Microbial Conversions and Transformations

of Vegetable Oils and their Components

Prof. Dr. Siegmund Lang

TU Braunschweig, Institut für Biochemie u. Biotechnologie
Spielmannstr. 7, 38106 Braunschweig
Tel. 0531-391 5737; e-mail: s.lang@tu-bs.de

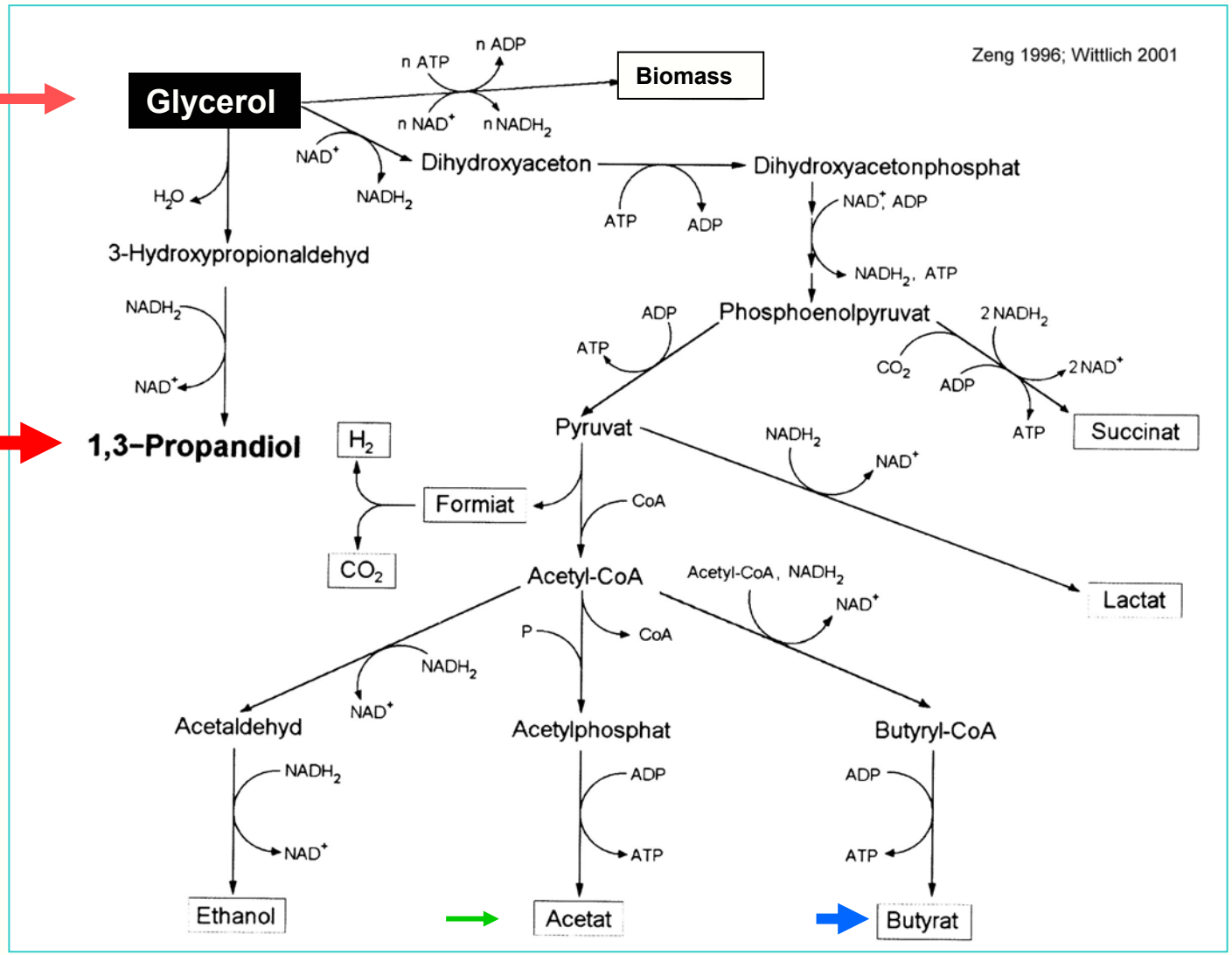
Microbial Conversion of Triglycerides of Glycerol of Fatty Acids

Products: - 1,3-Propanediol (► Polymers)
- Polyhydroxyalkanoates (Polymer)
- Glycolipids (= Biosurfactants)

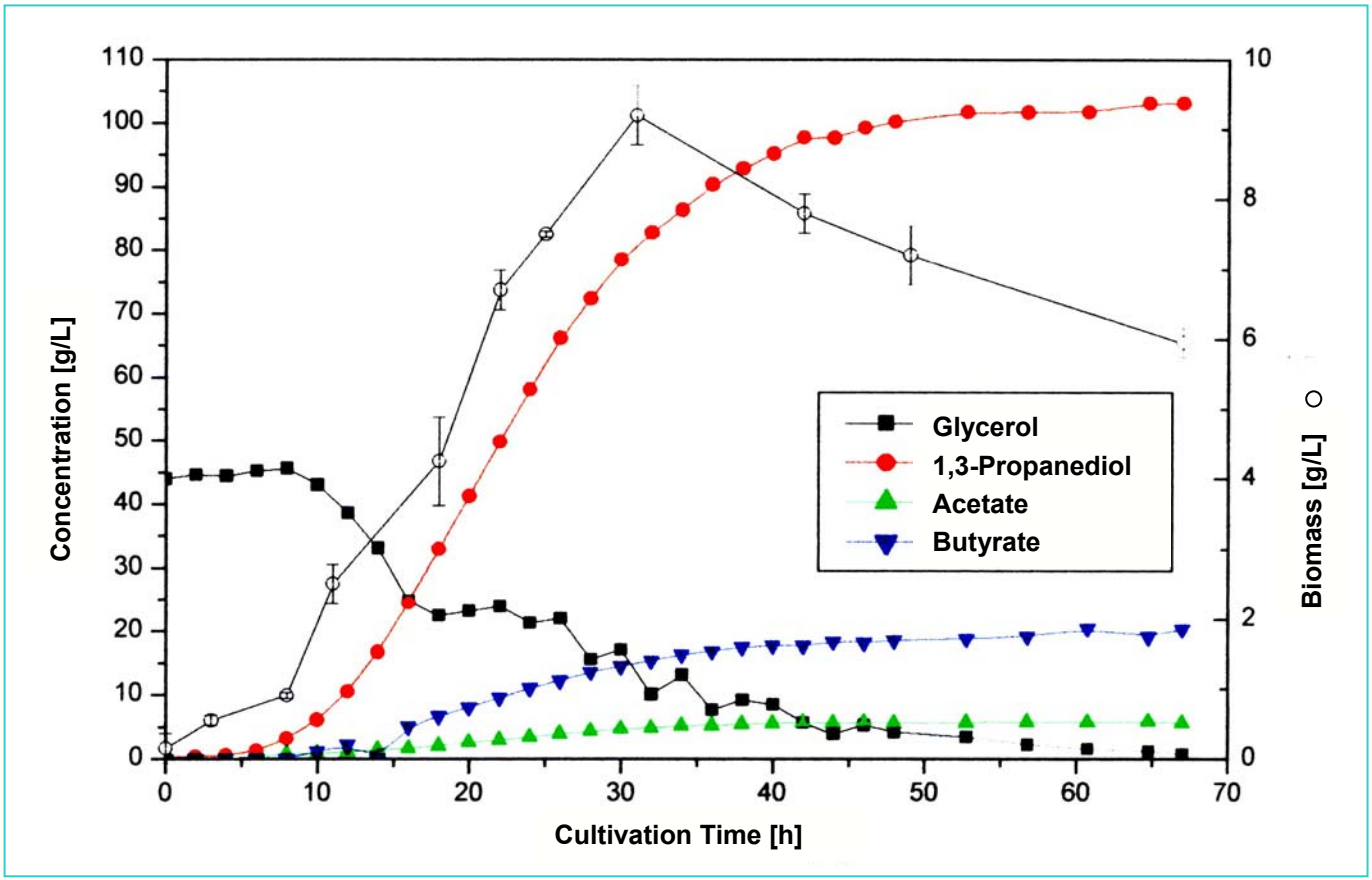
Microbial / Enzymatic Transformation of TAGs / Fatty Acids

Products: - Functionalized TAGs / Fatty Acids
(Cocoa-butter equivalents, PUFA into plant oils, functional food, etc...)

Glycerol Metabolisms of *Clostridium butyricum*



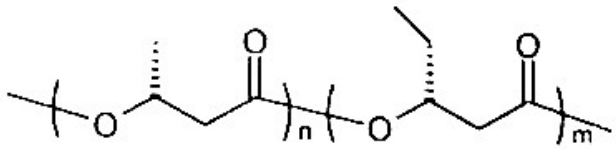
Microbial Conversion of Glycerol by *Clostridium butyricum*



10 L fed-Batch Cultivation: Mineral salts medium, 35°C;
Feed: Glycerol + 40 g/L yeast extract **[Bock, Vorlop 2004]**

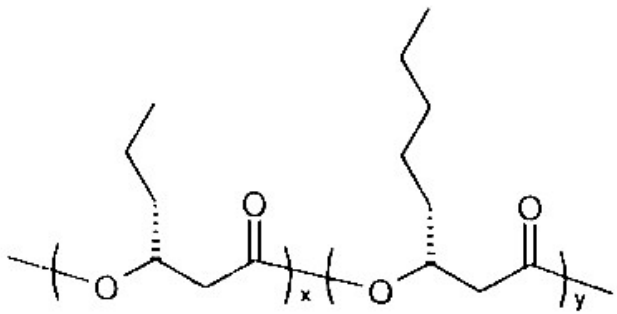
Polyhydroxyalkanoates from Triglycerides

scl - PHA
(4-5 C atoms)



Poly (3 HB - *co* - 3 HV)
[C4] [C5]

mcl - PHA
(6-14 C atoms)



Poly (3 HHx - *co* - 3 HO)
[C6] [C8]

Pseudomonas oleovorans,

Pseudomonas corrugata

Biodiesel Co-Product Stream (30 g/L):

40% glycerol
34% hexane-solubles
(FA soaps/FAME, MAG/DAG)
26% water

PHA yield: 2 g/L

Ashby et al. (2004), J. Polymers Environ. 12: 105-112

Polyhydroxyalkanoates from Triglycerides

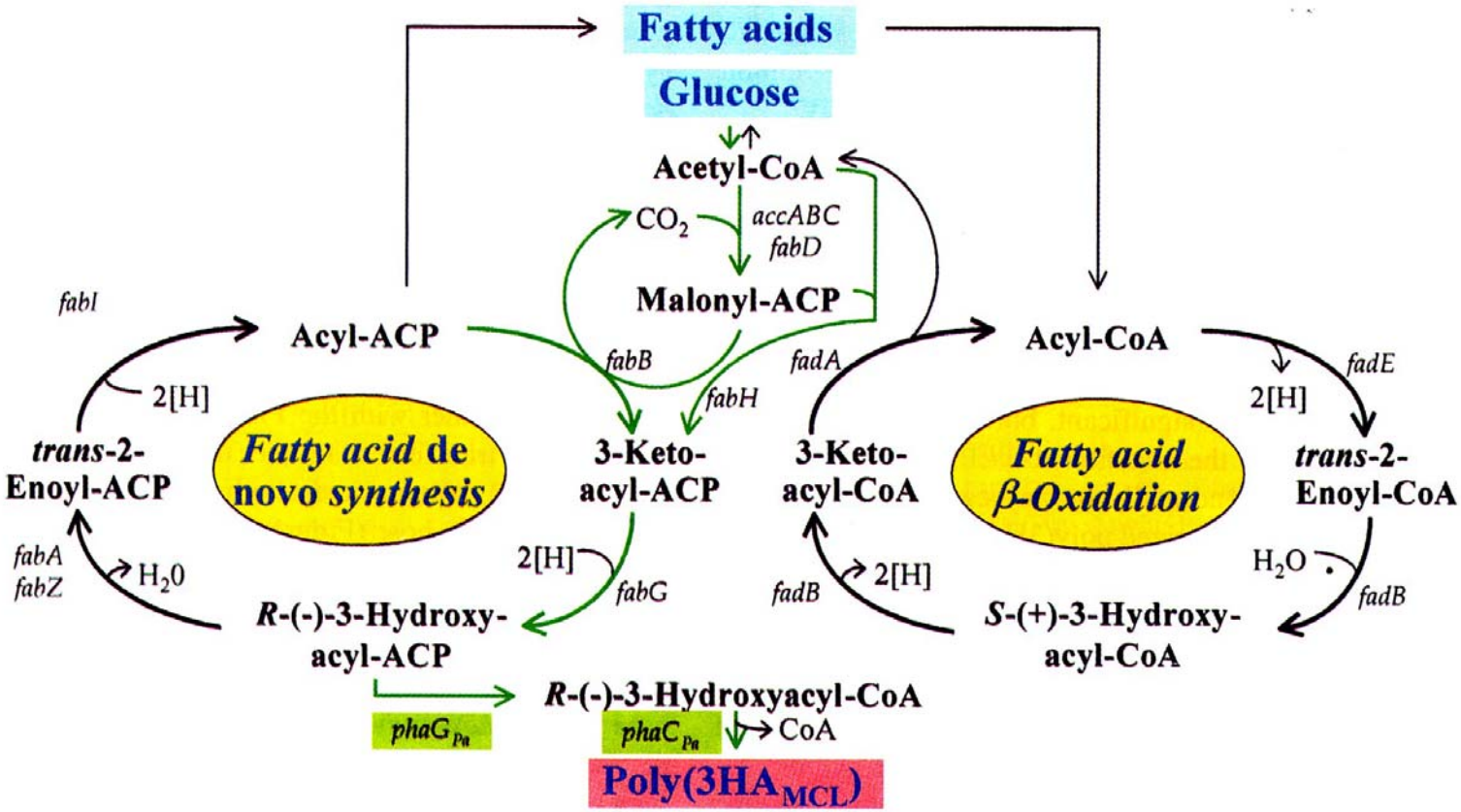


Figure 7. Metabolic link between fatty acid *de novo* biosynthesis and poly(3HA$_{MCL}$) biosynthesis.

[A. Steinbüchel (2001), Macromol. Biosci. 1: 1-24]

