Novel Production Strategies for Biosurfactants

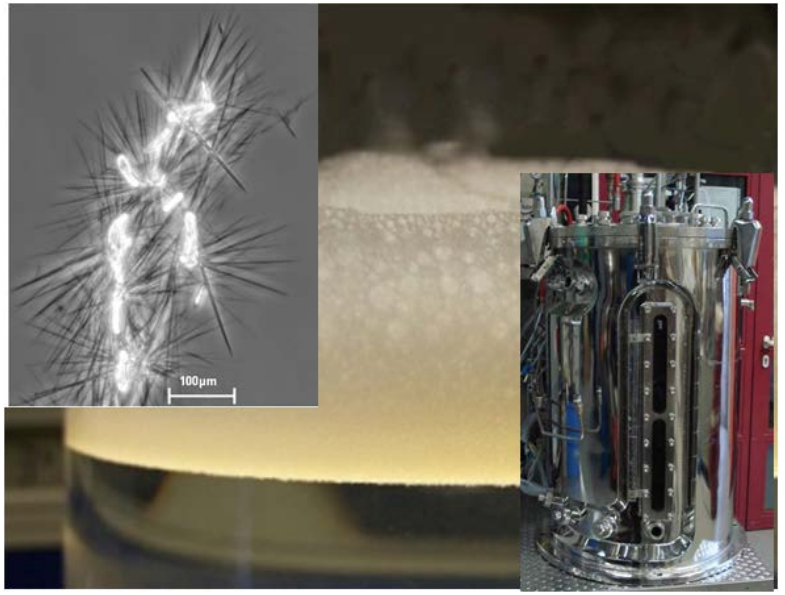
Project acronym: BioSurf.
Project no: EIB.10.039

ERA IB
European Research Area
Industrial Biotechnology

Project aim

Surfactants form an integral part of our everyday life with applications from food to fuel additives

BioSurf aims at an increased replacement of petro-based surfactants by biosurfactants generated from renewable resources



ECOVER
POWERED BY NATURE

TORMANS
Our people, your success

c-LEcta enzymes and strains

vito
vision on technology

Fraunhofer

LISBP

KIT
Karlsruhe Institute of Technology

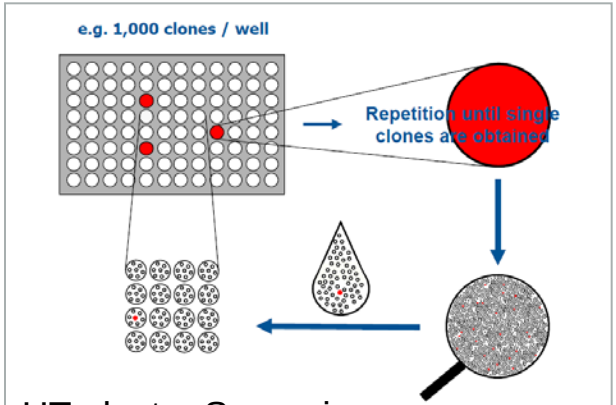
Overview of the WPs

BioSurf: Novel Production Strategies for Biosurfactants

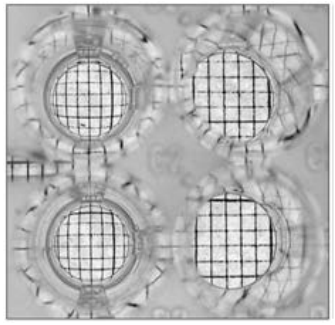
WP2	WP 3	WP 4	WP 5	WP 6	WP 7
Identifi-cation and preparation of new surfactants	Enzymatic synthesis and modification	Product characte-rization and selection	Metabolic engineering Improved strains for application	Bioprocess engineering and downstream processing	Dissemina-tion and Exploitation

WP2: Identification and preparation of new surfactants

- Development of modified or unexplored biosurfactants
- Identification of novel biosurfactants by screening of strain collections
- Characterisation of microorganisms and the produced biosurfactants



