



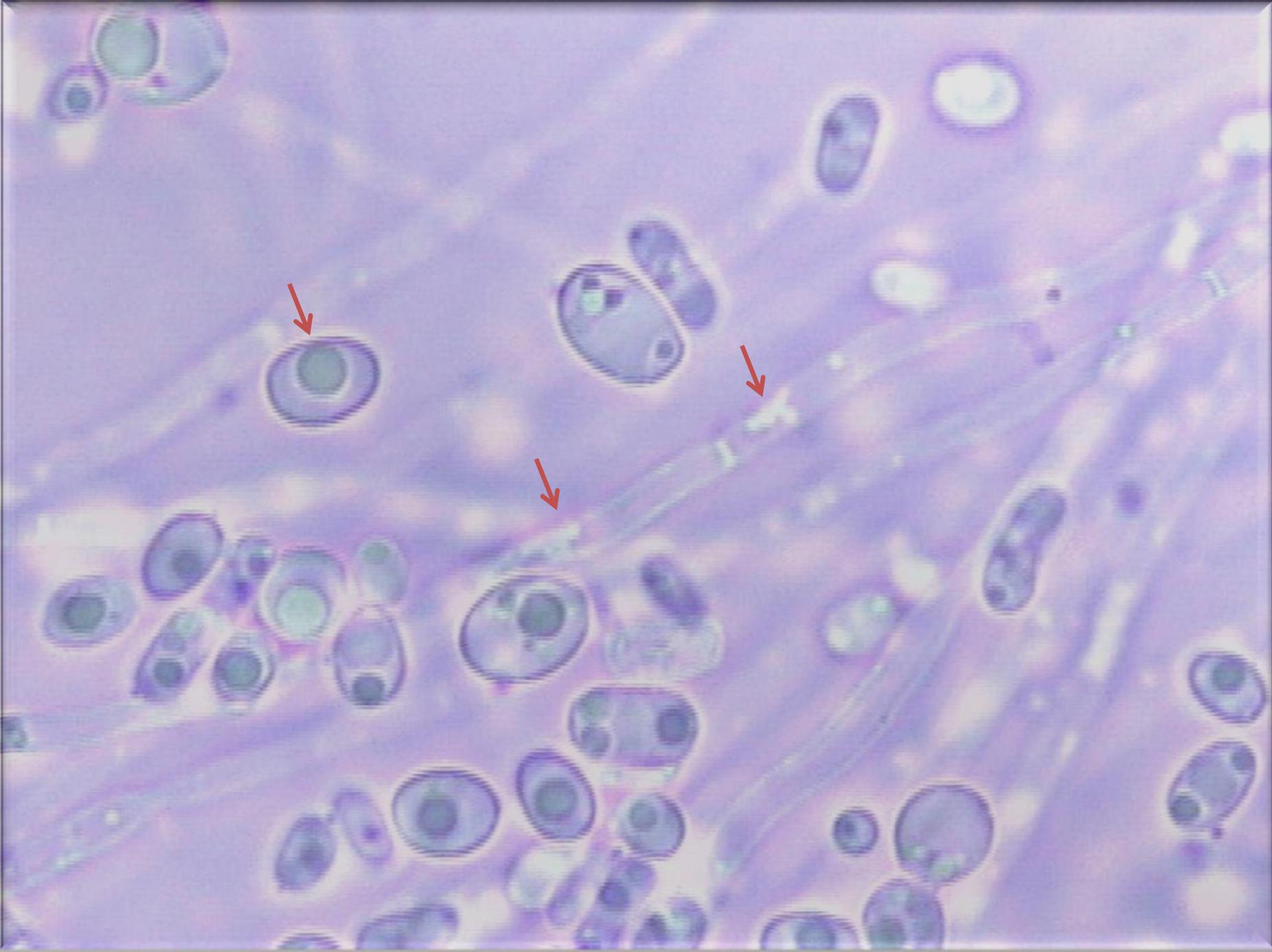
FEBS Workshop
Microbial Lipids From Genomics to Lipidomics
13-15 May 2010, Vienna, Austria

“Metabolic activities in *Yarrowia lipolytica* grown on glycerol”