Polyhydroxyalkanoates from Triglycerides

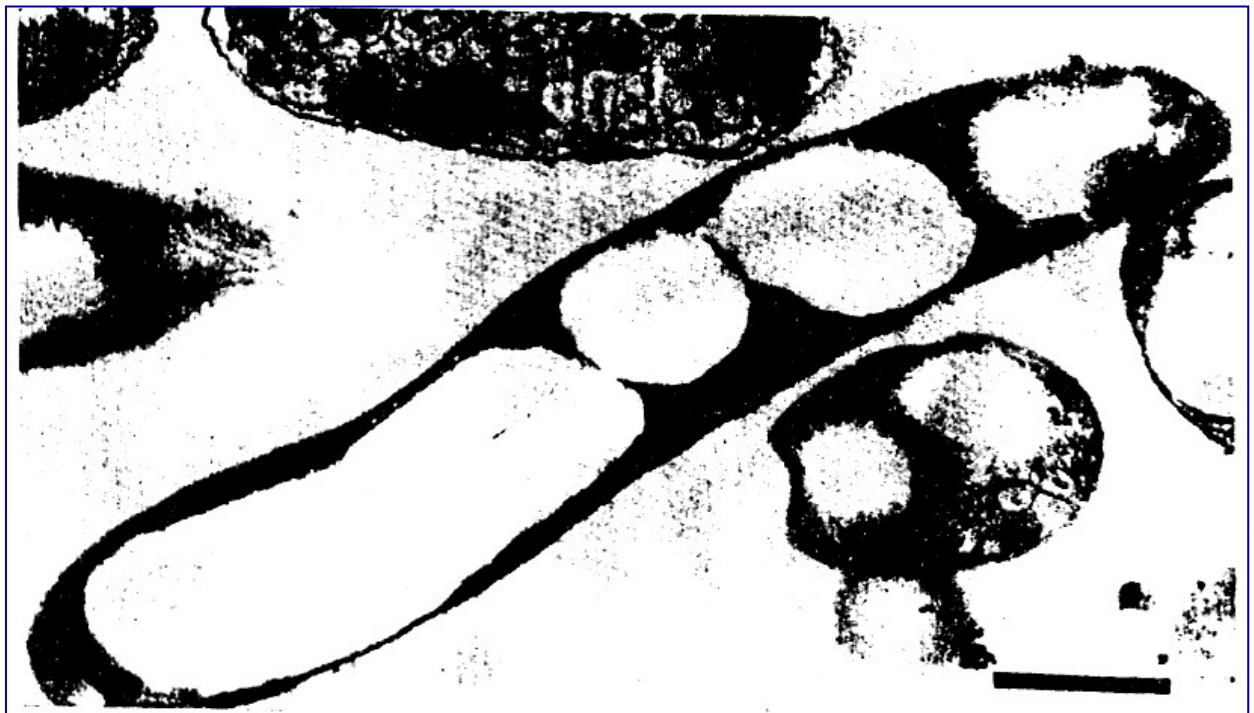
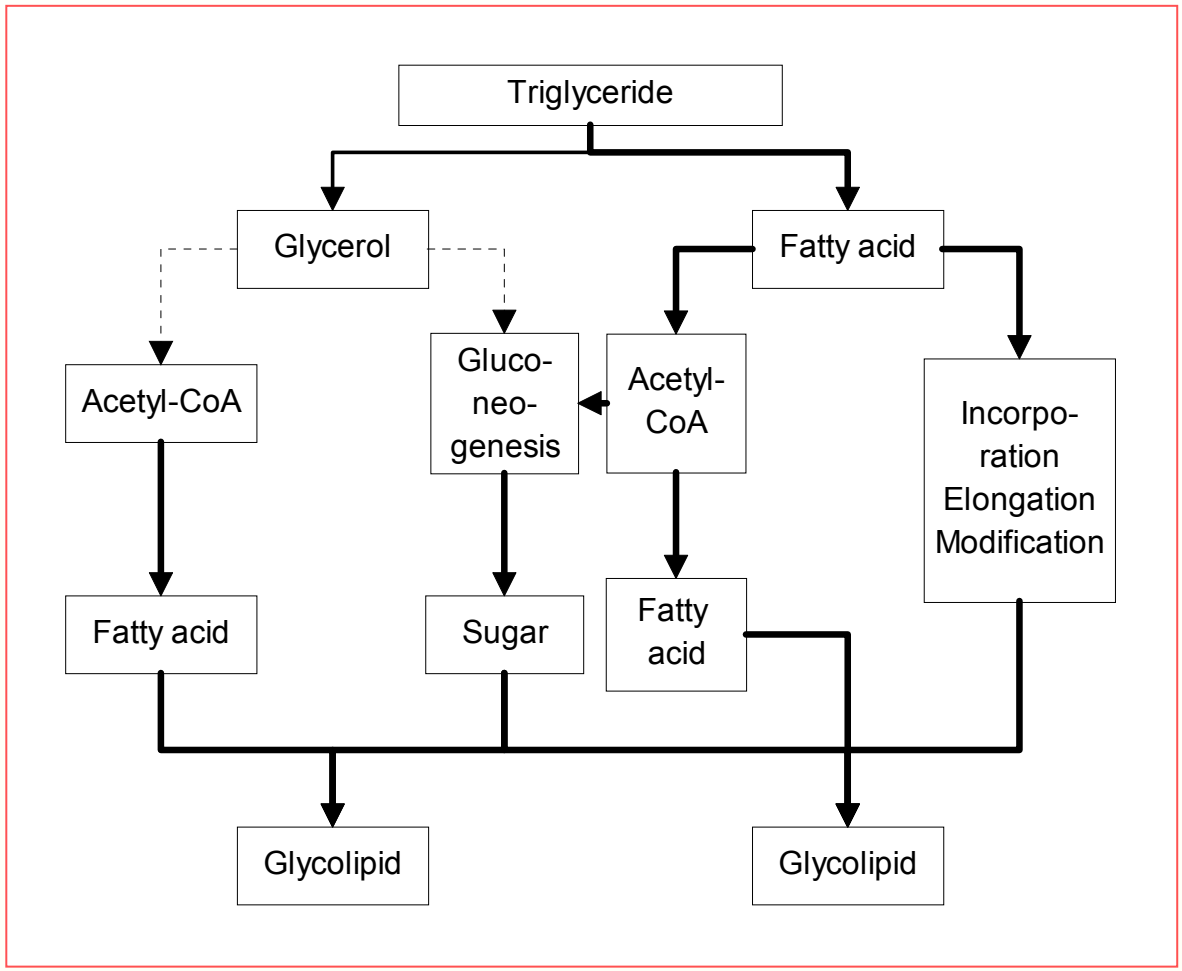
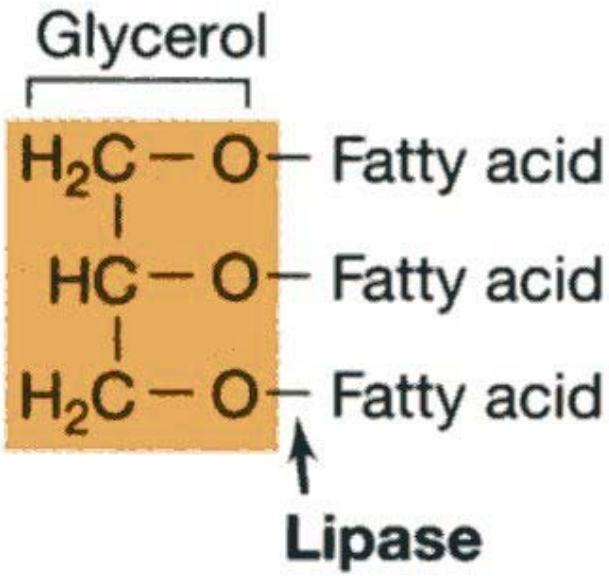


FIGURE 3. Transmission electron micrograph of the bacterium *Pseudomonas oleovorans* showing accumulation of electron-lucent PHA inclusions (courtesy of R. C. Fuller, University of Massachusetts, Amherst). Bar represents 0.5 μm.

Biosynthesis of Glycolipids from Triglycerides



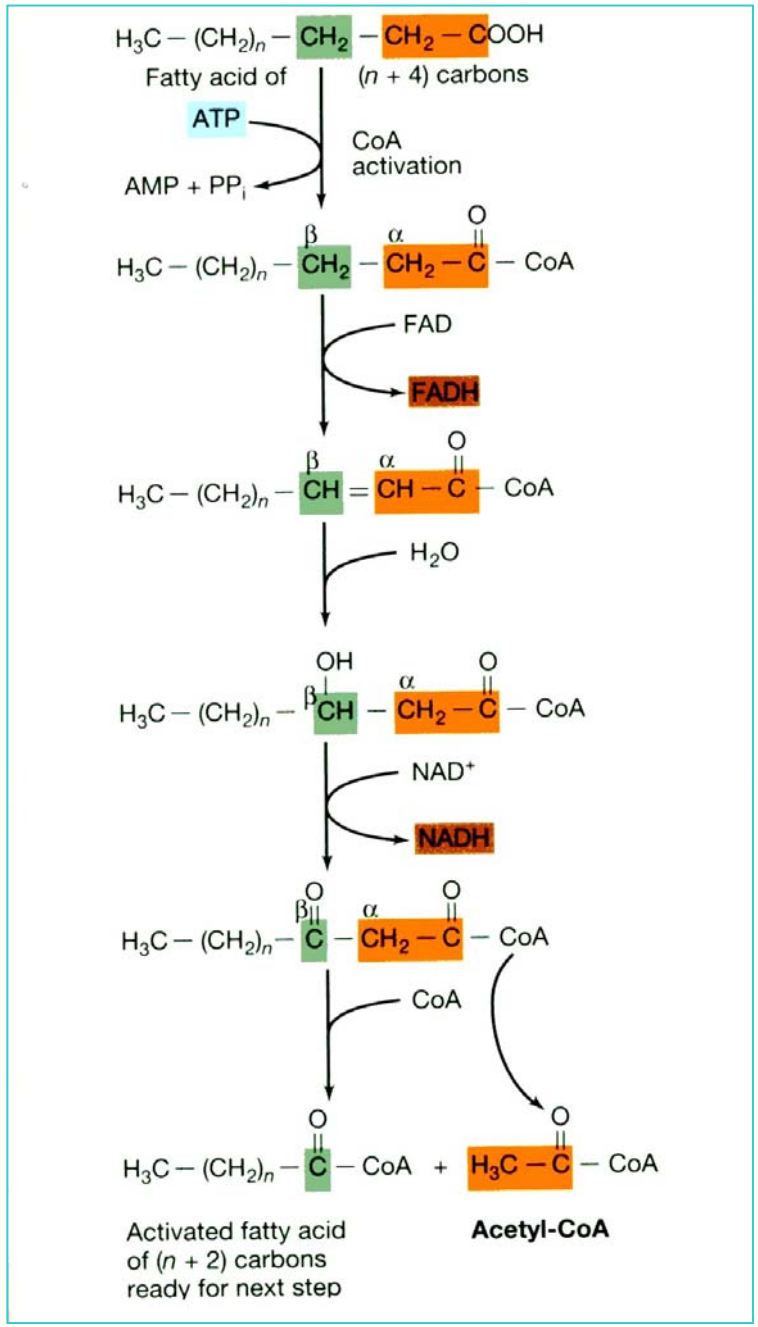
Ester Cleavage



ß-oxidation of a fatty acid:

► successive formation of

two-carbon fragments of acetyl-CoA



Biosynthesis of Fatty Acids

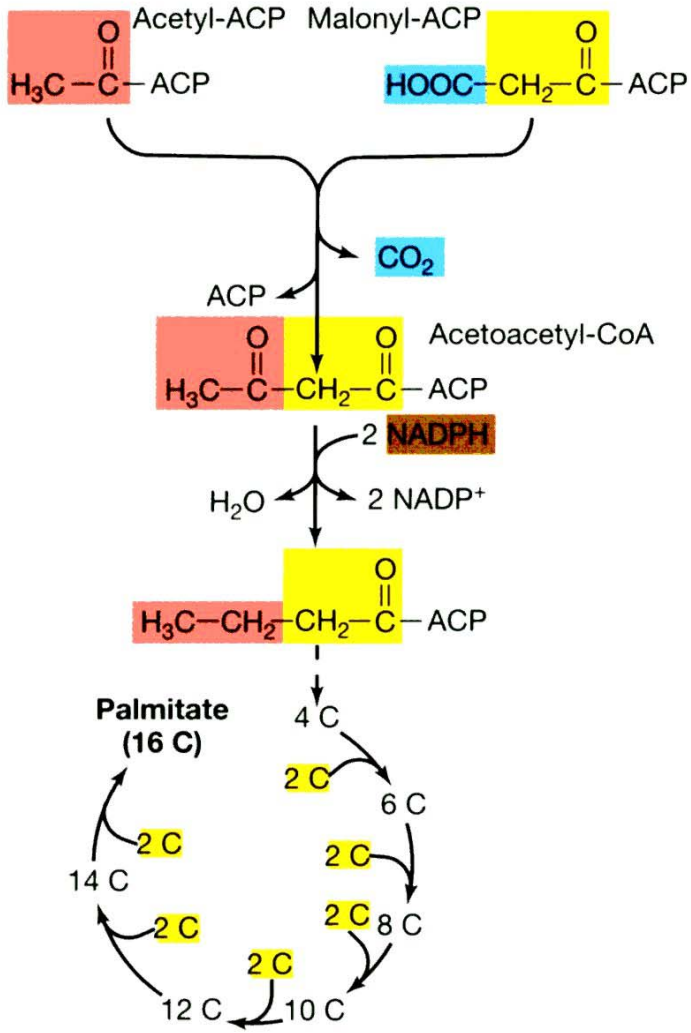


FIGURE 4.28 The biosynthesis of fatty acids; shown is the biosynthesis of the C_{16} fatty acid, *palmitate*. The condensation of acetyl-ACP and malonyl-ACP forms acetoacetyl-CoA. Each successive addition of an acetyl unit comes from malonyl-CoA.

Glycolipid Precursor Synthesis

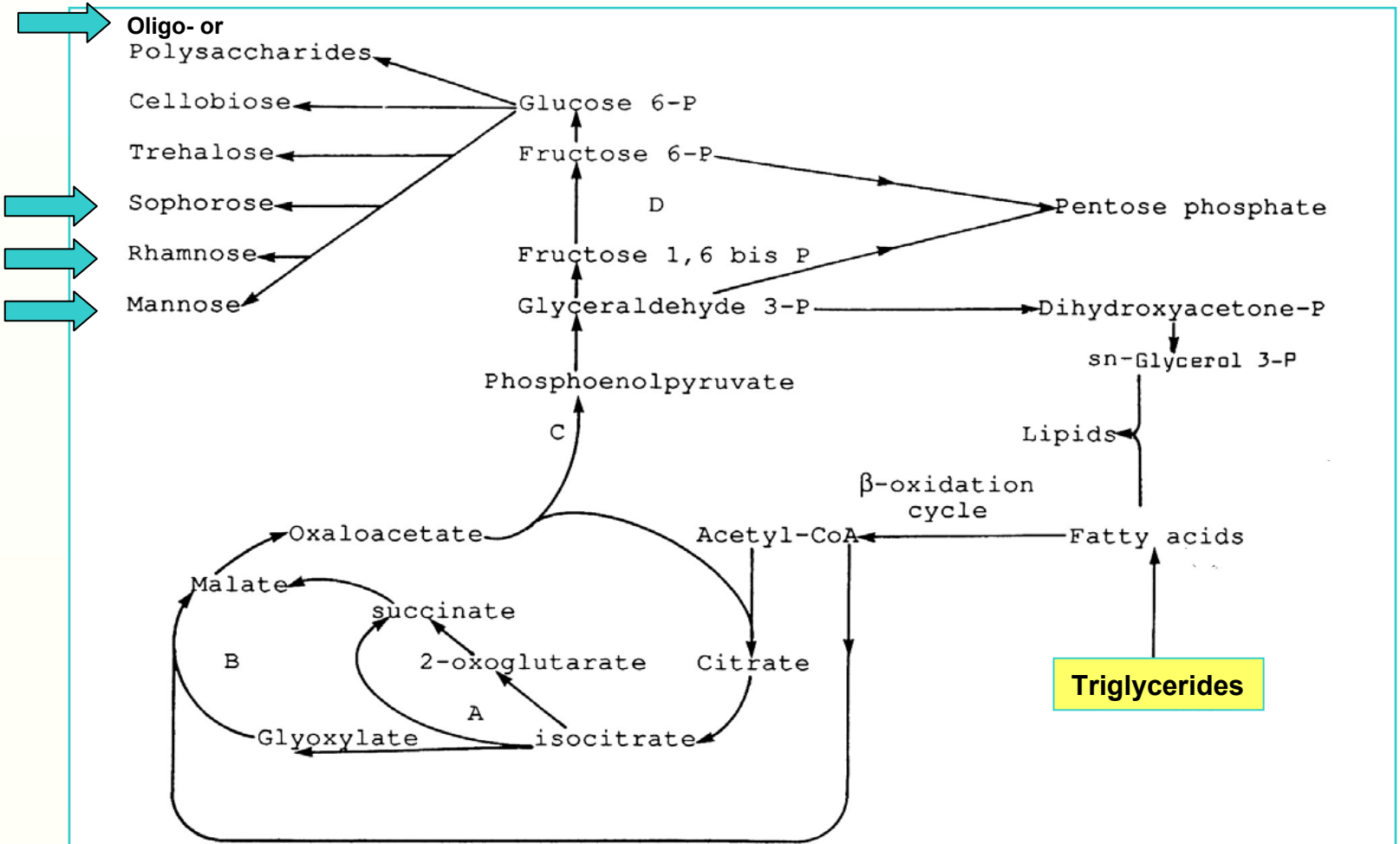


FIG. 24 Outline of intermediary metabolism relating to biosurfactant precursor synthesis from hydrophobic substrates. Key enzymes are A, isocitrate lyase; B, malate synthase; C, phosphoenolpyruvate carboxykinase; D, fructose-1,6-bisphosphatase.

Hommel & Ratledge, 1993

Overview

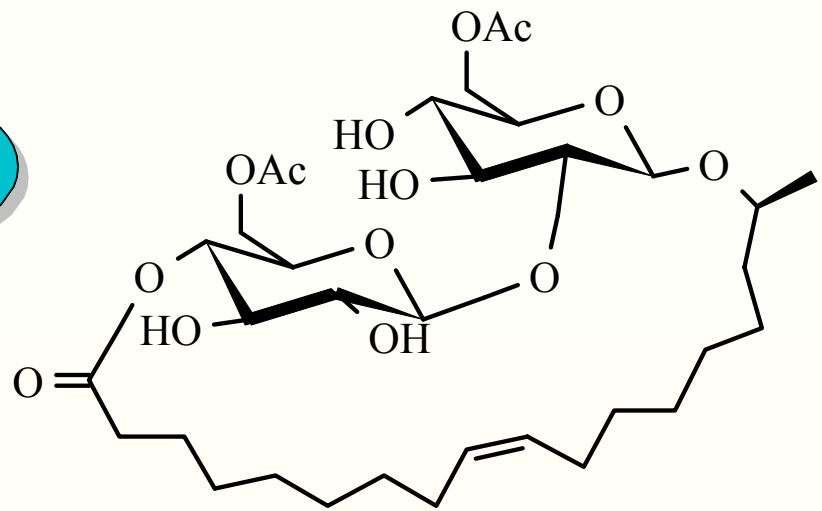
1. Fungal Sophorose Lipids
3. Bacterial Di- and Oligosaccharide Lipids
4. Bacterial Rhamnose Lipids