HT-cluster Screening

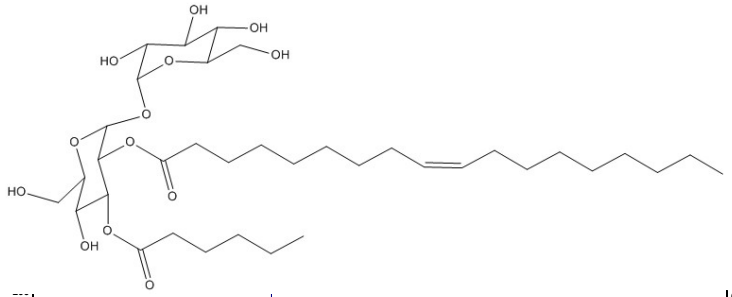

Microplate assay based on surface activity of BS

© Fraunhofer IGB

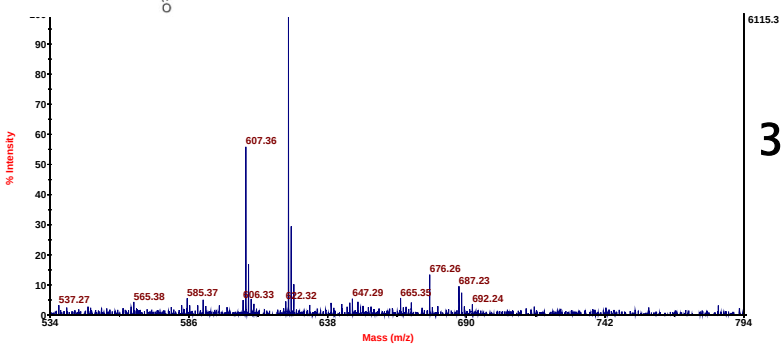
Fraunhofer IGB

WP2: Identification and preparation of new surfactants

1. Screening for new surfactant producers:
   - Establishment of high throughput screening methods
   - Isolation and screening of 377 microorganims (e.g. peat-bog soil, intestines of seawater animals, mediterranean sponges)
   - Identification of not yet described non-pathogenic biosurfactant producers (*Rahnella sp.*, *Citrobacter braaki*, *Serratia fonticola*, *Pseudomonas extremaustralis* and several *Psychrobacter sp.*).



2. Production and purification
   - Fermentation (L scale) of novel Trehalose-lipids from *Tsukamurella spumae* and *T. pseudospumae*
   - Production of unknown surfactants (584 and 600Da) from *Rahnella sp.* 323 (1,2 L scale)



3. Characterization and structure elucidation
   - Isolation by liquid chromatography structure elucidation using MALDI-ToF-MS-MS and NMR

WP2: Modified biosurfactants

cellobiose lipid (CL)

- antibiotic properties
- needle like crystals in acidic conditions

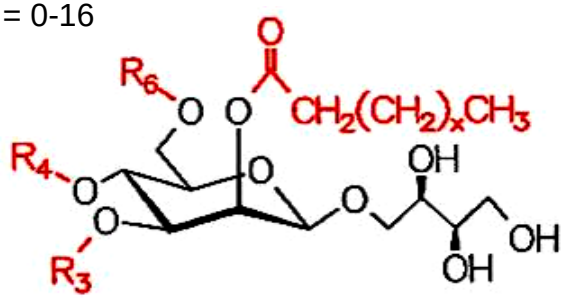
Ca. 80 different variants

mannosylerythritol lipid (MEL)

- strong surface activity,
- good applicability as emulgators
- secreted as oily, viscous substance