Anna Makri, Stylianos Fakas & George Aggelis

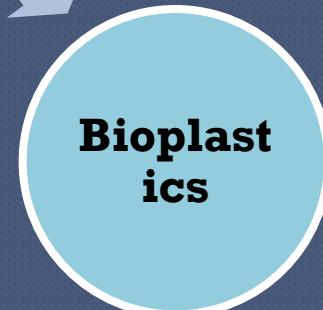
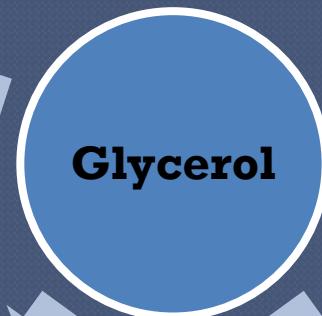
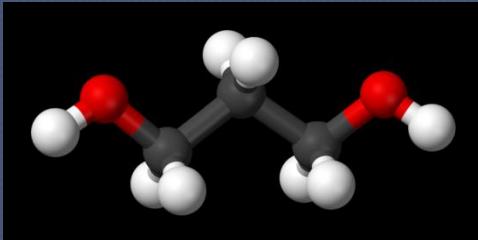
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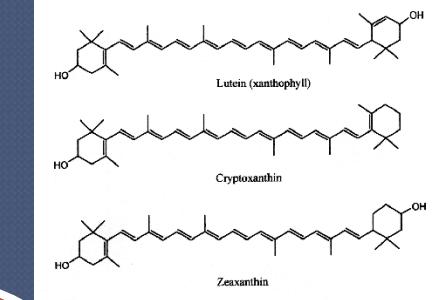
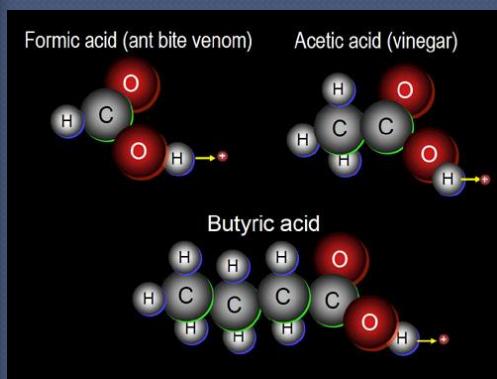




Why Glycerol?

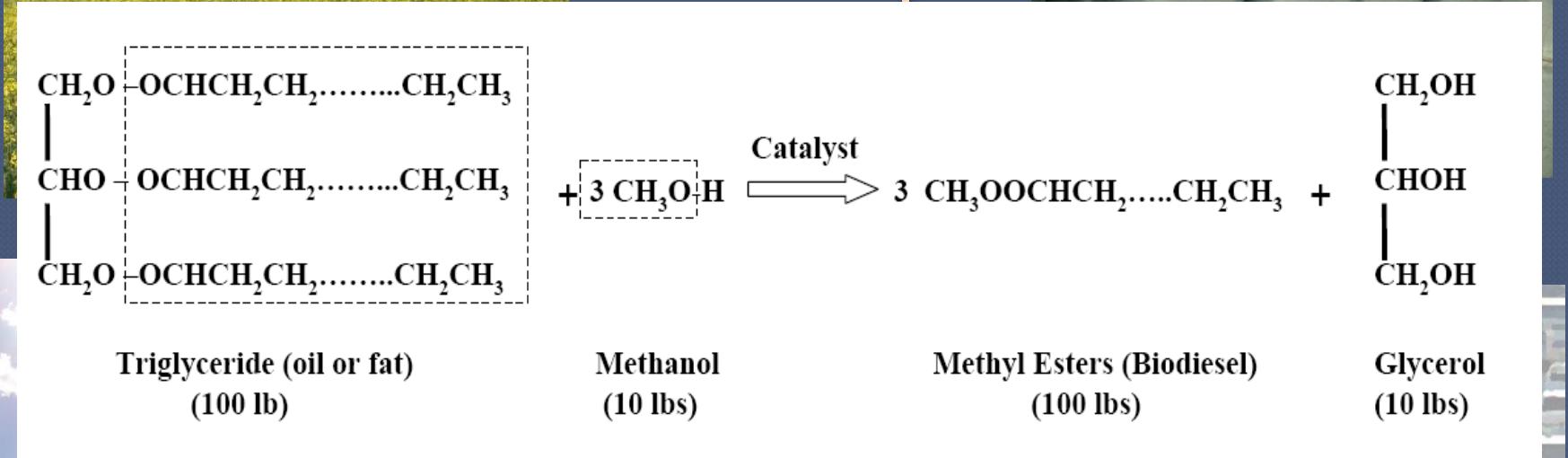
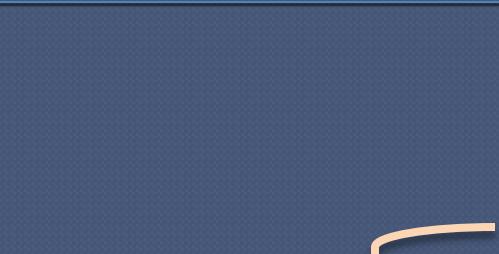