1. Fungal Sophorose Lipids

Sophorose Lipids

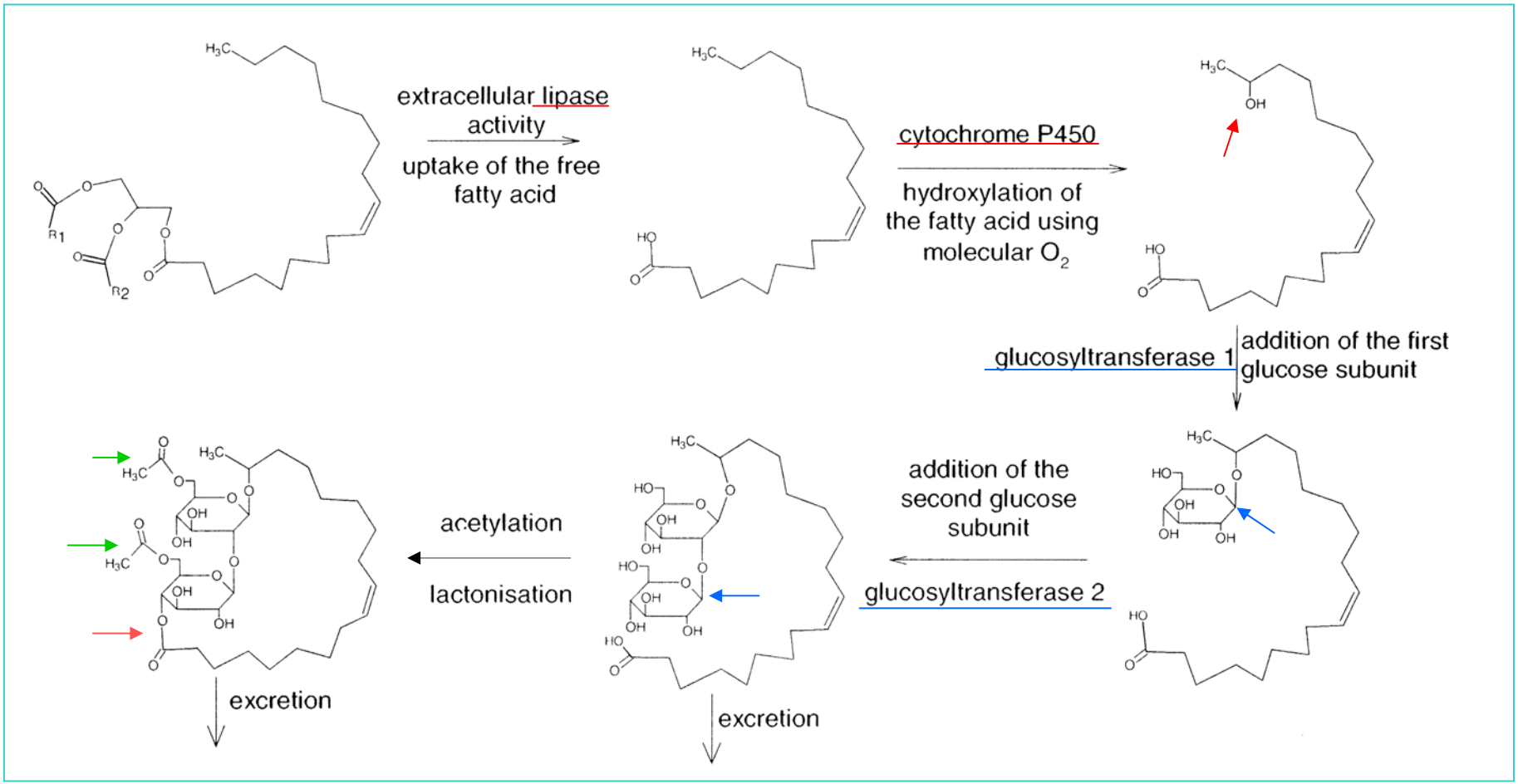
Glucose
+
Rapeseed Oil

Candida bombicola



C. bombicola: Biosynthesis of Sophorose Lipids

Carbon Sources: Glucose + Cosubstrates



SJJ Fleurackers (2006), Eur J Lipid Sci 108: 5-12

Sophorolipid Production with *C. bombicola*

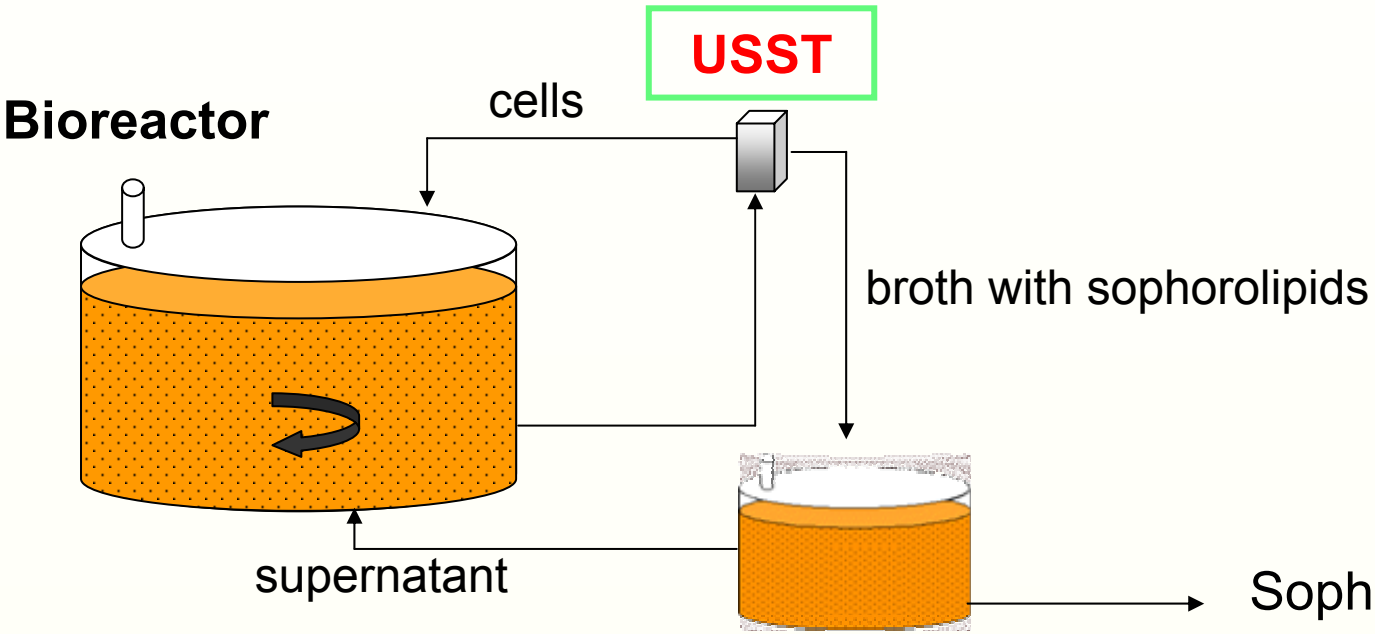
Discovered: 1961 [Gorin et al.]

State of the art: Fed batch fermentations

Carbon Sources [g/L]	Product [g/L]	Conversion [%]	Reference
Glucose / Rapeseed oil [490]	320	65	Davila et al. (1992) Appl. Microbiol. Biotechnol. 38: 6-11
More recently:			
Soy molasses / Oleic acid [120]	21	18	Solaiman et al. (2004) Biotechnol. Lett. 26: 1241-1245
Biodiesel co-product stream* [300]	60	20	Ashby et al. (2005) JAOCS 82: 625-630
Glycerol / Me-Soyate [145]	42	29	Ashby et al. (2006) Biotechnol. Lett. 28: 253-260
Glucose / Corn oil [670]	400	60	Pekin et al. (2005) Eng. Life Sci. 5: 357-362
Glucose / Frying oil [200]	50	25	Fleurackers (2006) Eur. J. Lipid Sci. Technol. 108: 5-12

* 40% glycerol, 34% hexane-solubles (92% FA soaps/FAME + 6% MAG/DAG), 26% water

Fermentation set-up using UltraSound Separation Technology



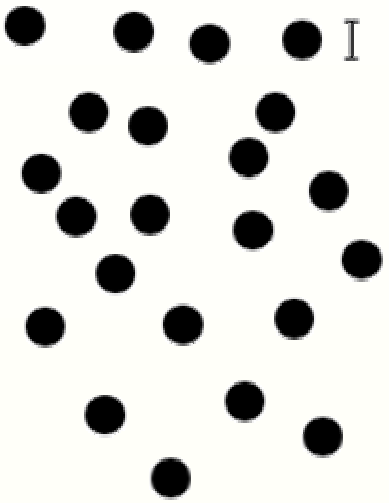
Small reactor: settling vessel for sophorolipids

Aims: - continuous product removal

- facilitated downstream processing

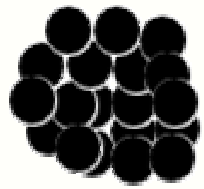
- limitation of broth viscosity

Principle of ultrasonically settling technology



agglomeration

by ultrasound



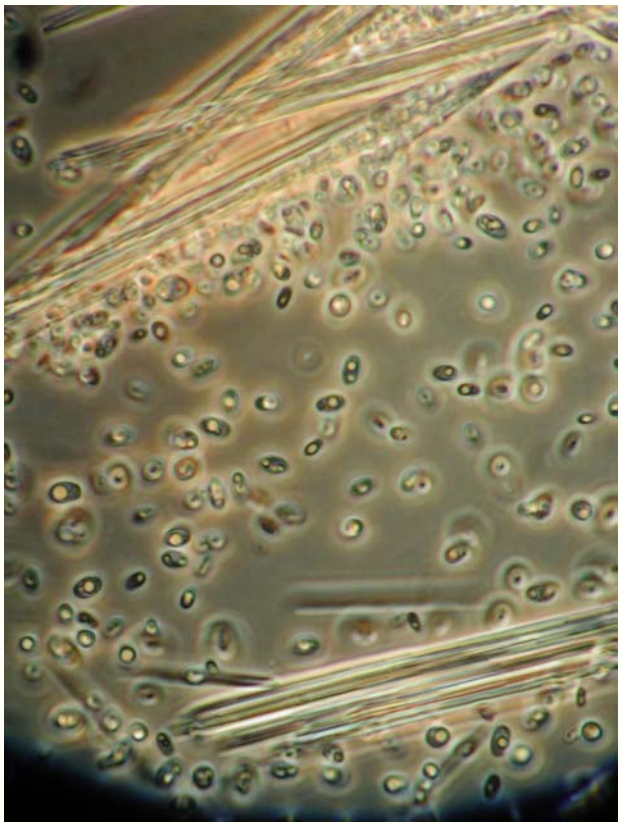
Practice

flow direction

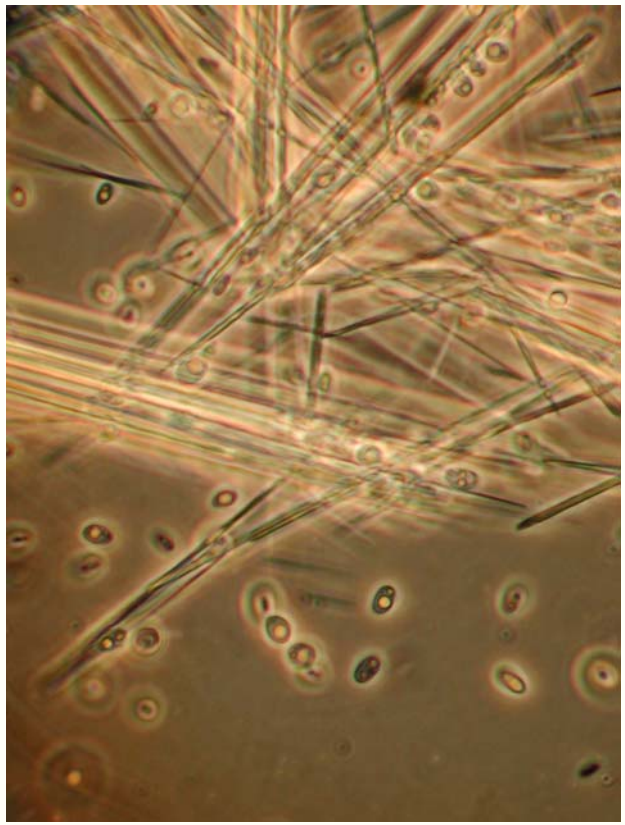
of feed stream

settling cells

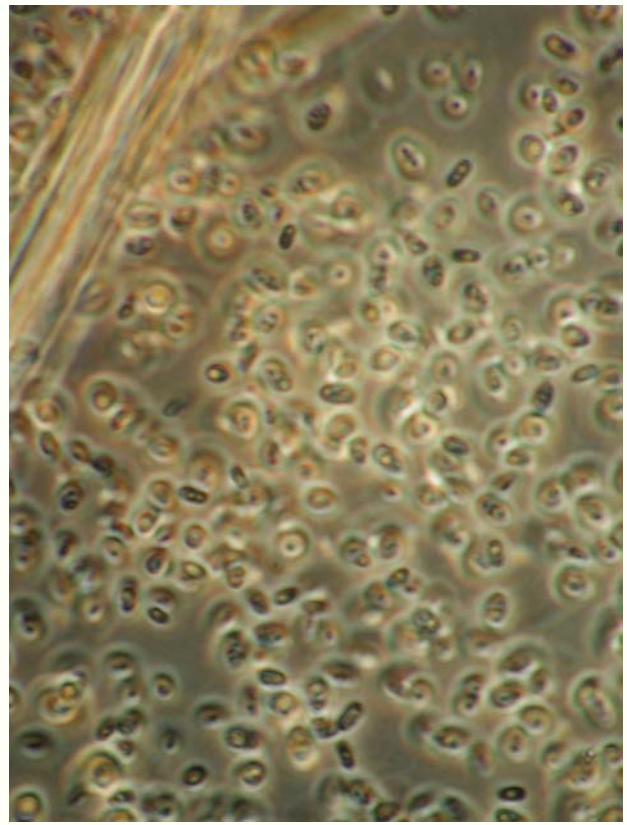
Separation efficiency using ultrasound separation



Feed

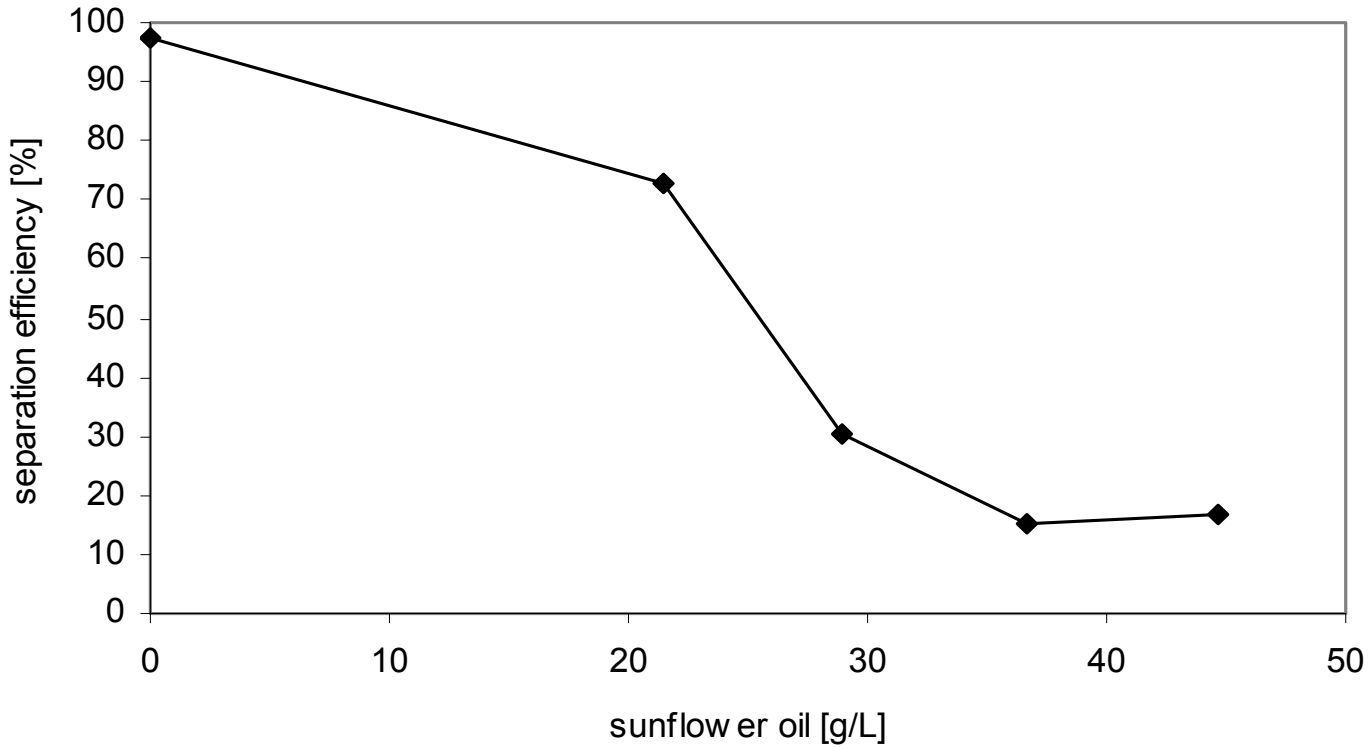


Permeate



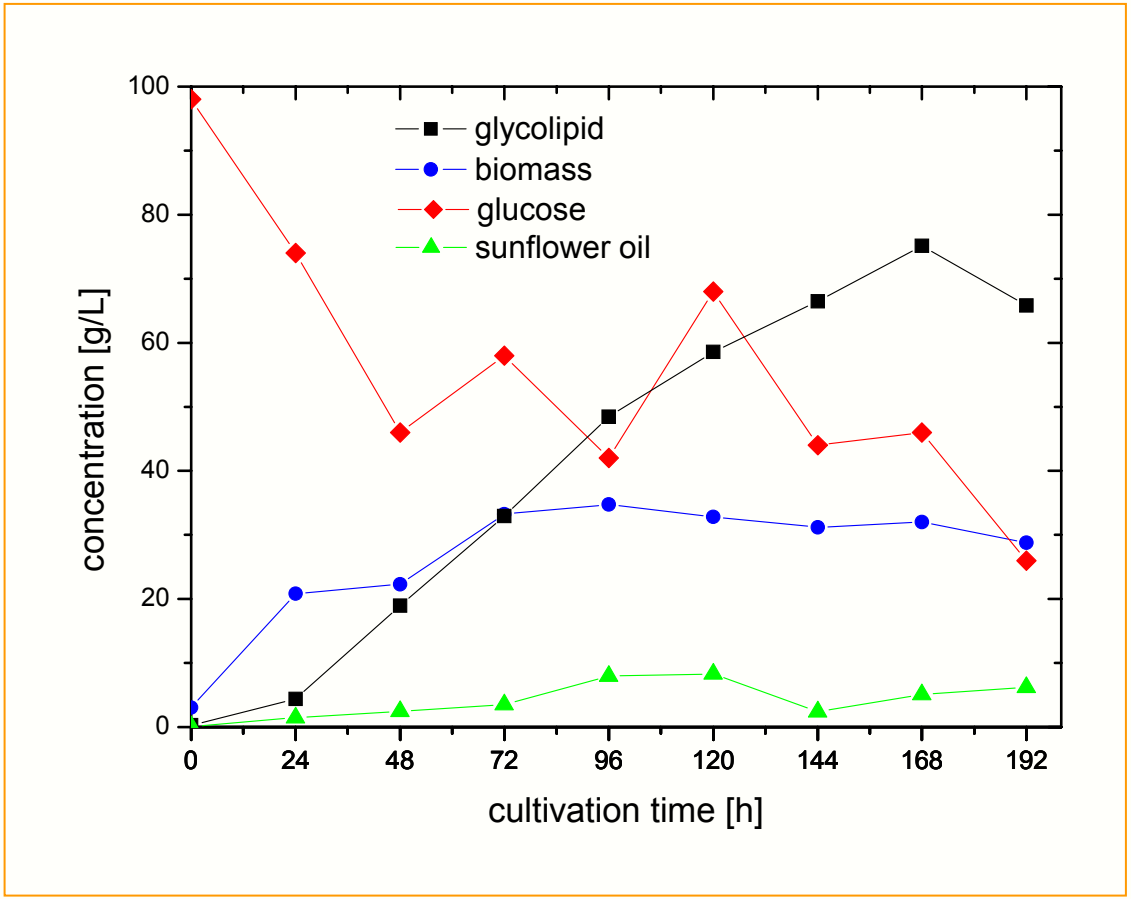
Retentate

Separation efficiency using ultrasound separation



Oil problem is currently worked at by adding a separation chamber to ultrasound separator