red = variable side groups

R_3= C2-C18 fatty acid residue
R_4= acetyl- oder H
R_6= acetyl- oder H
X = 0-16



> 240 different variants

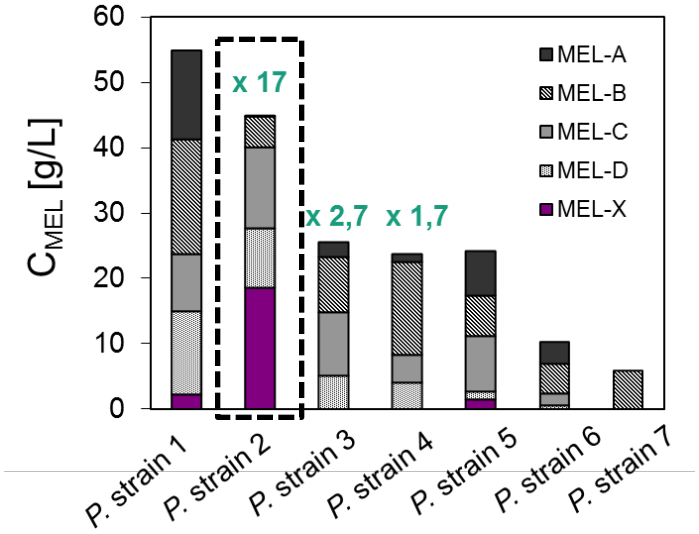
© Fraunhofer IGB

Fraunhofer IGB

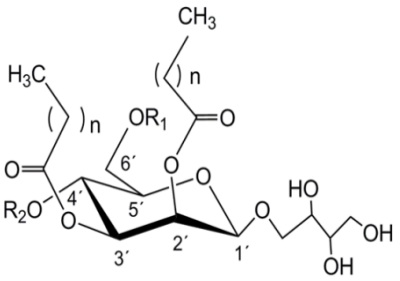
WP2: Production of rare hydrophilic MEL-structures

- Comparison of 7 *Pseudozyma* strains

➔ 5 produce abundant amounts of MEL-B, MEL-C MEL-D and with MEL-X greater hydrophilicity

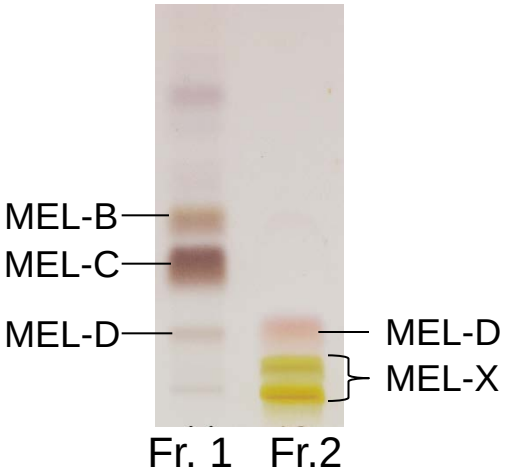


n= 5-16
MEL-A: R_1, R_2 = Acetyl
MEL-B: R_1 = Acetyl, R_2 = H
MEL-C: R_1 = H, R_2 = Acetyl
MEL-D: R_1, R_2 = H

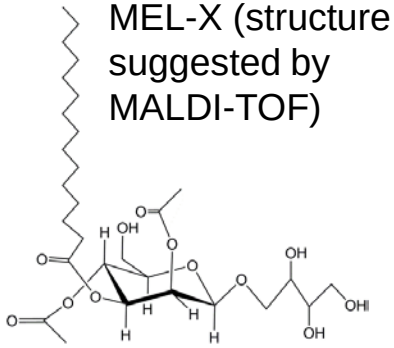


- *P. strain* 2 produces a 17-fold higher MEL-yield, compared to literature (2-fractions)

1. MEL-B,-C,-D-fraction (20 ± 5 g/L)
2. MEL-D, -X-fraction with high water solubility (25 ± 5 g/L)



monoacylated MEL-X (structure suggested by MALDI-TOF)



Fraunhofer IGB

WP3: Enzymatic synthesis and modification

- enzymatic modification of natural biosurfactants to enhance surfactant performance

- enzymatic synthesis of novel types of glycolipid-biosurfactants derived from fatty acids, alkanols or terpenes in combination with rare sugars or oligosaccharides