Organic acids





Glycerol



Industrial Biodiesel System 40.000 ton/year – Client: Brasil Ecodiesel



Biotechnological interest of SCO



Oleaginous
species

nutrient limitation

oil contents
>20% biomass
weight



Dietary and
pharmaceutical
importance

Conversion into
bio-diesel



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| Table I | | Table II | | |
|-------------|--------------------------|----------|----------------|-----------------------------|
| AIN NAME | IDENTIFIED AS: | S.I. (*) | STRAIN CODE | IDENTIFIED AS: |
| 1-Y | <i>Candida</i> sp. | 0.833 | A1-GP | <i>Actinomyces</i> sp. |
| 2-Y | <i>Candida</i> sp. (?) | 0.496 | A7-GP | <i>Actinomyces</i> sp. |
| 3-Y | <i>Candida</i> sp. | 0.818 | A9-GP | <i>Actinomyces</i> sp. |
| 5-Y | <i>Candida</i> sp. | 0.805 | A19-GP | <i>Actinomyces</i> sp. |
| 7-Y | <i>Cryptococcus</i> sp. | 0.616 | B23-GP | <i>Cassabacter</i> sp. |
| 1-Y | <i>Cryptococcus</i> sp. | 0.516 | B24-GP | <i>Cassabacter</i> sp. |
| 9-Y | <i>Debaryomyces</i> sp. | 0.597 | A2-GP | <i>Corynebacter</i> sp. |
| 5-Y | <i>Debaryomyces</i> sp. | 0.550 | A10-GP | <i>Corynebacter</i> sp. |
| 8-Y | <i>Pichia</i> sp. | 0.686 | A13-GP | <i>Corynebacter</i> sp. |
| 9-Y | <i>Pichia</i> sp. | 0.545 | A16-GP | <i>Corynebacter</i> sp. |
| 9-Y | <i>Pichia</i> sp. | 0.622 | B27-GP | <i>Corynebacter</i> sp. |
| 5-Y | <i>Rhodotorula</i> sp. | 0.519 | A8-GP | <i>Listeria</i> sp. (?) |
| 6-Y | <i>Rhodotorula</i> sp. | 0.507 | A4-GP | <i>Micromicrobacter</i> sp. |
| 6-Y | <i>Rhodotorula</i> sp. | 0.800 | A26-GP | <i>Rhodococcus</i> sp. |
| 2-Y | <i>Saccharomyces</i> sp. | 0.806 | A25-GP | <i>Sugarcoccus</i> sp. |
| S.I. sp. | | 0.769 | | all sp. |

Table 1 Yeast strains (Y), Table 2 Growth parameters and under industrial conditions.

| AS. | S.I. (*) | STRAIN CODE | Table 3 | |
|---------|----------|-------------|--------------------------|----------|
| | | | IDENTIFIED AS: | S.I. (*) |
| g. | 0.808 | A30-GN | <i>Achromobacter</i> sp. | 0.854 |
| g. | 0.733 | A31-GN | <i>Achromobacter</i> sp. | 0.596 |
| g. | 0.811 | B40-GN | <i>Alcaligenes</i> sp. | 0.870 |
| g. | 0.665 | B42-GN | <i>Franciscella</i> sp. | 0.672 |
| g. | 0.596 | A28-GN | <i>Pseudomonas</i> sp. | 0.770 |
| g. | 0.797 | B39-GN | <i>Pseudomonas</i> sp. | 0.951 |
| lum sp. | 0.714 | B43-GN | <i>Pseudomonas</i> sp. | 0.850 |
| lum sp. | 0.590 | B44-GN | <i>Pseudomonas</i> sp. | 0.561 |
| lum sp. | 0.736 | B45-GN | <i>Pseudomonas</i> sp. | 0.873 |
| lum sp. | 0.548 | B49-GN | <i>Pseudomonas</i> sp. | 0.703 |
| lum sp. | 0.554 | B50-GN | <i>Pseudomonas</i> sp. | 0.879 |
| lum sp. | 0.500 | B52-GN | <i>Pseudomonas</i> sp. | 0.582 |
| lum sp. | 0.683 | B47-GN | <i>Fibrio</i> sp. | 0.543 |
| lum sp. | 0.698 | B48-GN | <i>Fibrio</i> sp. (?) | 0.500 |
| lum sp. | 0.504 | B56-GN | <i>Fibrio</i> sp. | 0.700 |
| | | B57-GN | <i>Fibrio</i> sp. (?) | 0.500 |
| | | B33-GN | <i>Xanthomonas</i> sp. | 0.848 |
| | | B34-GN | <i>Xanthomonas</i> sp. | 0.848 |

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lips, decreased during growth. G+S comprised mainly of mono- and di-glycosylated (DGDG) diglycerides, while P comprised phosphatidyl-choline (PC), phosphatidyl-ethanolamine and phosphatidyl-serine [3]. DGDG followed by PG and GLA.

The major sugars of G+S fraction were glucose, mannose, and arabinose were found in lesser percentage proportion of glucose increased during major sterol (the rate of its synthesis increased during 5.7, 9.1(1), 22-tetraenol, ergosta-7,22-dienol, ergosta-episterol, and lanosterol were found in lesser percent.

Growth on N-limited media enhanced lipid accumulation by 25% of biomass [4]. The accumulated lipids contained high amounts of TAG (Fig 2). When the medium was exhausted the microorganism consumed the lipids and produced some biomass [4]. During lipid preferentially consumed and concomitantly some protein.

During growth lipids comprised mainly of oleic acid content was high (20% w/w) (Fig. 3). In the concentrations of C18:1 and palmitic acids (C16:0) linoleic (C18:2) and GLA contents decreased. However, the concentrations of C18:2 and GLA increased, while C16:0 and stearic (C18:0) acids decreased. The increase in the production of polar lipids that contained high g to the lower hydroxyl rates of GLA-containing

During growth GLA was transported from plasma membrane during lipid turnover. The flow of GLA was reversible, functioning as a storage of both energy and structural components. These PUFAs are mobilized when TAG degradation is used for membrane structure, meaning that the microorganism is able to respond quickly to environmental changes by altering its membrane structure using PUFAs stored in TAG.