Fermentation Results



5 L fermentation with sunflower oil and glucose feeding using integrated ultrasound separation:

70 g/L sophorose lipids; 70 g could be separated by USST

► totally 84 g/L sophorose lipids

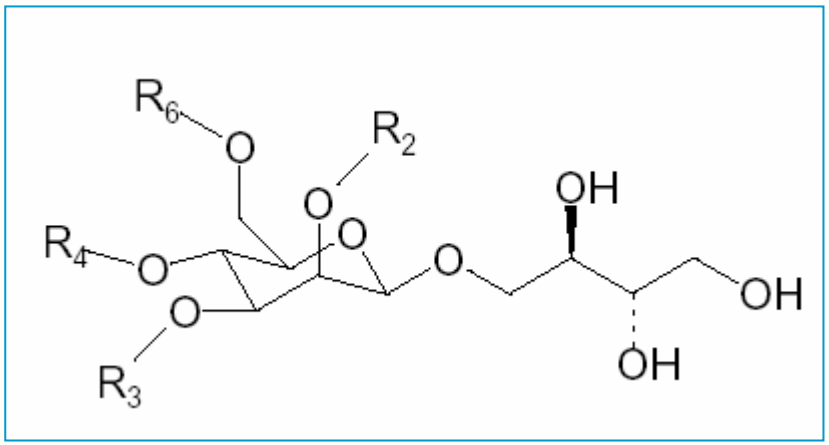
Overview

1. Fungal Sophorose Lipids

2. Fungal Mannosylerythritol Lipids

4. Bacterial Rhamnose Lipids

History of Mannosylerythritol Lipids (MEL)



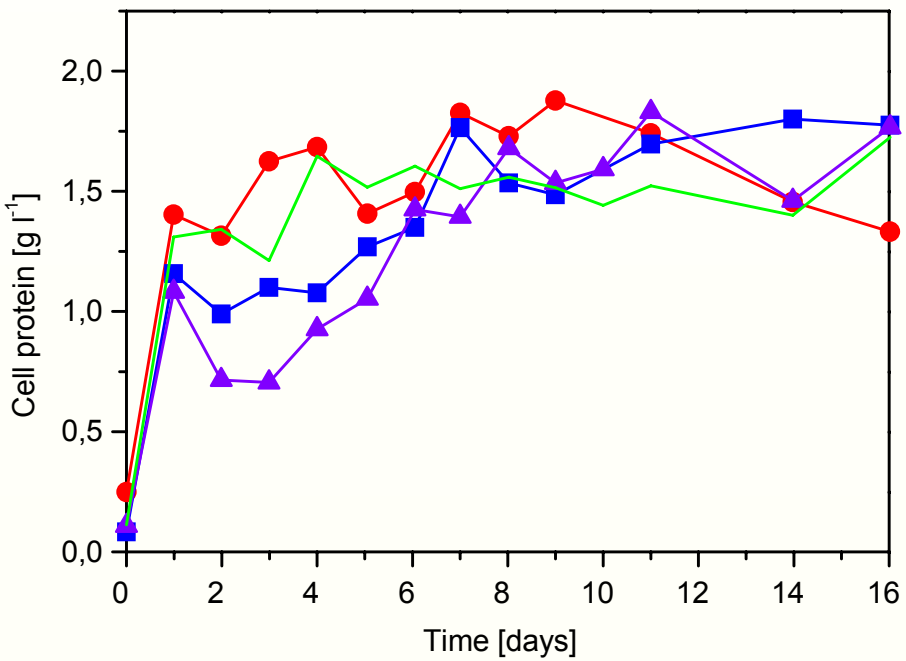
Properties:

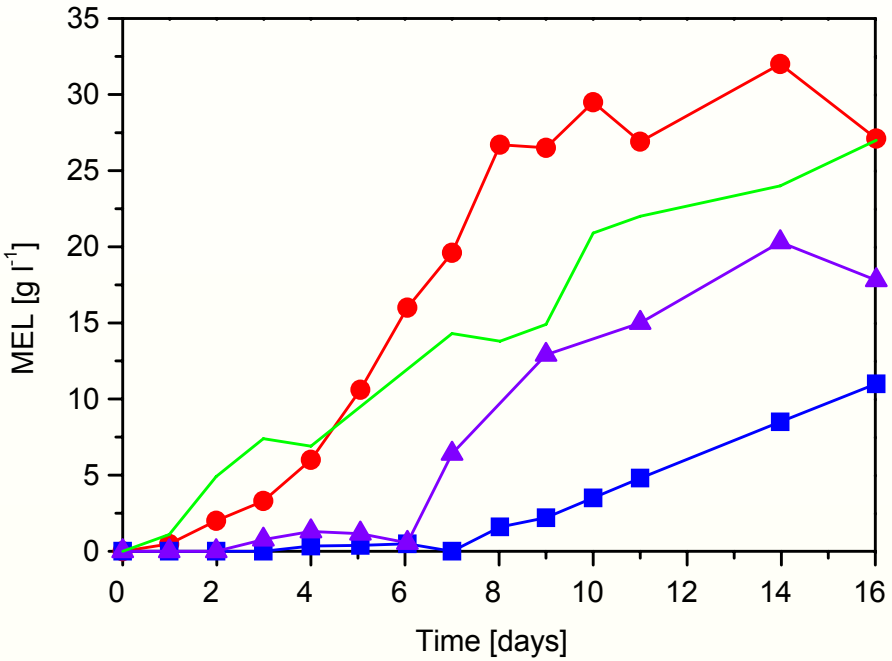
- Surface tension lowering of water: 72 → 28 mN m^{-1}
- Bioactivities (vs. leukemia cell lines etc.)

Shake flask experiments (30°C):

Strain	Carbon Source (g l^{-1})	Yield (g l^{-1})	Ref.
Ustilago nuda PRL-627	Glucose (100)	16	Haskins et al. 1955
Ustilago maydis ATCC 14826	Sunflower oil/FA (45)	30	Spoeckner et al. 1999
Candida antarctica T-34	Soybean oil (72)	47	Kitamoto et al. 1992
Pseudozyma (Candida) antarctica T-34	n-Octadecane (187)	140	Kitamoto et al. 2001
Candida antarctica ATCC 20509	Soapstock (100)	14	Bednarski et al. 2004

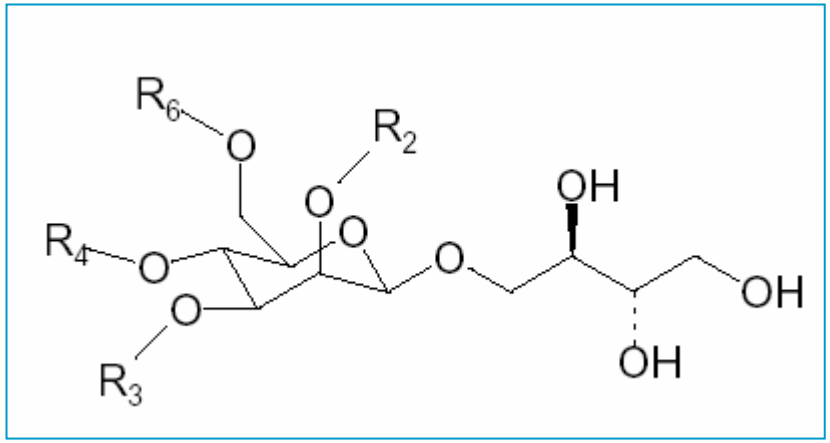
Growth (left) and MEL Production (right)





Strains:
***Pseudozyma aphidis*:** **(●) DSM 14930, (✶) CBS 6821**
***Pseudozyma antarctica*: (▲) CBS 6678, (■) CBS 5955**

Medium A: 67 g l^{-1} soybean oil, 2 g l^{-1} $NaNO_3$, etc., pH 6; 30°C, 100 rpm, **shake flasks**



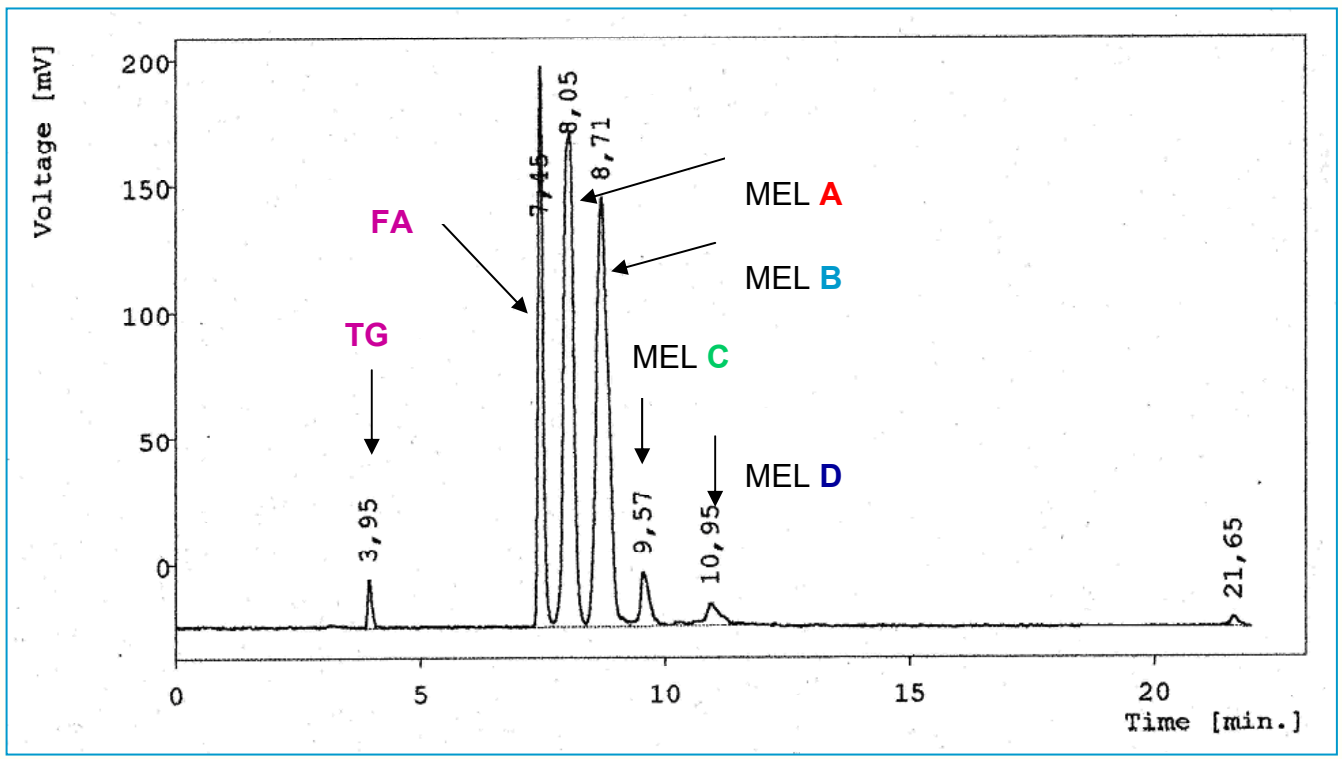
MEL **A-D**: R_2, R_3 = C_7-C_{14}

MEL **A**: R_4, R_6 = acetyl

MEL **B**: R_4 = acetyl, R_6 = H

MEL **C**: R_4 = H, **R_6 = acetyl**

MEL **D**: R_4 = H, R_6 = H



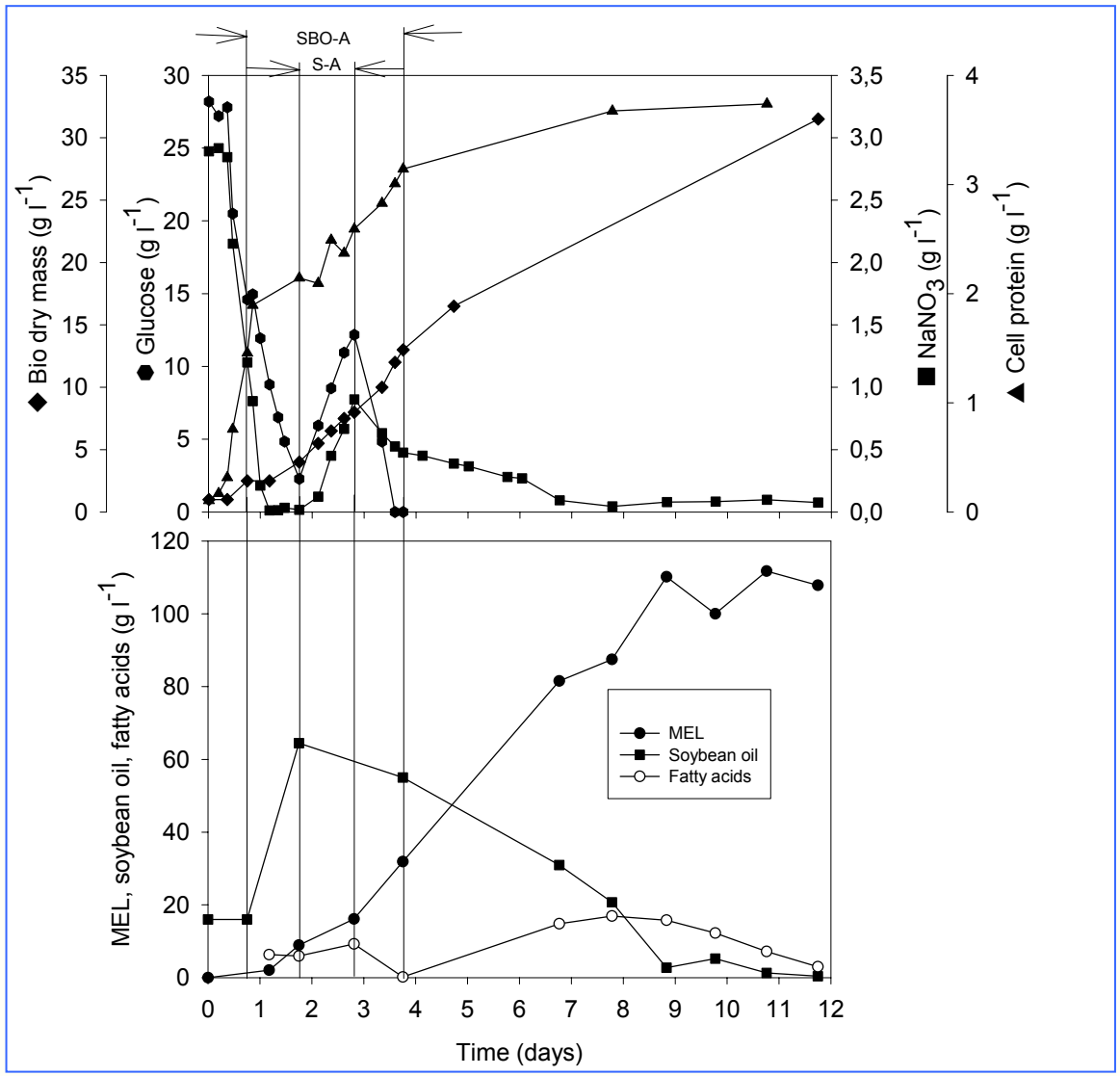
HPLC:

Silicagel (NP)

$CHCl_3/CH_3OH$

ELS detector

Pseudozyma aphidis DSM 14930: 30 l cultivation



30 L Cultivation:

300 rpm; aeration: 540 l h^{-1}

medium A:

incl. **30 g l^{-1} glucose**

17 g l^{-1} soybean oil

3 g l^{-1} $NaNO_3$

Feeding:

5.1 kg soybean oil [SBO-A]

0.9 kg glucose [S-A], add. Nitrate, YE

Yield:

3.8 g l^{-1} cell protein

165.0 g l^{-1} MEL [incl. flotated MEL]