© Fraunhofer IGB

WP3: Screening for new enzymes

Genomic Libraries

Genomic DNA of single strains with high potential is cloned

Micro-organisms

Screening of isolated microorganisms

Metagenomic and Microbial Libraries

Genomic DNA of environmental probes/strains is cloned

Recombinant expression libraries

high-throughput Cluster-Screening

→ Highly efficient identification of new enzymes in activity-based screens

Engineering

Recombinant expression libraries

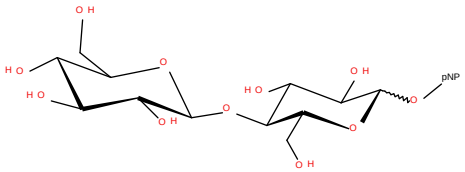
Lipases send out to Partners

→ Highly efficient identification of new enzyme variants in activity-based screens

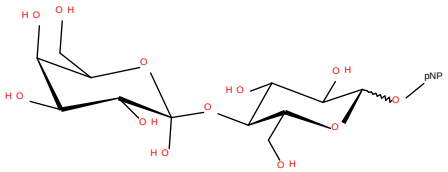
© Fraunhofer IGB

WP3: Enzymatic glycolipid modification

- screening for transglycosidases
- established with pNP-derivatives of cellobiose and lactose and with pNP-derivatives of palmitic acid for lipases



pNP derivative of cellobiose



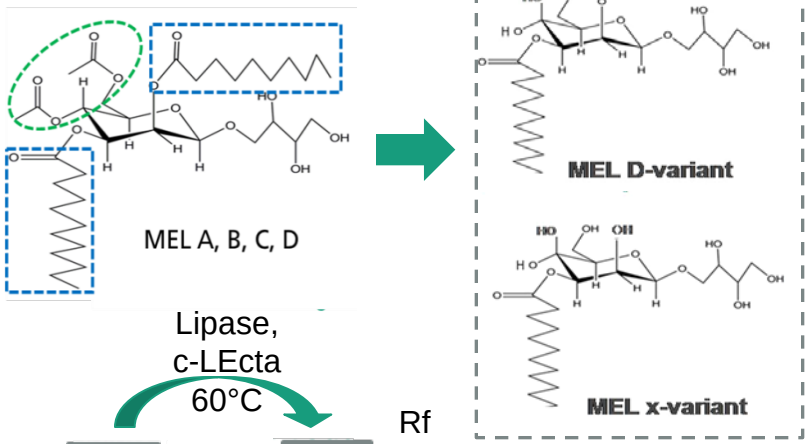
pNP derivative of lactose

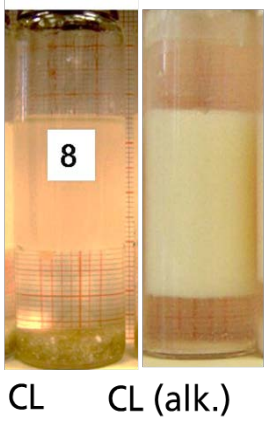
- screening of different genomic and metagenomic libraries (altogether 300,000 clones)
- four new glycosidases able to hydrolyze cellobiose- and lactose derivatives
- similarities to known glucosidases, glycosyl-hydrolase and rhamnosidases
- identities to known database enzymes between 48% and 96%
- enzymes are available

© Fraunhofer IGB

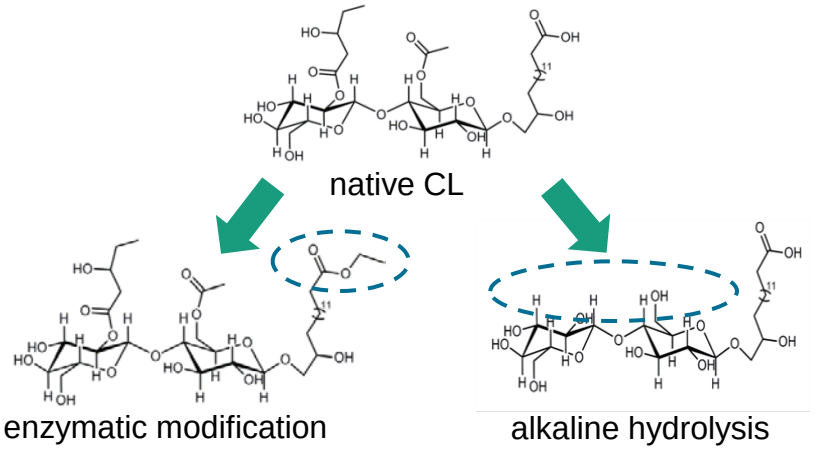
WP3: Modification of glycolipids from fungi