Mold

Fusarium
Penicillium spp., *Aspergillus* sp
Rhizopus nigricans, *Paecilomyces*
sp. *Verticillium* sp.

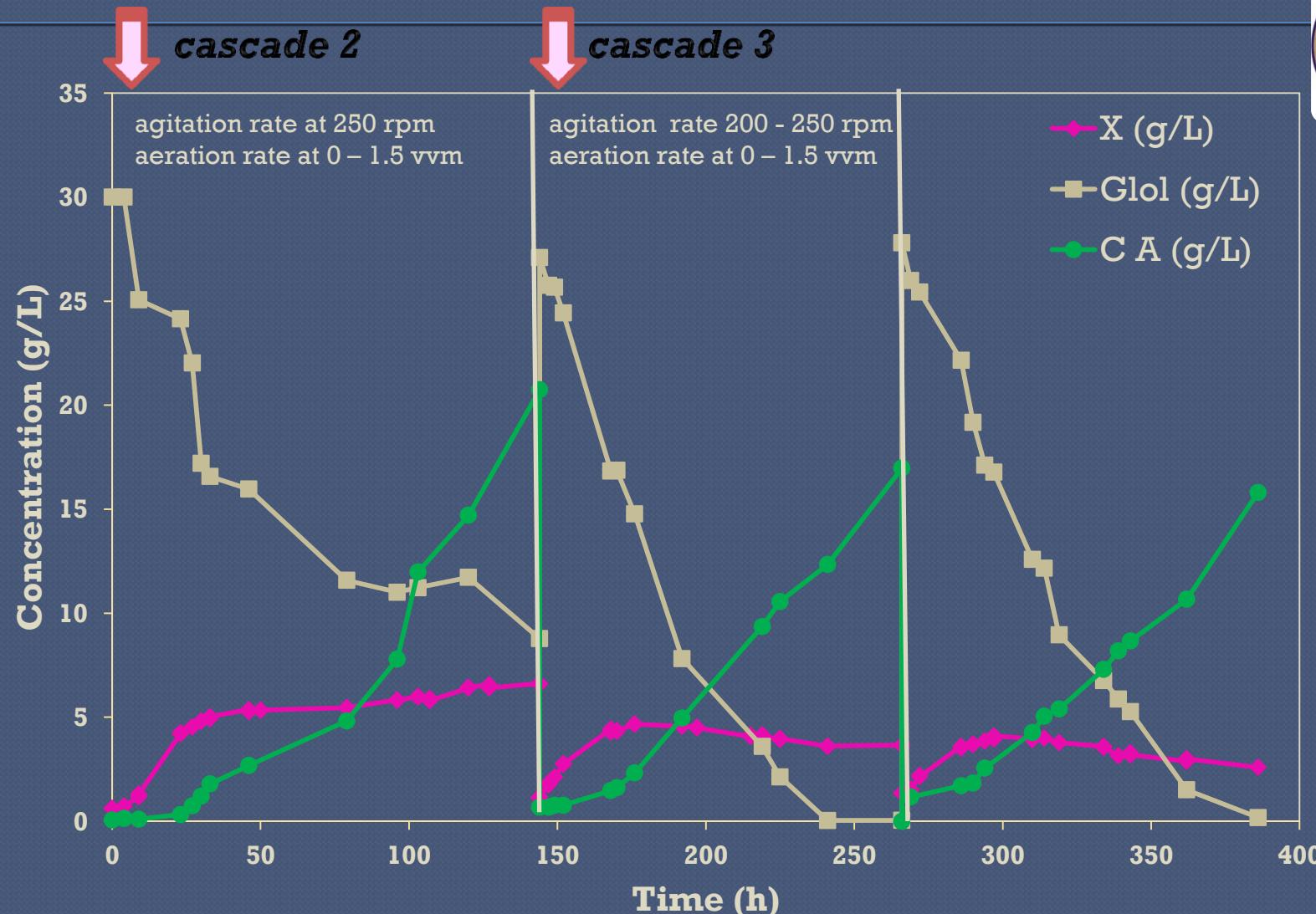
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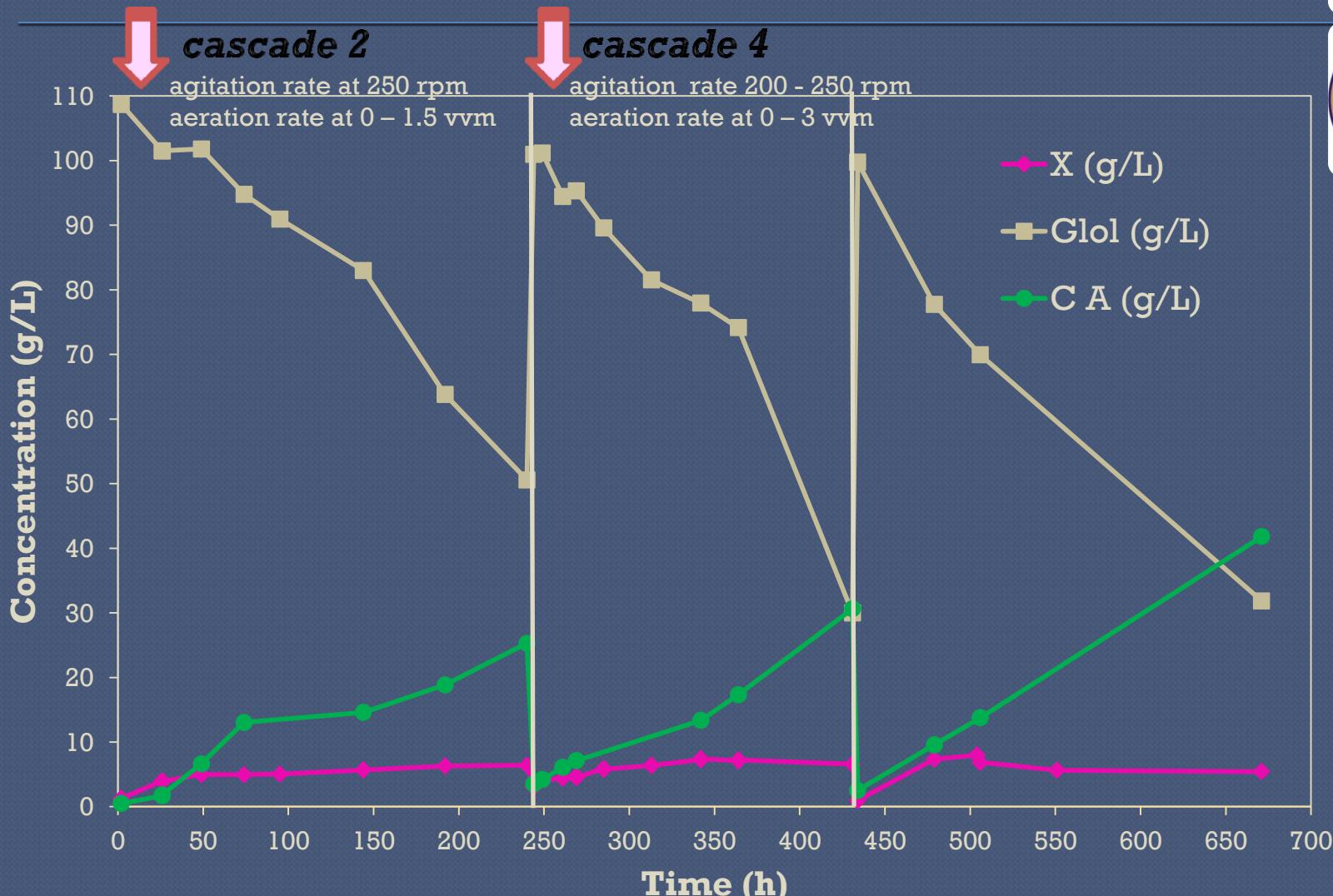
Kinetics of biomass (x), substrate (Glol) and citric acid (CA) in repeated batch cultures of *Y. lipolytica*.