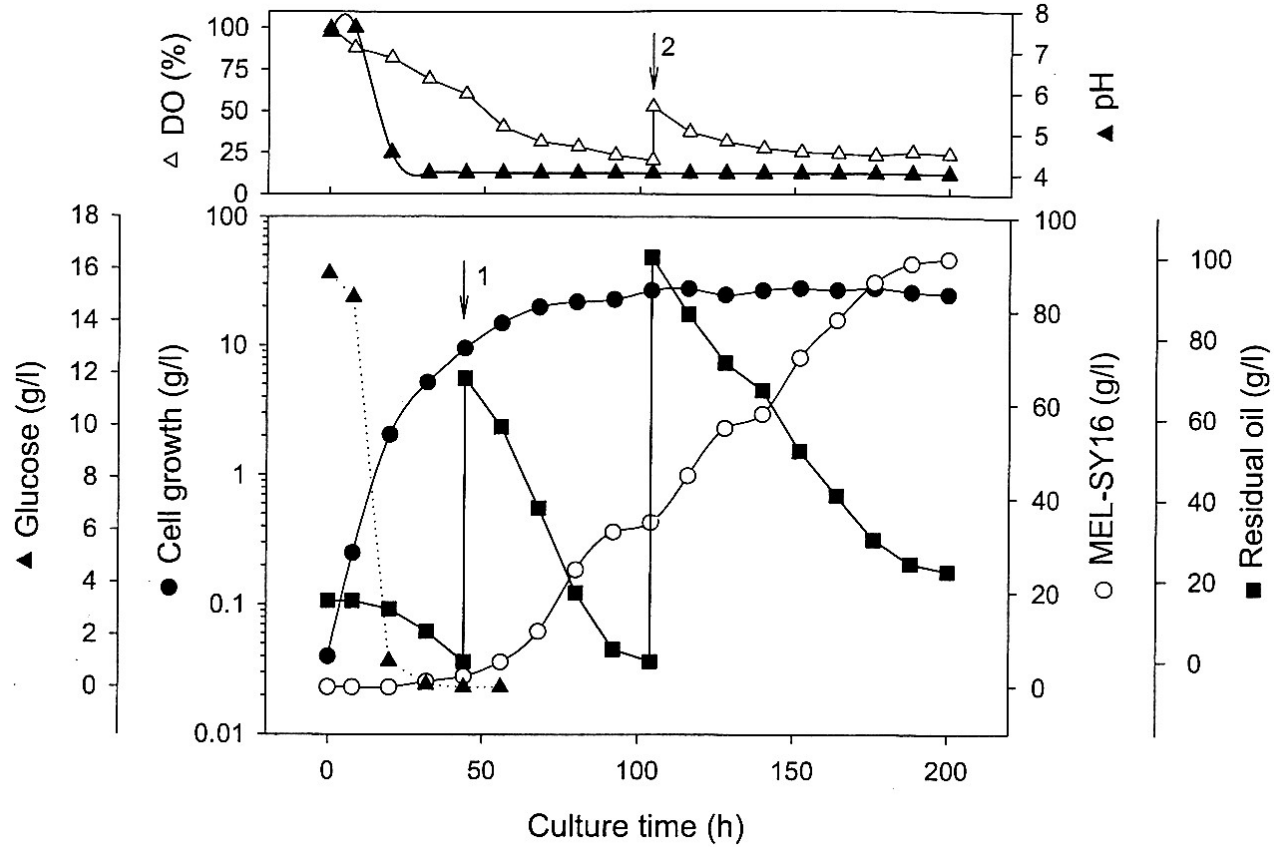
Conversion: 66%

[Rau, Lang et al. (2005), Appl. Microbiol. Biotechnol. 70: 391-396]

MEL production by *Candida* sp. SY16

[Kim et al. (2006), Appl. Microbiol. Biotechnol. 70: 391-396]

Fig. 4 Fed-batch fermentation for MEL production by *Candida* sp. SY16. The fermentation was performed at 30°C with an agitation of 500 rpm and aeration of 1 vvm in a 5-l jar fermentor (initial working volume of 2 l). The culture pH was controlled at 4.0 (±0.2) using a 2N NaOH solution. *Arrow 1* indicates the feeding of soybean oil to a concentration of 70 g l^{-1}, and *arrow 2* indicates the soybean oil feeding to 100 g l^{-1} and changes in the aeration and agitation to 0.3 vvm and 700 rpm, respectively, from the initial conditions (1 vvm and 500 rpm)



Conversion: 45%

MEL production by *Candida* sp. SY16

[Kim et al. (2006), Appl. Microbiol. Biotechnol. 70: 391-396]

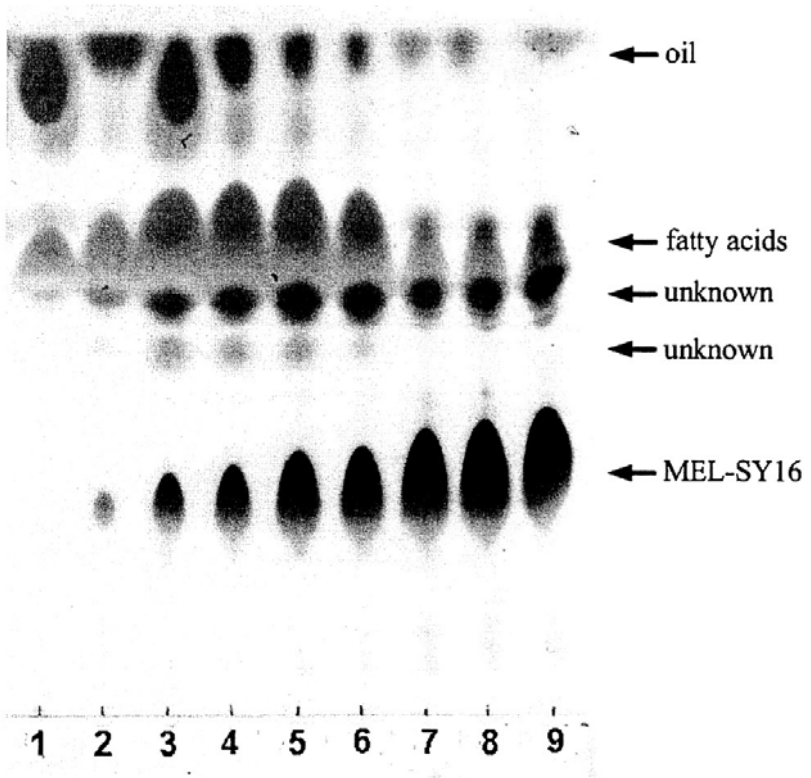
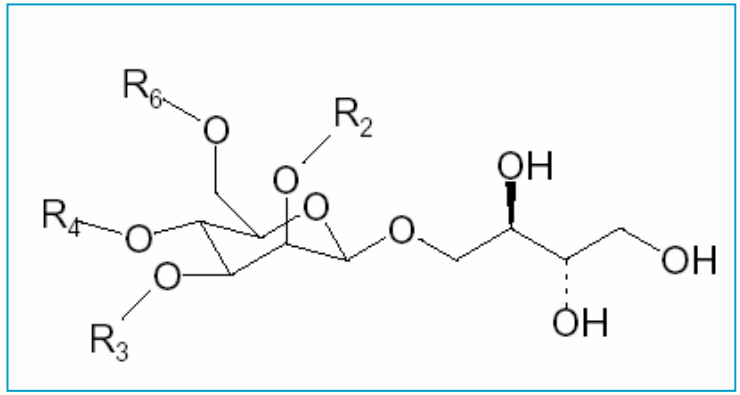


Fig. 5 TLC patterns of lipid mixture isolated from fed-batch fermentation of *Candida* sp. SY16. Samples were extracted from the fed-batch culture (Fig. 4) using ethyl acetate, and the organic solvent fraction spotted onto a TLC plate. The spots were visualized with 30% H_2SO_4. *Lanes 1* to *9* are the samples cultured after 44, 68, 104, 128, 140, 152, 164, 176, and 200 h, respectively



R_2, R_3 = C_6, C_{12}, C_{14}

R_6 = acetyl

R_4 = H

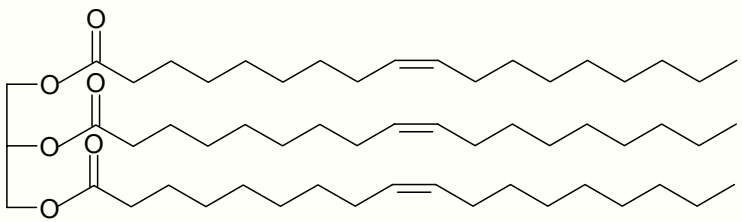
Overview

1. Fungal Sophorose Lipids
2. Fungal Mannosylerythritol Lipids

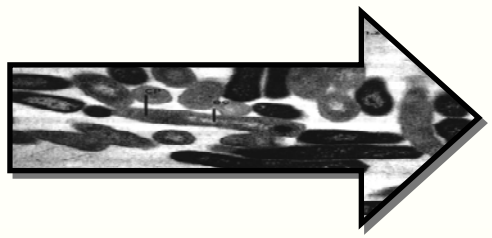
3. Bacterial Di- and Oligosaccharide Lipids

4. Bacterial Rhamnose Lipids

Glycolipids from *Tsukamurella* sp.



Carbon source: vegetable oils

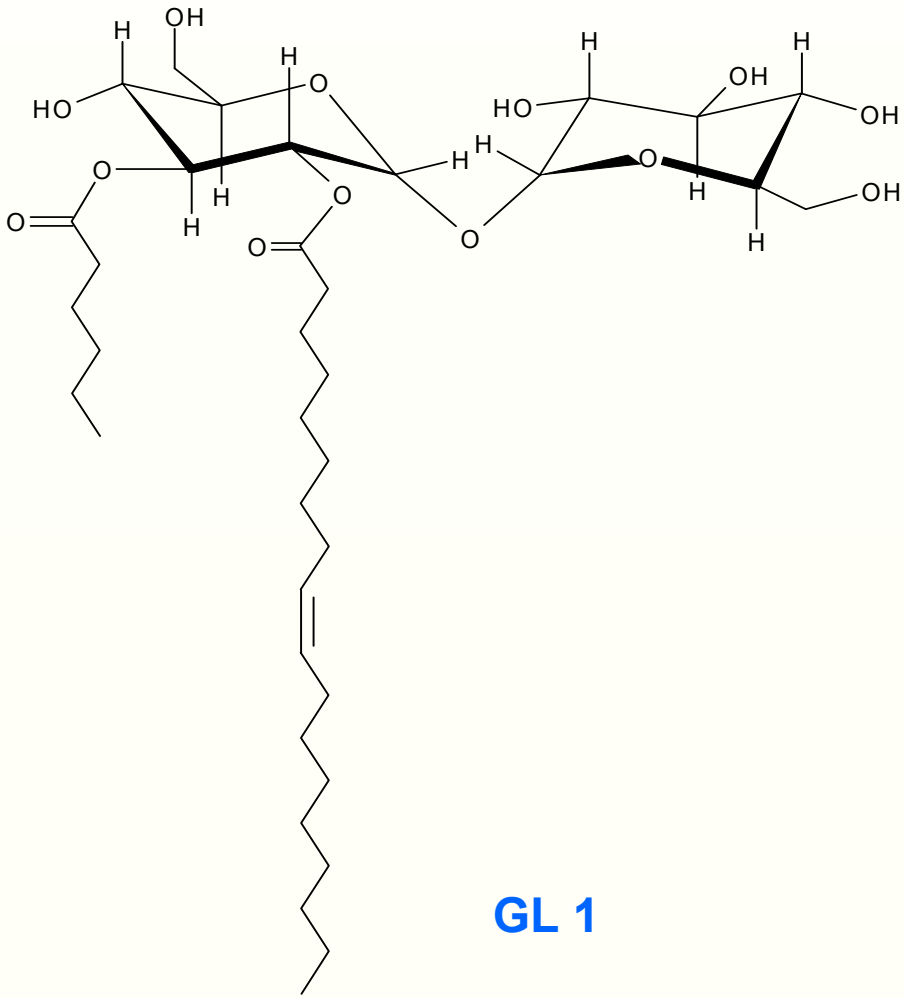


***Tsukamurella* spec.**
30°C, pH 7.5

Thin Layer Chromatography:

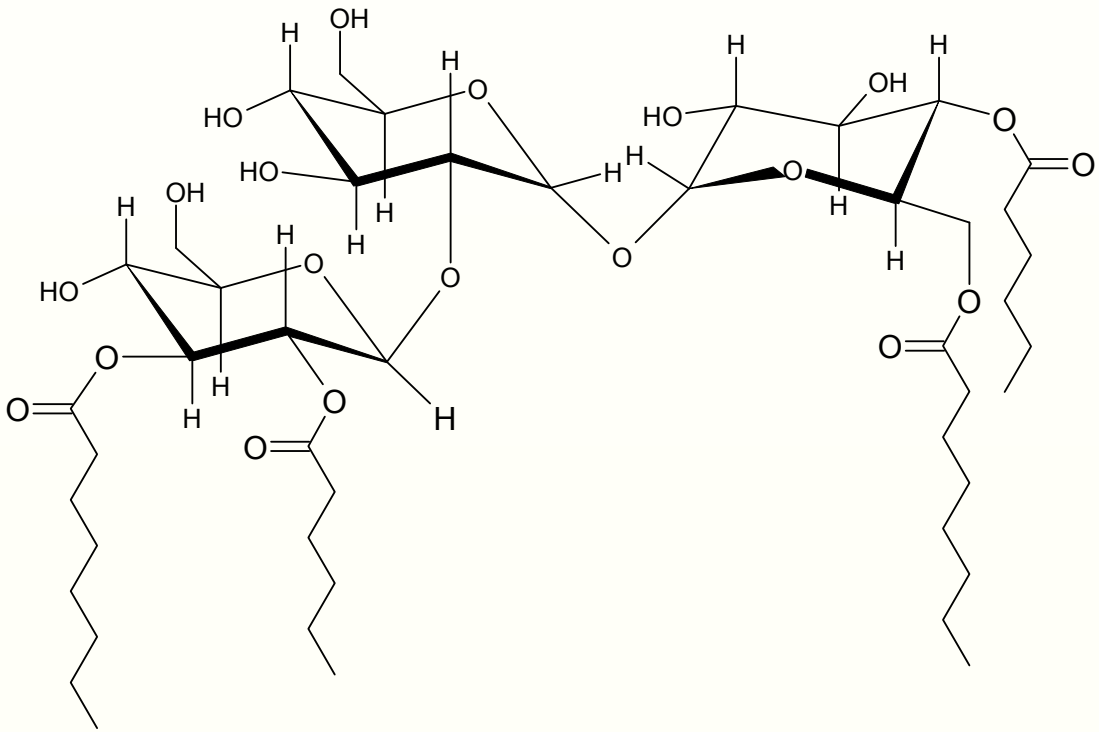
Glycolipids

Molecular structures of *Tsukamurella* glycolipids

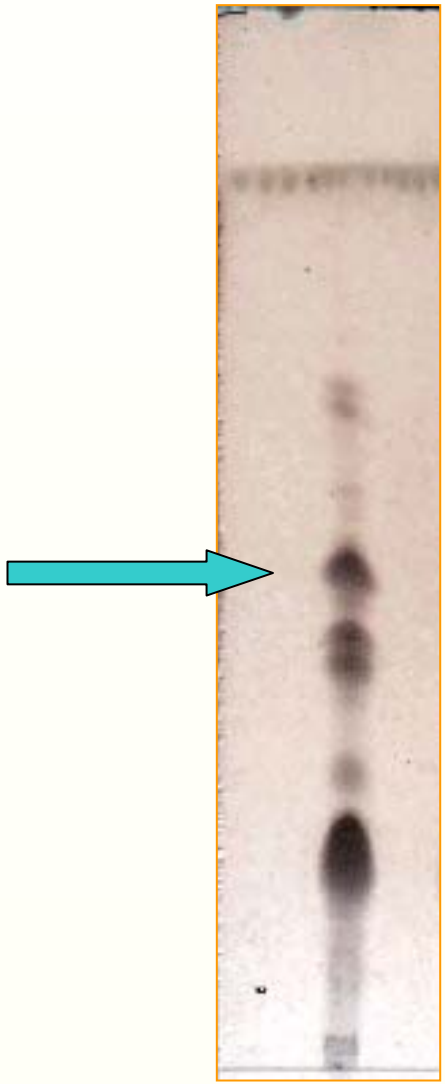


GL 1

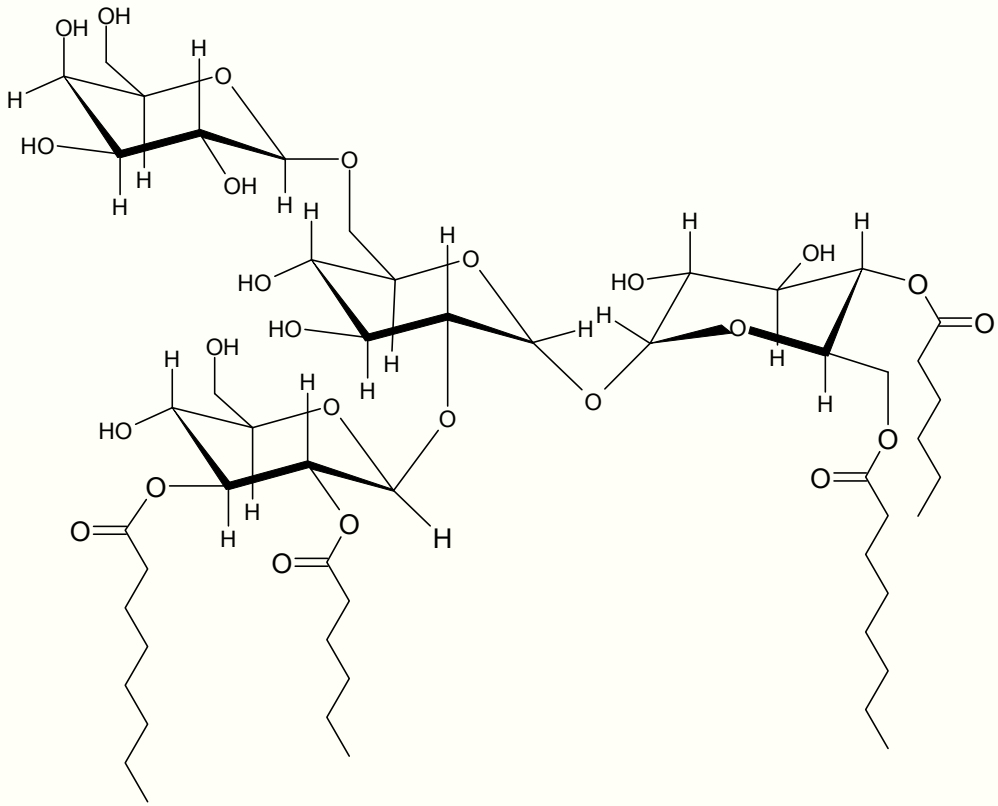
Molecular structures of *Tsukamurella* glycolipids