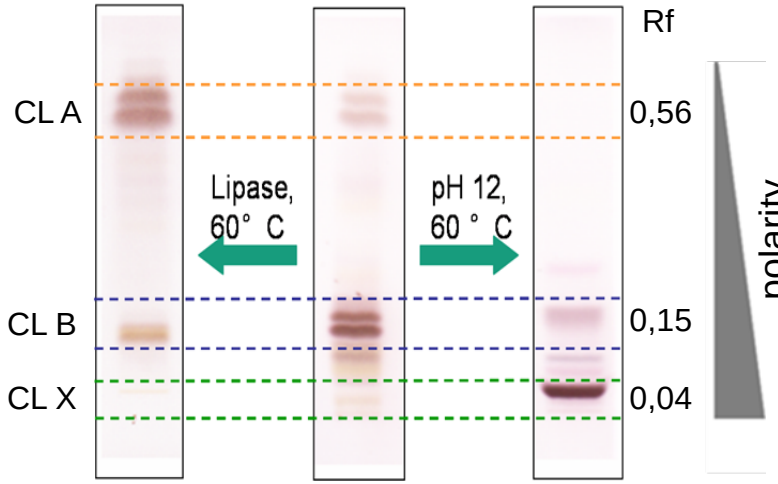
Modification of mannosylerythritol lipids





Modification of cellobioselipids



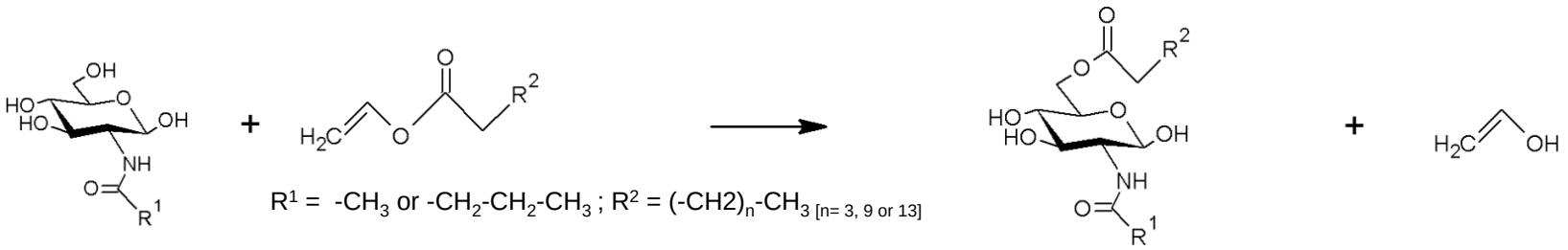


© Fraunhofer IGB

Fraunhofer IGB

WP3: Enzymatic synthesis of novel glycolipids

Different amino sugar fatty acid esters synthesized via lipase-catalyzed transesterification



- Experiments carried out for 48h in 2m2b with Novozyme 435™ at 40°C and 300 rpm

- Structures elucidated via NMR
- Quantification using high performance liquid - and gas chromatography
- Purification via MPLC