Culture conditions: initial glycerol concentration 27.8 g/L; dissolved oxygen concentration 30 % of saturation; pH 6 ± 0.03; T=28 °C



Kinetics of biomass (x), substrate (Glol) and citric acid (CA) in repeated batch cultures of *Y. lipolytica*.

Culture conditions: initial glycerol concentration 104.9 g/L; dissolved oxygen concentration 30 % of saturation; pH 6 ± 0.03; T=28 °C



Growth of *Y. lipolytica* in repeated batch culture and conversion yields in different initial glycerol concentration.



Culture conditions: dissolved oxygen concentration 30 % of saturation; pH 6 ± 0.03; T=28 °C; agitation rate 200 – 250 rpm; aeration rate 0 – 1.5 and 0 – 3.0 vvm for initial glycerol concentration 27.8 and 104.9 g/L, respectively. Abbreviations: initial substrate (S₀); remaining substrate (S); biomass produced (x); lipid content (L/x, %); citric acid produced (CA); and conversion yields (Y) in the various growth phases.

| S ₀ (g/L) | Growth phase | Time (h) | S (g/L) | x (g/L) | L/x (%) | CA (g/L) | Y _{x/S} | Y _{L/S} | Y _{CA/S} |
|----------------------|--------------------|----------|-------------|------------|-------------|-------------|------------------|------------------|-------------------|
| 27.8 | Biomass Production | 30 | 23.09 ±1.06 | 4.37 ±0.17 | 13.90 ±0.10 | 0.53 ±0.21 | 0.97 ±0.18 | 0.13 ±0.03 | 0.11 ± 0.02 |
| | Lipogenic | 50 | 14.07 ±1.90 | 4.68 ±0.68 | 22.30 ±0.39 | 3.87 ±1.19 | 0.36 ±0.10 | 0.08 ±0.02 | 0.28 ± 0.05 |
| | CA Production | 130 | 0.44 ±0.36 | 3.18 ±0.27 | 11.83 ±0.66 | 13.95 ±1.47 | 0.17 ±0.10 | 0.01 ±0.01 | 0.51 ± 0.05 |
| 104.9 | Biomass Production | 30 | 94.86 ±0.43 | 4.57 ±0.17 | 12.13 ±0.03 | 6.63 ±0.51 | 0.46 ±0.04 | 0.06 ±0.01 | 0.66 ± 0.08 |
| | Lipogenic | 50 | 78.29 ±2.56 | 6.71 ±0.52 | 20.41 ±1.31 | 13.83 ±2.27 | 0.25 ±0.01 | 0.05 ±0.01 | 0.53 ± 0.09 |
| | CA Production | 240 | 41.22 ±9.37 | 5.92 ±0.49 | 13.40 ±0.33 | 33.55 ±8.26 | 0.10 ±0.02 | 0.01 ±0.01 | 0.52 ± 0.05 |