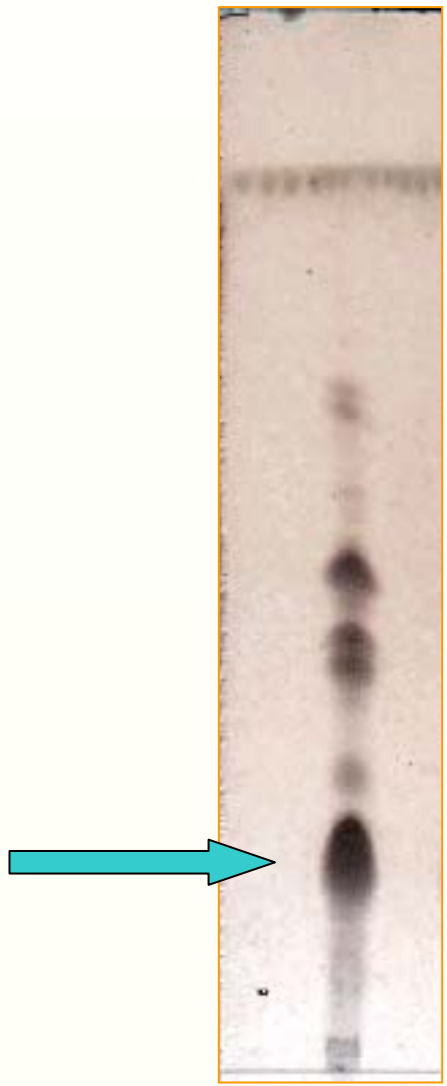
GL 2



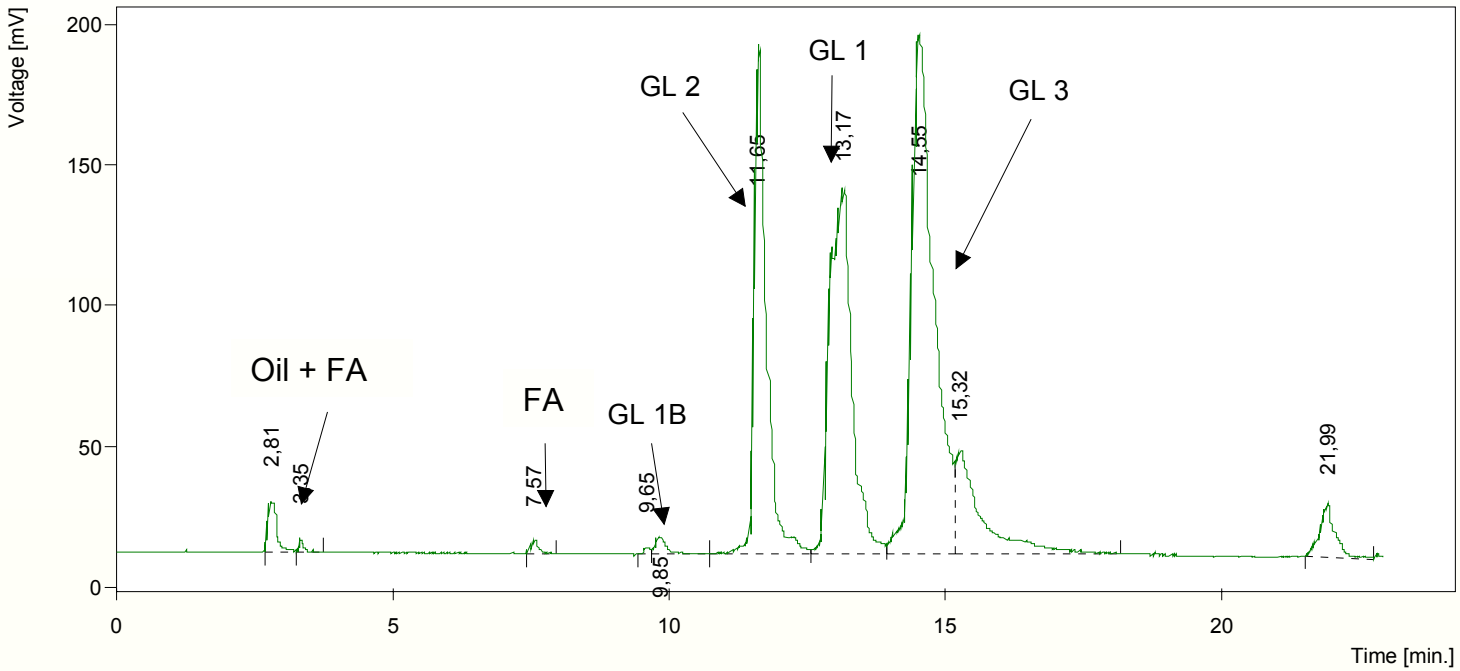
Molecular structures of *Tsukamurella* glycolipids



GL 3



HPLC – Glycolipid analysis



System:

Silicagel (NP), Chloroform/Methanol - Gradient elution system, evaporative light scattering detector

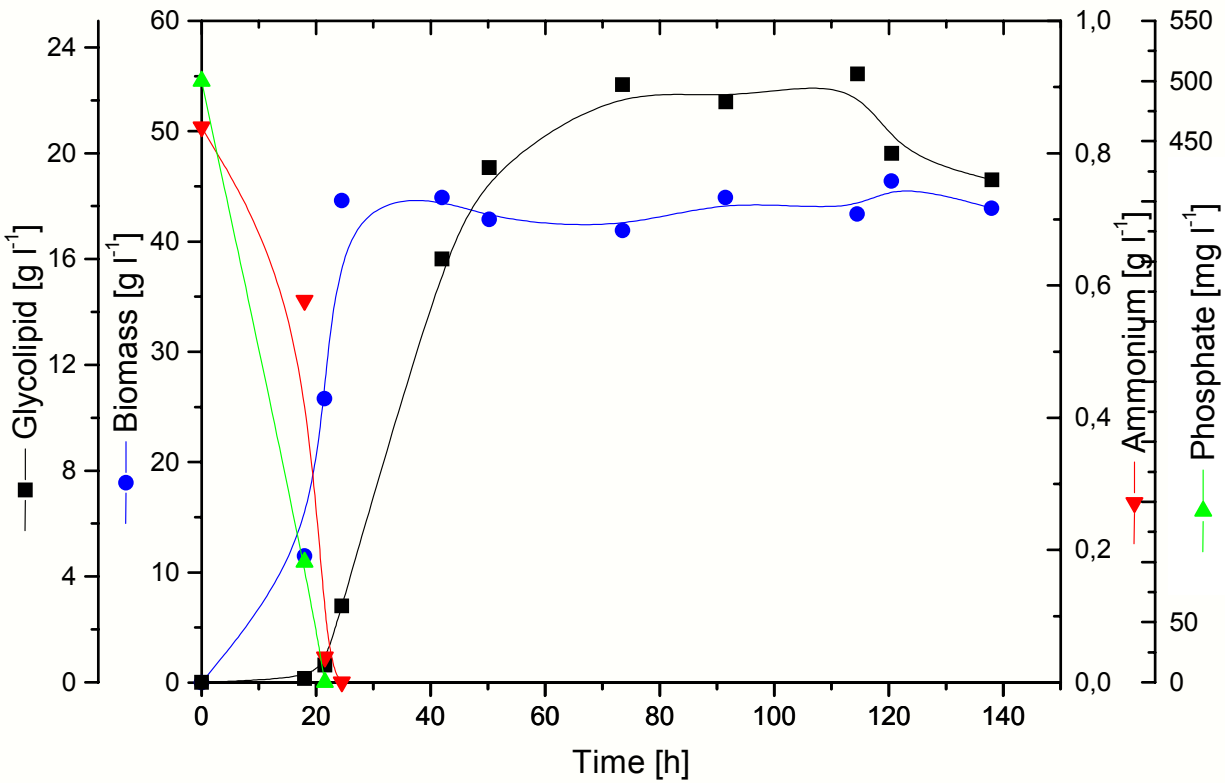
Bioreactor Cultivations

Production of the Glycolipids

Conditions:

- Volume	20 l working volume
- Stirrer speed	550 rpm
- Stirrer type	Rushton turbines
- Temperature	30°C
- pH	adjusted to 7.5
- Aeration	0.4 v/vm
- Nutrient	Mineral salts medium incl. K_2HPO_4, $(NH_4)SO_4$, Sunflower oil, e.g.

20 l - Cultivation on Sunflower Oil

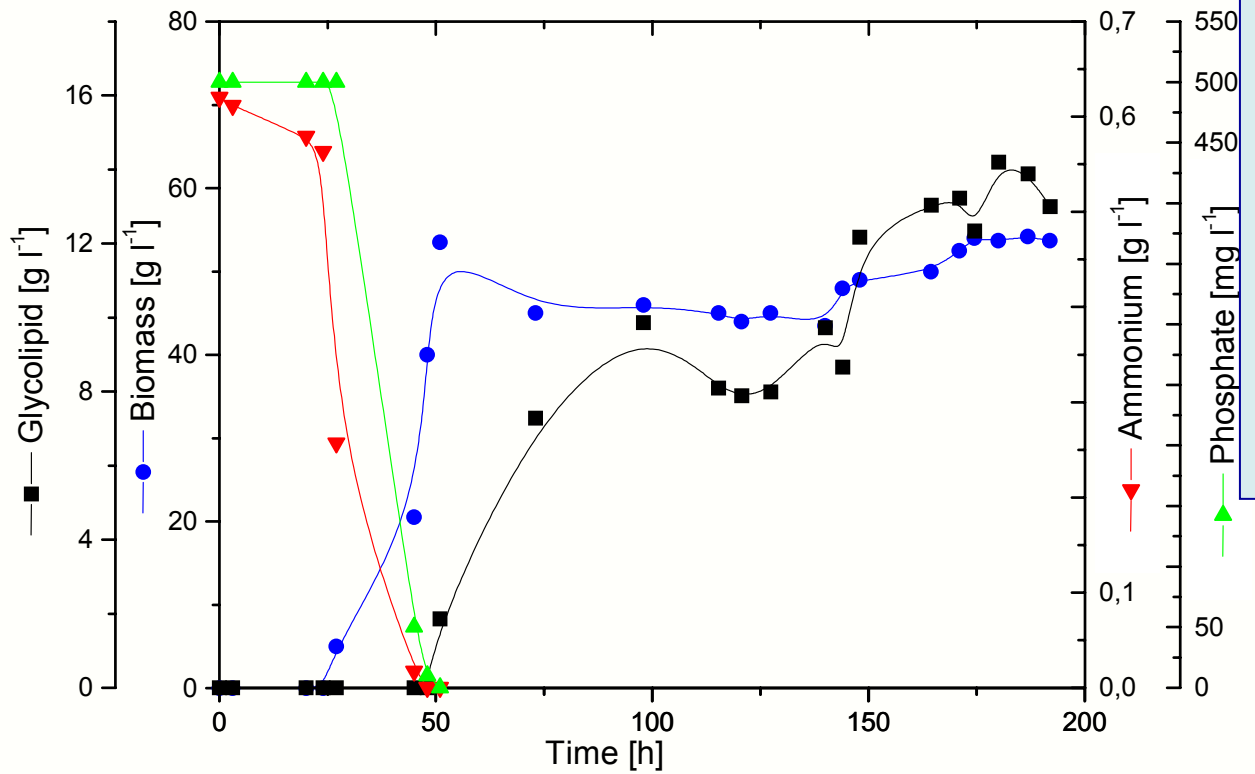


Nutrient:
- Mineral salts medium
- 1.78 g l^{-1} K_2HPO_4
- 7.44 g l^{-1} $(NH_4)_2SO_4$
- 120+70 g l^{-1} Sunflower oil

Analysis:
- GL: 22.5 g l^{-1}
- BDW: 40 g l^{-1}
- d[S]: 130 g l^{-1}
- P_V: 0.16 g l^{-1} h^{-1}
- $Y_{P/X}$: 0.56
- $Y_{P/S}$: 0.17

5 L-Cultivation on Calendula Oil

[Thanks: F. Pudel (Pilot Plant Oil Technology Magdeburg e.V.)]



Nutrient:

Mineral salts medium

1.78 g l^{-1} K_2HPO_4

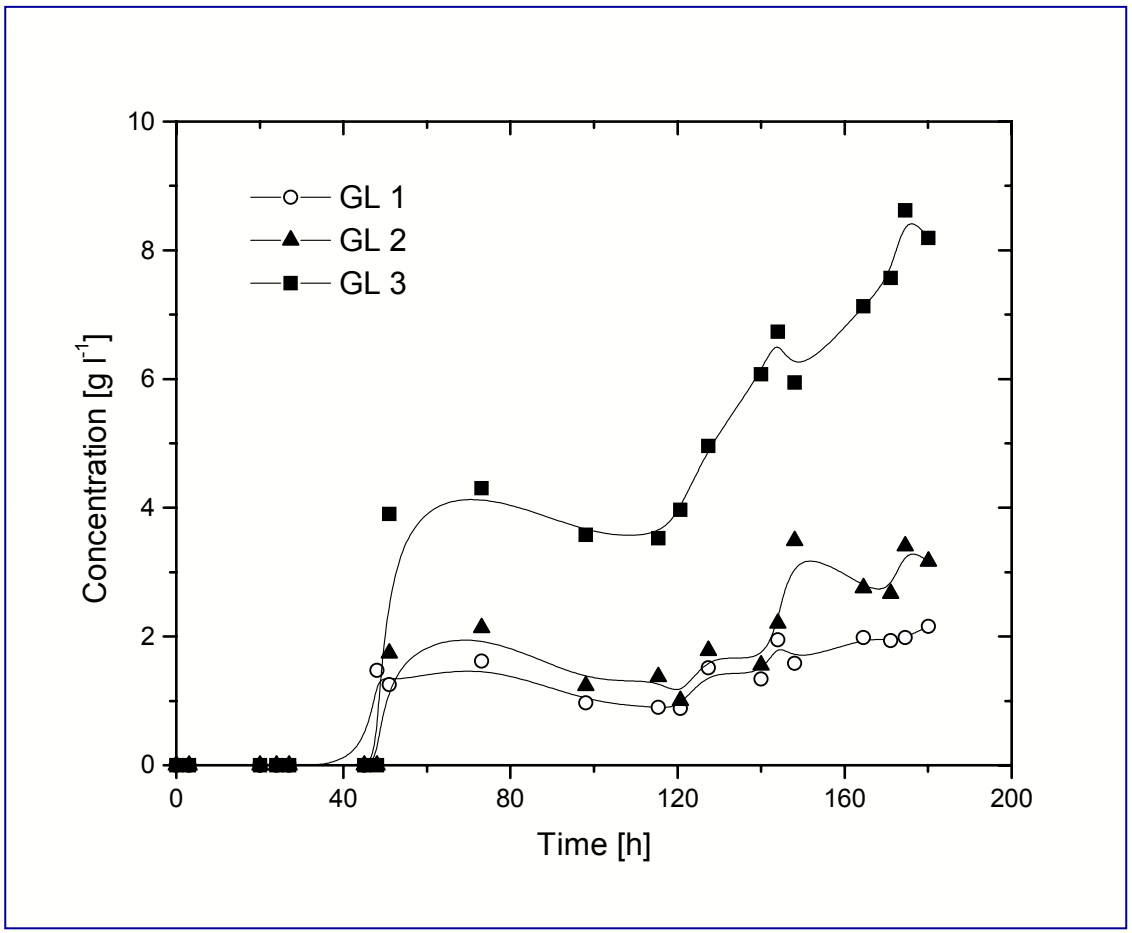
7.44 g l^{-1} $(NH_4)_2SO_4$

120.00 g l^{-1} Calendula oil

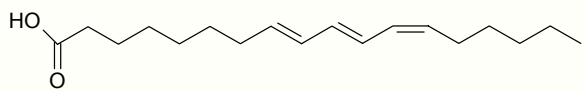
Analysis:

GL:	14 g l^{-1}
BDW:	50 g l^{-1}
d[S]:	100 g l^{-1}
P_V:	0.07 g l^{-1} h^{-1}
$Y_{P/X}$:	0.28
$Y_{P/S}$:	0.14

