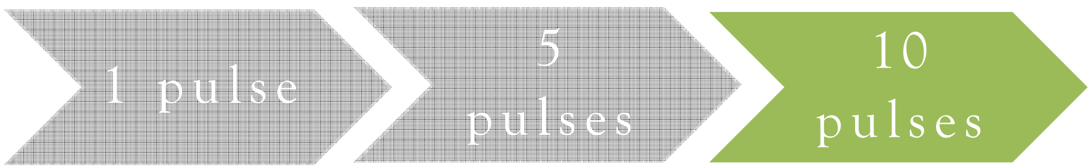
Time evolution of FAME yield and oleic acid amount when ten pulses of methanol were added.



Re-utilisation cycles taking the first reaction yield as 100% of yield, when ten pulses of methanol were added.

10 pulses of metanol, 66%

15% activity loss in 30 hours.



Banet-Raqel et al., Fuel 161, 12-17. (2015)

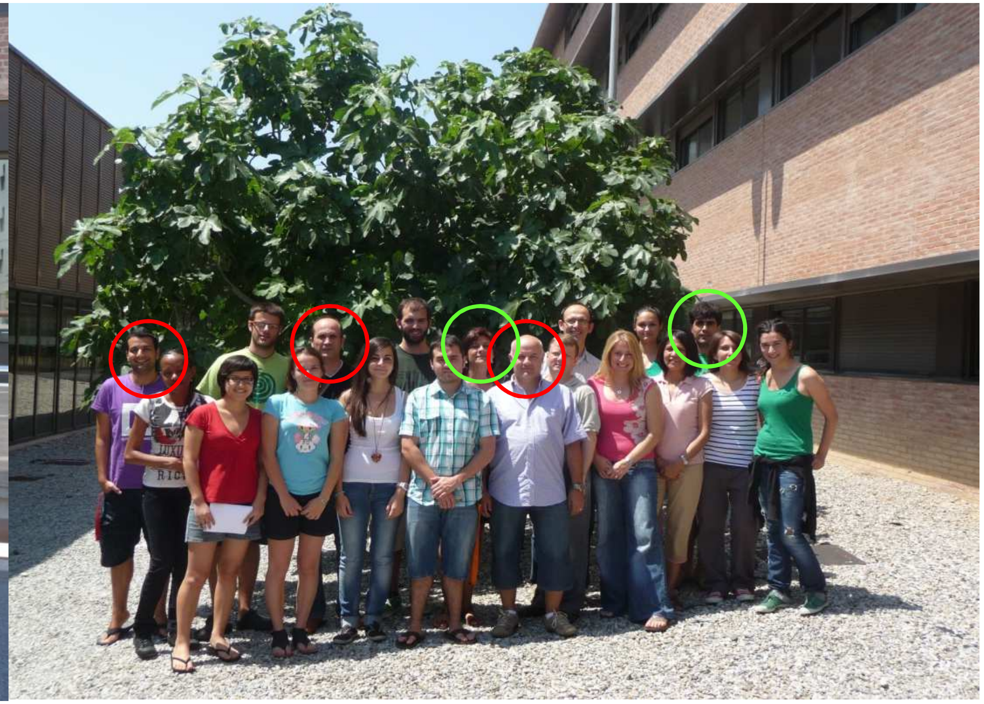
ENZITEC

It has been shown that *alperujo* is a good candidate for biodiesel production

High concentrations of FFAs provided a higher initial rate

FFAs enhanced biocatalyst stability

The key point is the way metanol was added



Thanks for your attention

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2014-SGR-452, and Reference Network in Biotechnology (XRB) (Generalitat de Catalunya).