Enzymatic activities in *Y. lipolytica* grown on glycerol in repeated batch culture.



Culture conditions: initial glycerol concentration 27.8 g/L; dissolved oxygen concentration 30 % of saturation; pH 6 ± 0.03; T=28 °C; agitation rate 200 – 250 rpm; aeration rate 0 – 1.5 vvm.



Abbreviations: **NADP⁺-ICDH** – NADP⁺ dependent isocitric dehydrogenase; **NAD⁺-ICDH** – NAD⁺ dependent isocitric dehydrogenase; **GK** – glycerol kinase; **NAD⁺-G-3-P DH** – glycerol -3- phosphate dehydrogenase; **Mit.** – mitochondrial; **Cyt.** – cytoplasmic; Units/mg dry weight – U/mg DW.

| Growth phase | Fermentation Period (h) | NADP ⁺ -ICDH (U/mg DW) | NAD ⁺ -ICDH (U/mg DW) | GK (U/mg DW) | NAD ⁺ -G-3-P DH (U/mg DW) |
|--------------------|-------------------------|-----------------------------------|----------------------------------|--------------|--------------------------------------|
| Biomass Production | 0 - 24 | 192.55 ± 4.61 | 45.53±0.89 | 39.40±6.23 | 14.93±8.68 |
| | | Mit./Cyt. 10 %/ 90 % | | | |
| Lipogenic | 30 - 52 | 187.82±13.68 | 13.66±0.94 | 49.48±6.99 | 17.67±6.48 |
| | | Mit./Cyt. 0 %/ 100 % | | | |
| CA Production | 55-130 | 208.05±0.15 | 0.39±0.01 | 18.00±0.10 | 18.00±0.07 |
| | | Mit./Cyt. 0 %/ 100 % | | | |