Composition of Glycolipids / Calendula Oil Cultivation



- Calendula oil:
  rich in calendula acid



Calendula acid

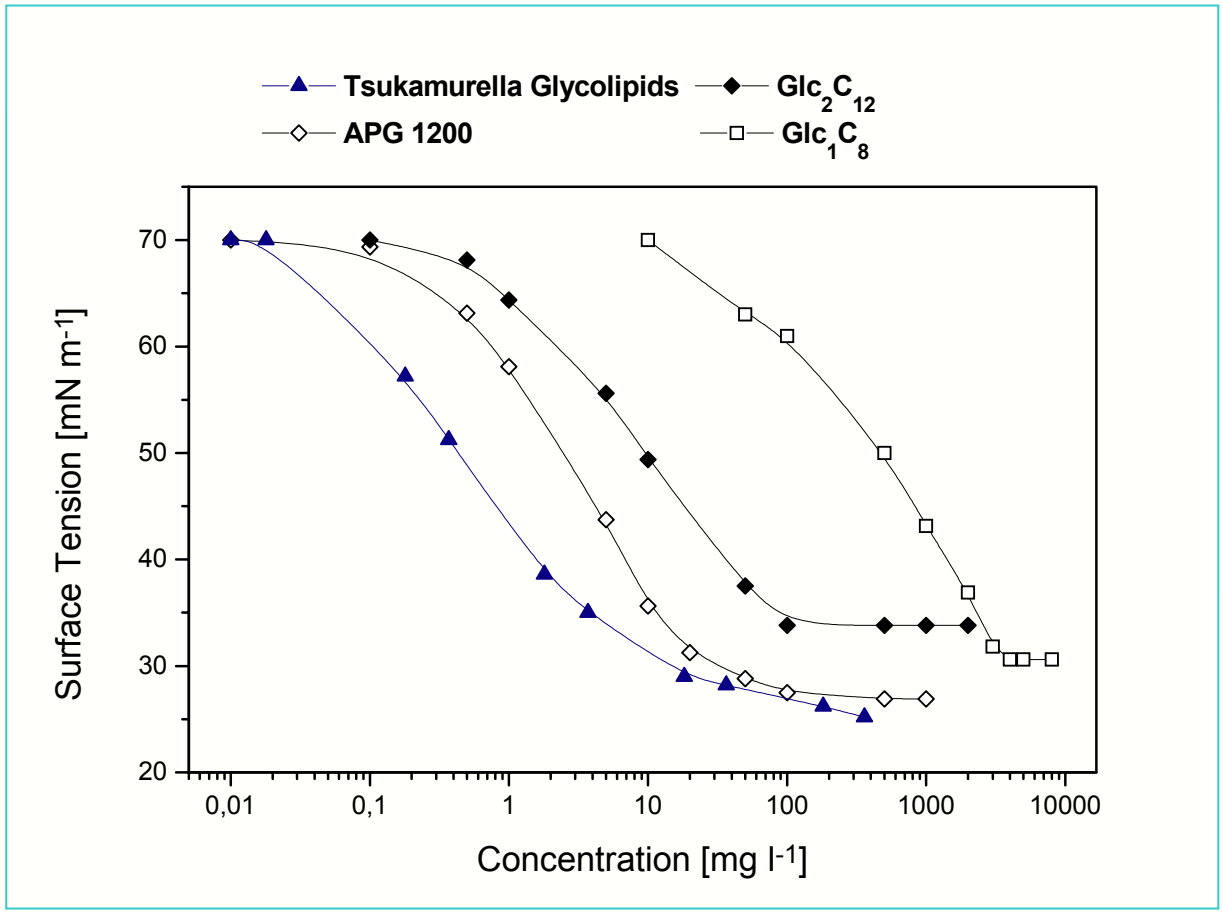
Bioconversion of Oils by *Tsukamurella* sp.

Conditions: **Bioreactor cultivations (5 L, 20 L), mineral salts medium, pH 7.5, 30°C, 500 – 900 rpm, 0.4 v/vm**

C – Source [g/L]	OSL [g/L]	Biomass [g/L]	$Y_{P/S}$ [g/g]	$Y_{P/X}$ [g/g]	P_V [g/L h]
Sunflower Oil* [130]	**22.5**	40.0	**0.17**	**0.56**	**0.16**
Rapeseed Oil* [110]	13.0	39.0	0.12	0.33	0.09
Calendula Oil** [100]	14.0	50.0	0.14	0.28	0.07

* C18:1; ** C18:3 (8-trans, 10-trans, 12-cis) [≤ 60%]

Comparison with commercially available carbohydrate surfactants



Influence of the glycolipid mixture of *Tsukamurella* spec. and of different commercially available carbohydrate surfactants on the surface tension of water at 25° C.
(APG 1200: Plantaren®, Henkel; Glc1C8: ß-Octylglucoside, Sigma; Glc2C12: ß-Dodecylmaltoside, Sigma)

Overview

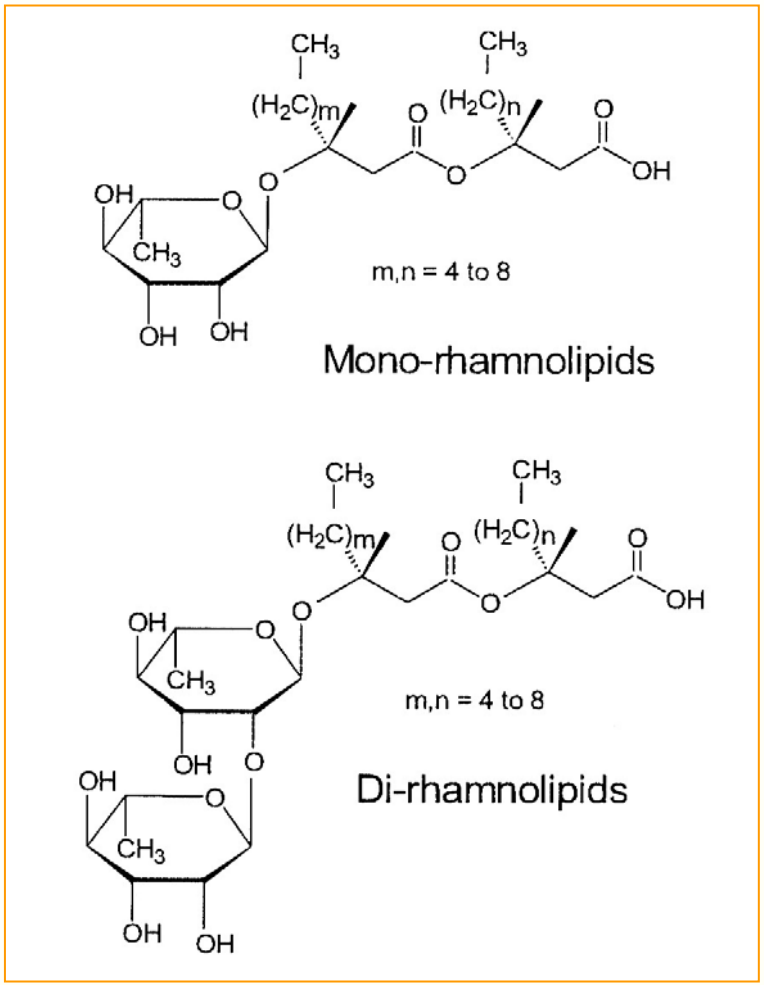
1. Fungal Sophorose Lipids
2. Fungal Mannosylerythritol Lipids

4. Bacterial Rhamnose Lipids

4. Bacterial Rhamnose Lipids

Rhamnose Lipids from *Pseudomonas aeruginosa*

Discovered: 1949 [Jarvis and Johnson]



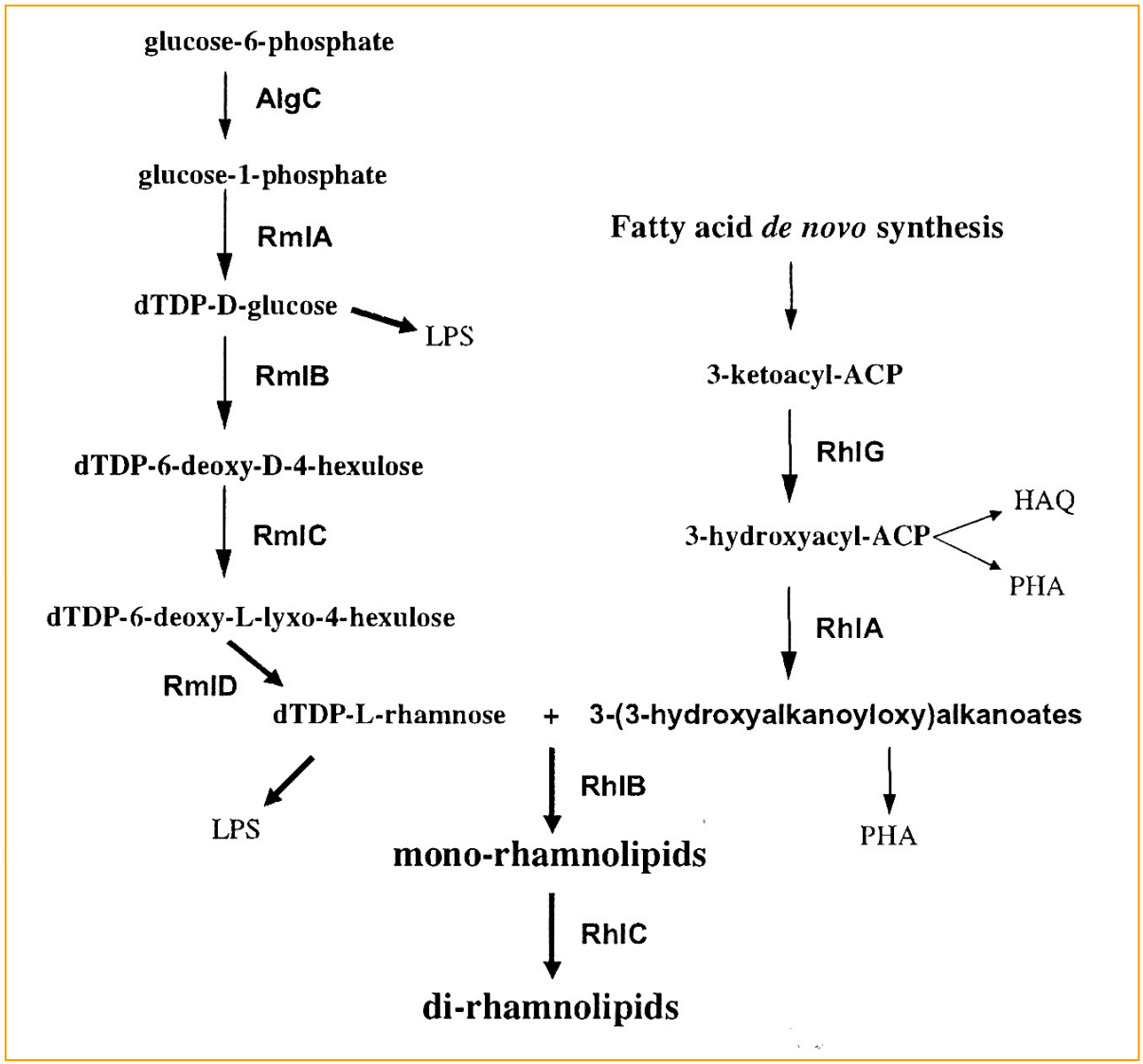
Properties:

- Surface tension lowering of water: 72 → 27 mN m^{-1}
- Bioactivities (vs. leukemia cell lines etc.)
- Potential use for environmental protection purposes

Producer Strain:

opportunistic human **pathogen**

Rhamnose Lipids from *Pseudomonas aeruginosa*



Sobéron et al. (2005), Appl. Microbiol. Biotechnol. 68: 718-725

Rhamnose Lipids from *Pseudomonas aeruginosa*

State of the art:

Carbon Sources [g/L]	Product [g/L]	Conversion [%]	Reference
Soybean oil [163]	112	68	Giani et al. (1997) U.S. Patent 5,658,793
<u>More recently:</u> Soapstock (50% C18:2) [20]	12	60	Benincasa et al. (2004) Antonie van Leeuwenhoek 85: 1-8
Canola oil [20]	9	45	Raza et al. (2006) Biodegradation DOI 10.1007/s10532-006-9047-9
Brazilian Nut oil [20]	10	50	Costa et al. (2006) Process Biochem. 41: 483-488

Rhamnose Lipids from *Pseudomonas chlororaphis* (nonpathogenic bacterium):

1 g/L from 20 g/L glucose

[Gunther IV et al. (2005), Appl. Environ. Microbiol. 71: 2288-2293]

Surfactant Properties

Tab.: Surface and interfacial tension data in aqueous systems at 25-40 °C.

Biosurfactant	σ_{min} [mN/m]	γ_{min}* [mN/m]	cmc [mg/L]
Sophorose lipids	36	n. d.	10
Mannosylerythritol lipids	28	2	60
Di/Oligosaccharide lipids	26	< 1	50
Rhamnose lipids	27	< 1	25

* Towards n-C16

Potential Applications for Biosurfactants

Recent Studies

Biosurfactant	Tests	Reference
SL, RL, Surfactin	environmental applications	**Mulligan (2005)** Environ. Poll. 133: 183-198
Sophorose lipids	anti-HIV, sperm-immobilizing activity	**Shah et al. (2005)** Antimicr. Ag. Chemother. 49: 4093-4100
	apoptosis induction/liver cancer cells	**Chen et al. (2006)** Appl. Microbiol. Biotechnol. DOI 10.1007/s00253-005-0243-z
Mannosylerythritol lipids	biodegradation/petroleum compounds	**Hua et al. (2004)** World J. Microbial Biotechnol. 20: 25-29
Di/Oligosaccharide lipids	inhibition/Epstein Barr virus EA activation	**Langer et al. (2006)** Process Biochem., in press
Rhamnose lipids	bioremediation/chloroaromatic contaminated soil	**Berselli et al. (2004)** Biotech. Bioeng. 88:111-120
	enhanced healing/burn wounds	**Stipcevic et al. (2006)** Burns 32: 24-34

Microbial / Enzymatic Transformation of TAGs / Fatty Acids

Table 1 Enzymes useful for lipid modification

Enzyme	Applications	Examples
Lipase	Synthesis of structured triglycerides	Cocoa-butter equivalent, Betapol
	Enrichment of specific fatty acids	PUFA from fish oils
	Incorporation of specific fatty acids	PUFA into plant oils
	Synthesis of fatty acid derived products	Emollient esters
Phospholipase	Removal of fatty acids in *sn*1- or *sn*2-position (PLA_1 or PLA_2)	Degumming of oils
		Lyso-phospholipids
	Removal of phosphate group (PLC)	Chiral diglycerides
	Head group exchange (PLD)	Phosphatidylserine
Monooxygenase	Hydroxylation of fatty acids	Precursor for polyesters/lactones
Epoxidase	Epoxidation of double bonds	–
Lipoxygenase	Synthesis of FA-hydroperoxides	–

Metzger and Bornscheuer (2006), Appl. Microbiol. Biotechnol. 71: 13-22

Lipase

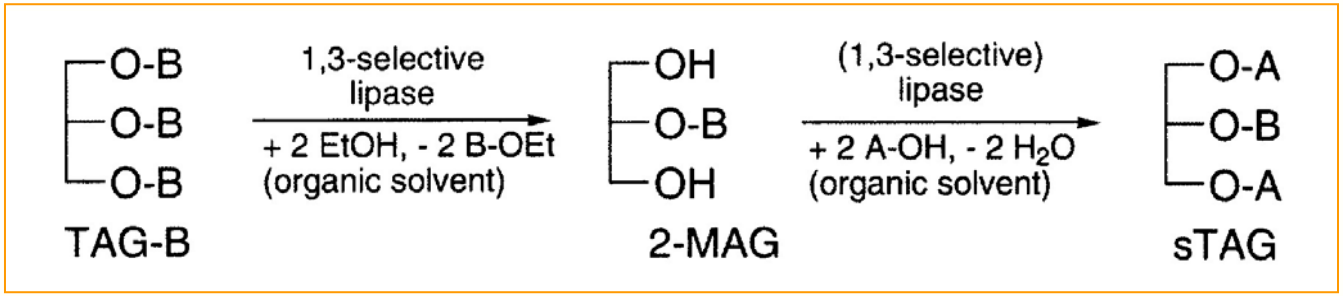


Fig. 9 Principle of the lipase-catalyzed two-step synthesis to obtain sTAG in high purity. *A* and *B* denote different fatty acids

Metzger and Bornscheuer (2006), Appl. Microbiol. Biotechnol. 71: 13-22

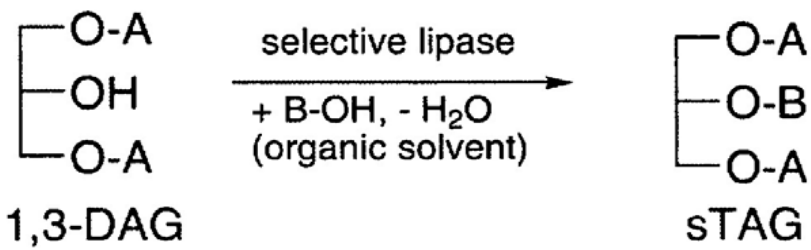
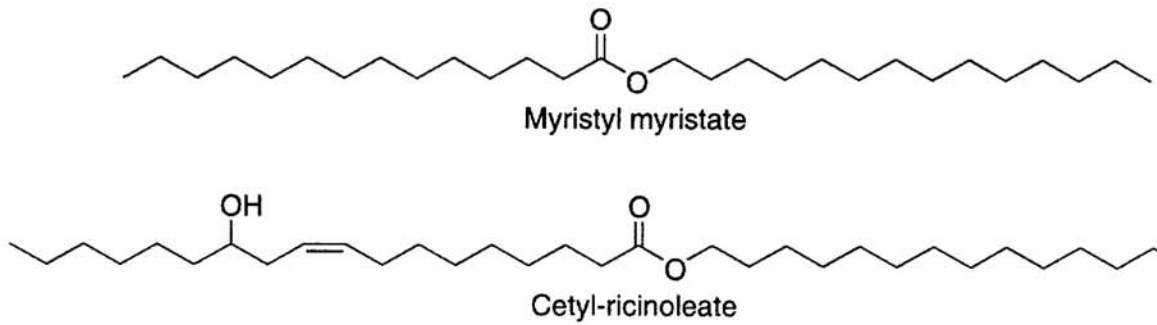