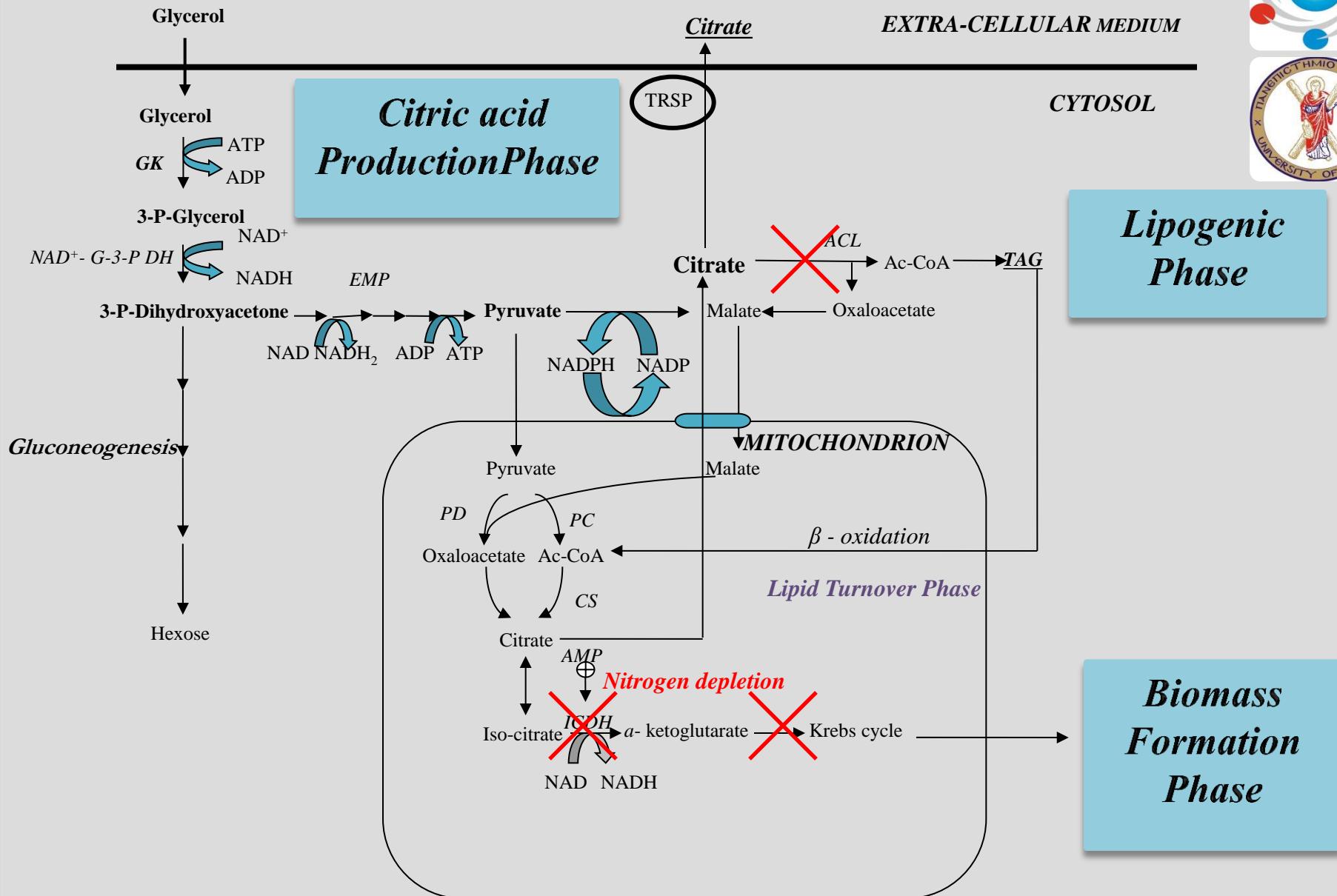


Enzymatic activities in *Y. lipolytica* grown on glycerol in continuous culture.

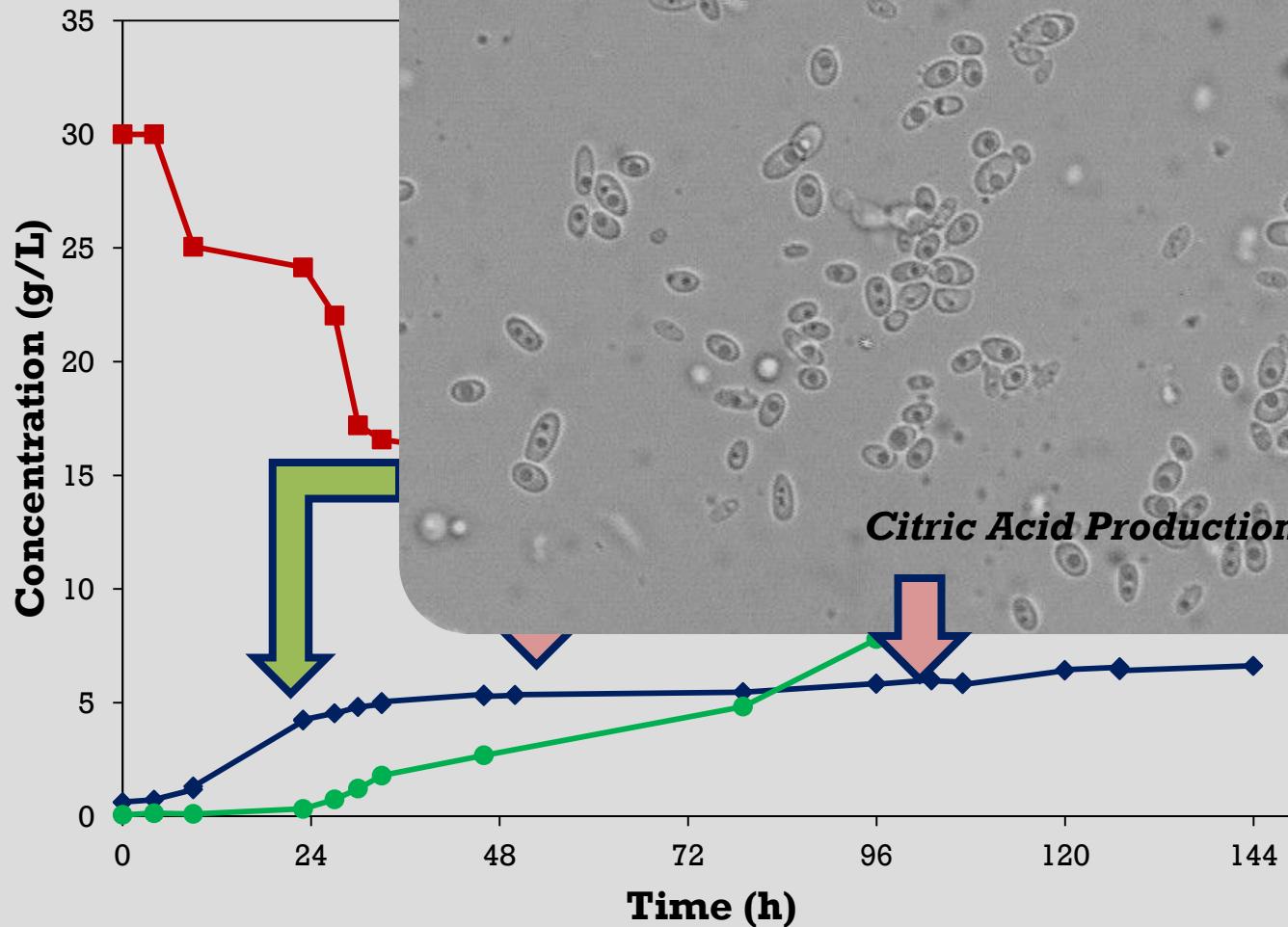
Culture conditions: initial glycerol concentration 50 g/L; dissolved oxygen concentration 20 % of saturation; pH 6 ± 0.03; T=28 oC; agitation rate 200 – 250 rpm; aeration rate 0 – 1.5 vvm.