Fig. 10 Principle of the lipase-catalyzed two-step synthesis to obtain sTAG in high purity. *A* and *B* denote different fatty acids

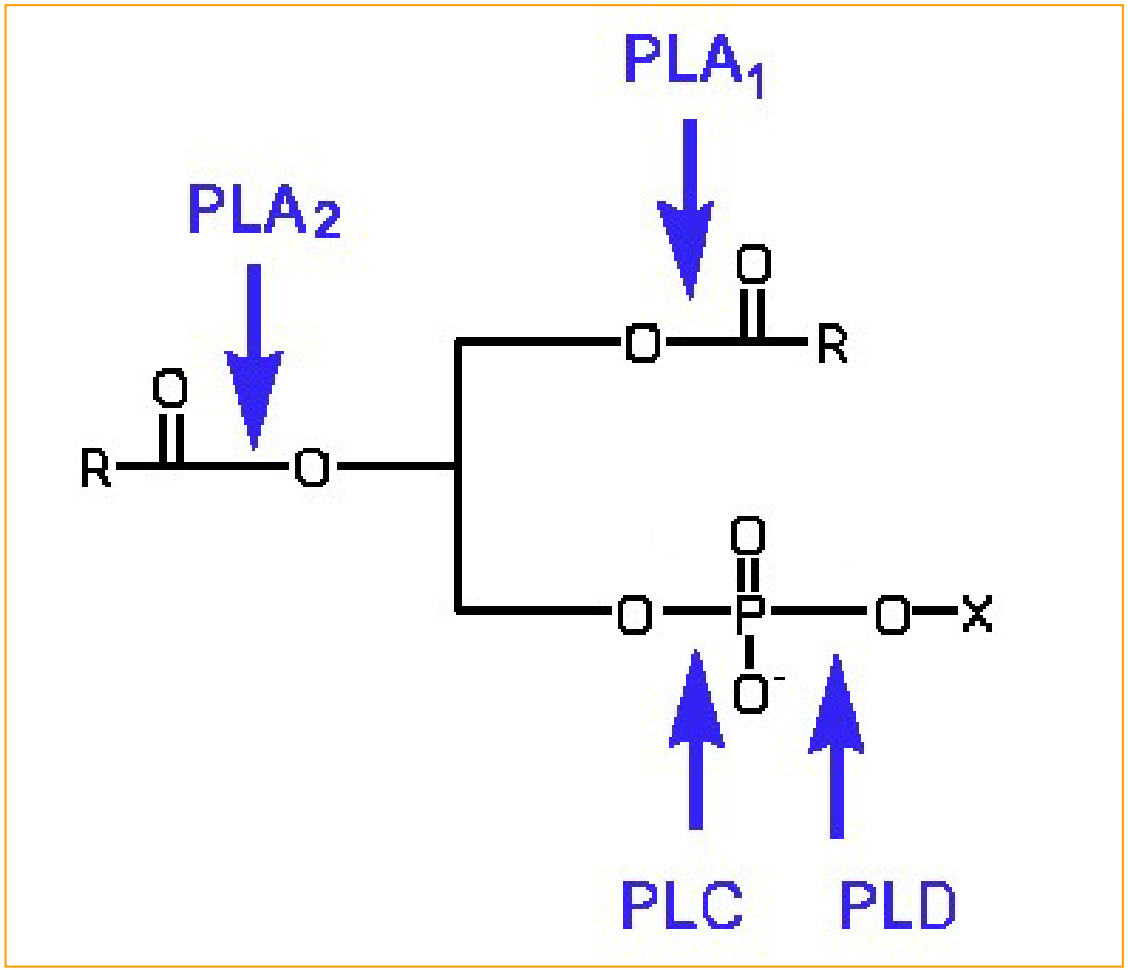
Esterification Reaction

Fig. 11 Myristyl myristate and cetyl ricinoleate are produced by lipase-catalyzed esterification



Metzger and Bornscheuer (2006), Appl. Microbiol. Biotechnol. 71: 13-22

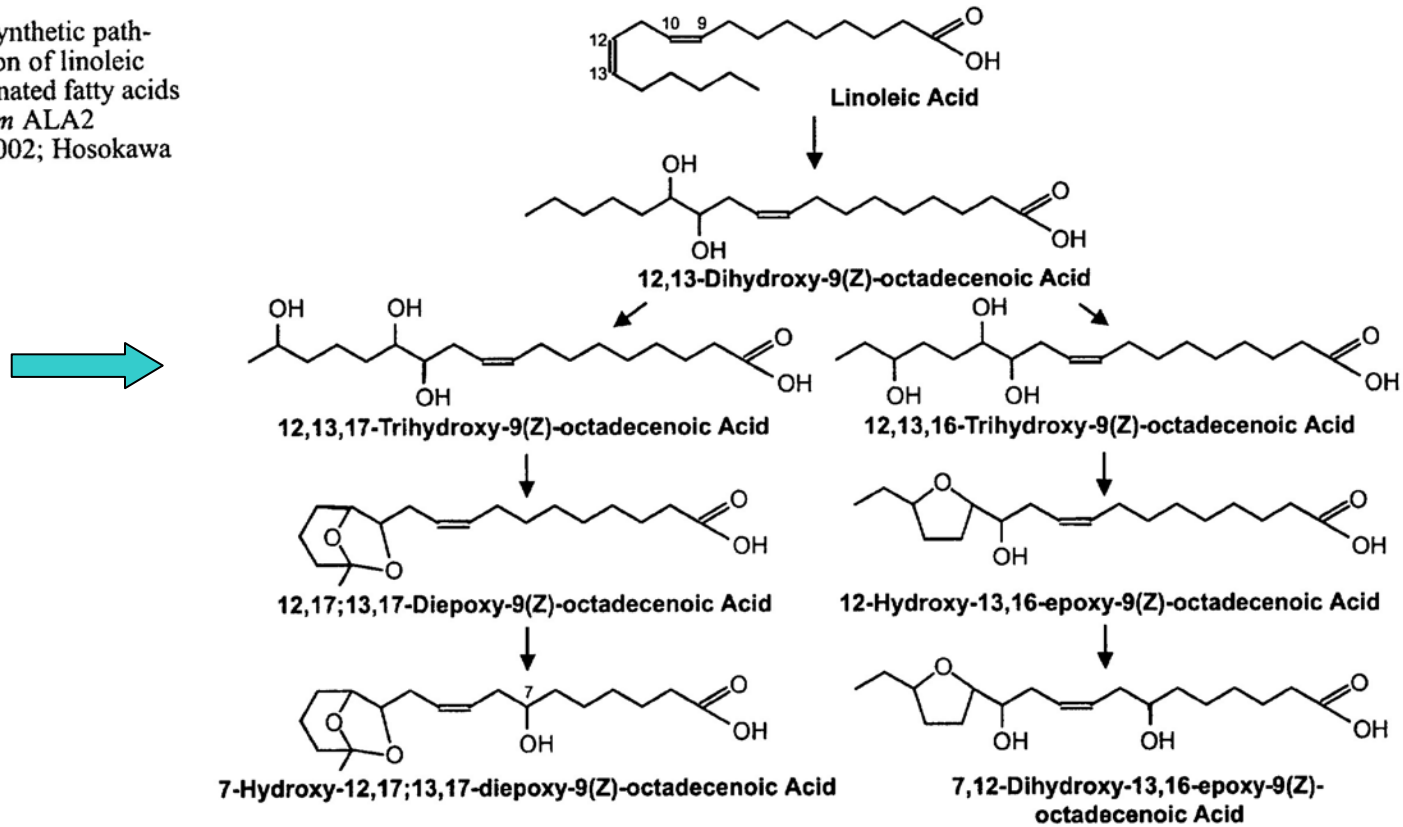
Phospholipases



U. Bornscheuer (2004), GDCH-Kurs "Nachwachsende Rohstoffe: Neue Synthesen mit Ölen und Fetten", Oldenburg

Monooxygenases

Scheme 1 Biosynthetic pathway of conversion of linoleic acid to its oxygenated fatty acids by *B. megaterium* ALA2 (Iwasaki et al. 2002; Hosokawa et al. (2003a,b))



C.T. Hou (2005), Appl. Microbiol. Biotechnol. 69: 463-468

Monooxygenases

Experiment:

50 mL cultures

(C-sources: Glucose, YE, Tryptone)

160 mg Linoleic acid

Bioactivities:

antifungal, anticancer, e.g.

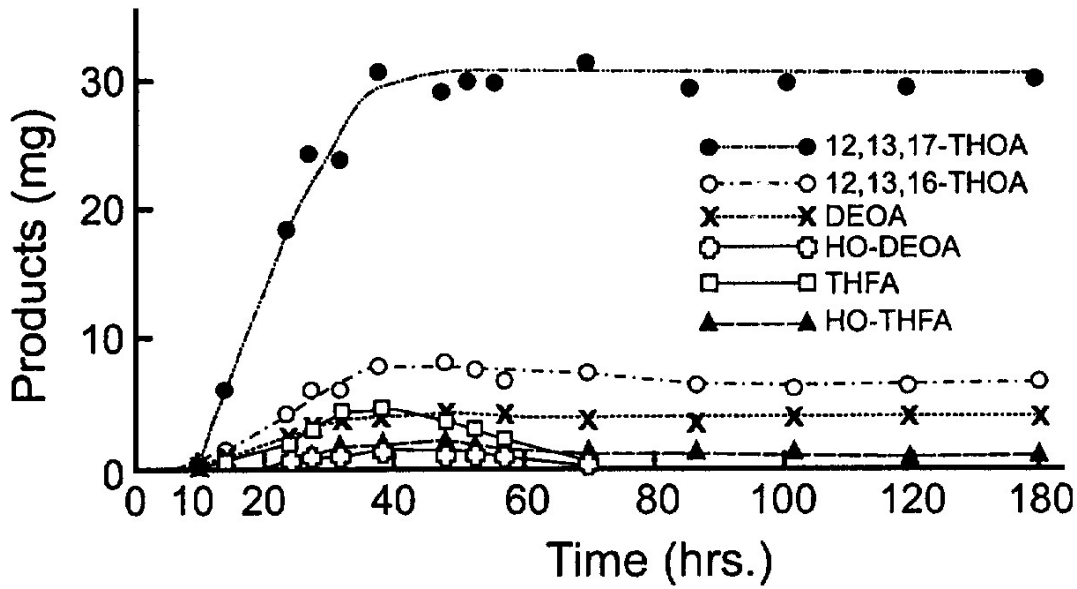
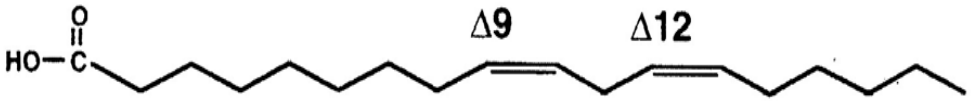


Fig. 1 Time course studies of the production of oxygenated fatty acids from linoleic acid by strain ALA2. *12,13,17-THOA* 12,13,17-Trihydroxy-9(Z)-octadecenoic acid, *12,13,16-THOA* 12,13,16-trihydroxy-9(*Z*)-octadecenoic acid, *DEOA* 12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid, *HO-DEOA* 7-hydroxy-12,17;13,17-diepoxy-9(*Z*)-octadecenoic acid, *THFA* 12-hydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid, *HO-THFA* 7,12-dihydroxy-13,16-epoxy-9(*Z*)-octadecenoic acid

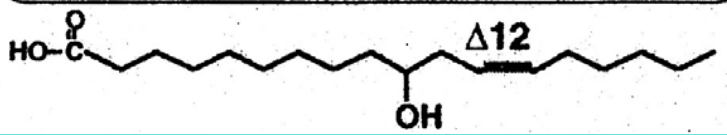
C.T. Hou (2005), Appl. Microbiol. Biotechnol. 69: 463-468

1. Hydratation, 2. Isomerization of Double Bonds

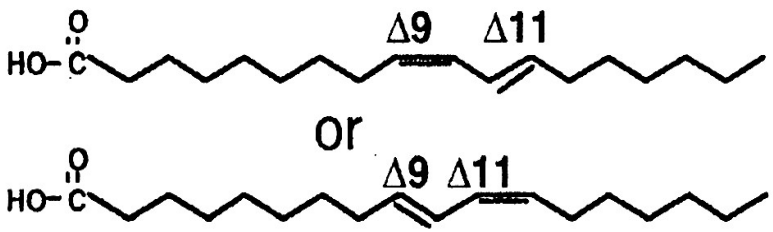
Linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid)



HY2 (10-hydroxy-*cis*-12-octadecaenoic acid)



CLA 1 (*cis*-9,*trans*-11- or *trans*-9,*cis*-11-octadecadienoic acid)



Bioactivities:

- **Anticarcinogenic**
- **Enhancing the immune system**
- **Decreasing in body fat content**

Conjugated Linoleic Acid Production

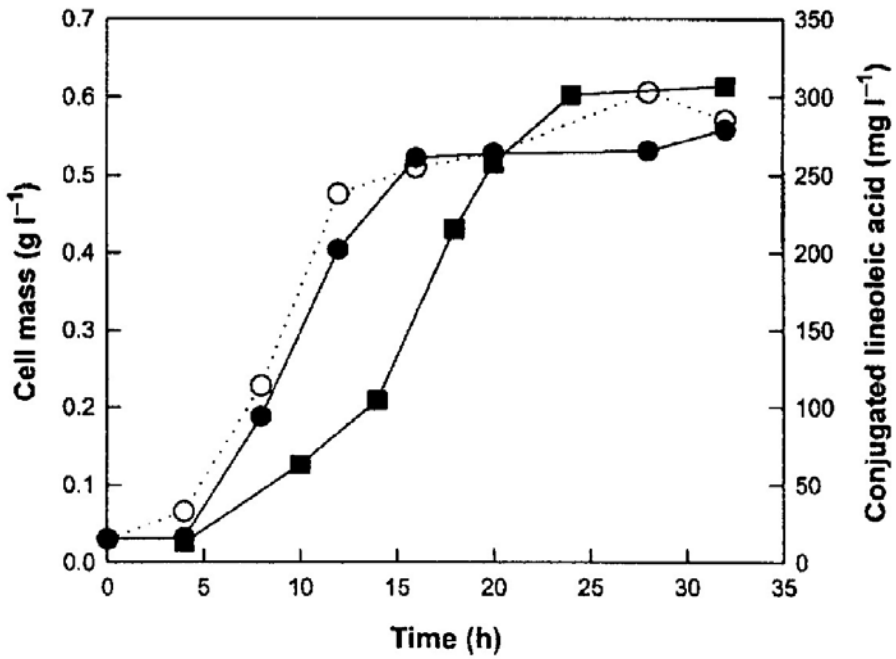


Fig. 1. Effect of LA on the growth of *Lactobacillus reuteri*. Cell growth on MRS medium without LA (○) or with 0.9 g LA l^{-1} and 1.67% (v/v) Tween 80 (●); CLA production (■). The pH value of the growth media was initially 6.1 and then remained at 4.5 after 32 h culture in MRS medium either with or without LA.

MRS-medium:

Tryptone, Meat extract, YE, Glucose

S.O. Lee et al. (2003), Biotechnol. Letters 25: 935-938

Conclusion

Microbial Conversion of Renewable Resources
(vegetable oils, glycerol, fatty acids; carbohydrates):

• ***Clostridium butyricum:***	1,3-Propanediol	100 g/l
• ***Pseudomonas corrugata:***	Polyhydroxyalkanoates	2 g/l
• ***Candida bombicola:***	Sophorose Lipids	400 g/l
• ***Pseudozyma aphidis*:**	Mannosylerythritol lipids	165 g/l
• ***Tsukamurella* sp.:**	Di- and Oligosaccharide lipids	22 g/l
• ***Pseudomonas aeruginosa:***	Rhamnose Lipids	100 g/l

Microbial / Enzymatic Transformation of TAGs / Fatty Acids

- **Lipases, Phospholipases:** Structured triglycerides/phospholipids
- **Whole-cell Catalysts (*Bacillus megaterium, Lactobacillus reutereri*, e.g.):**
  - Oxygenated fatty acids
  - Conjugated fatty acids

Acknowledgements

- L.A. Nguyen, O. Langer, O. Palme, U. Rau
  (TU Braunschweig, Inst. of Biochemistry and Biotechnology)
- S. Schulz (TU Braunschweig, Inst. of Organic Chemistry)
- V. Wray, M. Nimtz (Helmhotz Centre for Infection Research, Braunschweig)
- S. Radel, E. Benes (Vienna University of Technology, Inst. of General Physics)
- F. Pudel (Pilot Plant Oil Technology Magdeburg e.V.)
- H. Koch, H. Röper (Cerestar R&D Centre, Vilvoorde, Belgium)
- German Ministry for Nutrition, Agriculture and Forestry (Berlin)
- European Community