Abbreviations: **NAD⁺-ICDH** – NAD⁺ dependent isocitric dehydrogenase; **GK** – glycerol kinase; **NAD⁺-G-3-P DH** – glycerol -3- phosphate dehydrogenase; **ATP:CL**- ATP citrate lyase; **ME**- malic enzyme Units/mg dry weight – U/mg DW.

| D (h ⁻¹) | GK (U/mg DW) | NAD ⁺ -G-3-P DH (U/mg DW) | NAD ⁺ -ICDH (U/mg DW) | ATP:CL (U/mg DW) | ME (U/mg DW) |
|----------------------|-----------------|---|-------------------------------------|---------------------|-----------------|
| 0.017±0.001 | 101.84 | 3.42 | 15.95 | 218.33 | 142.93 |
| 0.104±0.003 | 161.55 | 33.07 | 26.93 | 18.84 | 648.95 |



The morphology of the unconventional yeast *Y. lipolytica* grown on glycerol is growth phase-dependent.



Lipid fractions and individual phospholipid classes during growth of *Y. lipolytica* on glycerol in repeated batch culture.



Analyses were performed in duplicate.

Culture conditions: initial glycerol concentration 27.8 g/L; dissolved oxygen concentration 30 per cent of saturation; pH 6 ± 0.03; T=28 °C; agitation rate 200 – 250 rpm; aeration rate 0 – 1.5 vvm.

Abbreviations : NL – Neutral lipids; G+S – glycolipids plus sphingolipids; P – phospholipids; PC – phosphatidylcholine; PE – phosphatidylethanolamine; PI – phosphatidylinositol; PA - phosphatidic acid; others - unidentified phospholipids.

| Time (h) | Lipid Fractions [% in total lipids] | | | Lipid Fractions [% in x] | | | P classes [% (w/w) in P] | | | | | |
|-------------|--|-------|-------|-----------------------------|------|------|-----------------------------|-------|------|-------|-------|--------|
| | NL | G+S | P | NL | G+S | P | PS | PI | PA | PE | PC | others |
| 28 | 82.81 | 12.20 | 4.99 | 11.51 | 1.70 | 0.69 | 2.38 | 24.35 | 1.10 | 11.66 | 29.38 | 31.11 |
| 48 | 92.45 | 4.35 | 3.20 | 20.61 | 0.97 | 0.71 | 0.22 | 20.45 | 5.88 | 15.57 | 30.11 | 27.76 |
| 130 | 60.96 | 24.48 | 14.55 | 7.21 | 2.90 | 1.72 | n.d.* | n.d. | n.d. | n.d. | n.d. | n.d. |

*n.d.: not determined



Fatty acid composition of lipids fractions of *Y. lipolytica* grown on 27.8 g/L glycerol in repeated batch culture.

Analyses were performed in duplicate.

Culture conditions: initial glycerol concentration 27.8 g/L; dissolved oxygen concentration 30 per cent of saturation; pH 6 ± 0.03; T=28 oC; agitation rate 200 – 250 rpm; aeration rate 0 – 1.5 vvm.

| | Time (h) | C16:0 | C16:1 | C18:0 | C18:1 | C18:2 | others |
|-----|----------|-------|-------|-------|-------|-------|--------|
| NL | 28 | 14.71 | 14.05 | 6.96 | 55.65 | 7.96 | 0.67 |
| | 48 | 13.07 | 16.97 | 5.96 | 55.88 | 7.06 | 1.05 |
| | 130 | 17.06 | 14.13 | 7.70 | 43.50 | 16.94 | 0.67 |
| G+S | 28 | 16.52 | 13.30 | 2.95 | 50.80 | 13.29 | 3.14 |
| | 48 | 13.65 | 17.28 | 4.66 | 53.24 | 9.62 | 1.55 |
| P | 28 | 8.38 | 17.00 | 0.73 | 50.14 | 21.64 | 2.10 |
| | 48 | 7.31 | 23.00 | 1.45 | 48.93 | 15.87 | 3.45 |
| | 130 | 10.62 | 19.92 | 1.07 | 34.47 | 30.66 | 3.25 |



Conclusions

Growth

- Three distinct phases

Regulation

- GK
- NAD⁺-ICDH
- ME
- ATP:CL

Lipids

- NL
- P

Unit of Microbiology





- FEBS Workshop
- Project K. Karatheodori, University of Patras,
Kinetics of growth of oleaginous
microorganisms and biosynthesis dynamics of
Polyunsaturated Fatty Acids (PUFAs),
(2007-2010)
- DRACOIL SA,
Biodiesel production from agricultural
by- products, (2006-2010)



A microscopic image showing numerous small, oval-shaped microorganisms, likely paramecia, distributed across a light purple background. Some individuals are in pairs or small groups, while others are isolated. The organisms have a distinct double-walled structure.

Thank you!