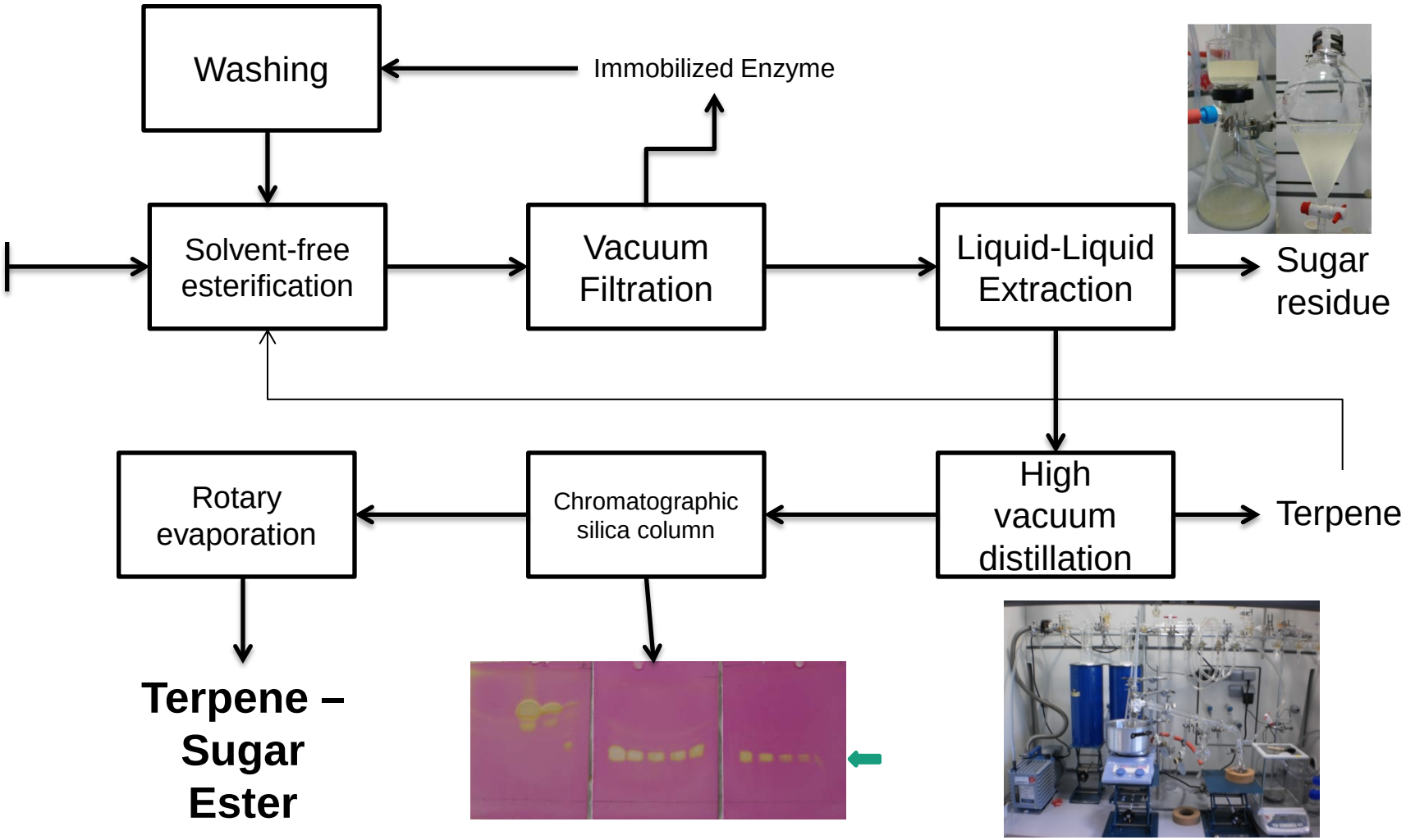
- Influence on the synthesis of different parameters tested:
  - Substrate solubility
  - Water content
  - Substrate ratio
  - Temperature

WP3: Enzymatic synthesis of novel terpenoid sugar esters

- Solvent-based and solvent-free process for novel, not yet characterized biosurfactants from terpenes and sugars
- Reaction kinetics determined
- Residual substrate and bio-catalyst recovery possible
- Analytical validation of products
- Promising surfactant properties of products
- Product stability at higher pH critical (hydrolysis)

© Fraunhofer IGB

Solvent-free process



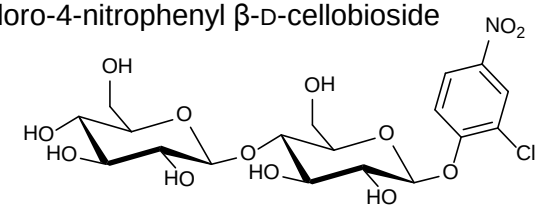
© Fraunhofer IGB

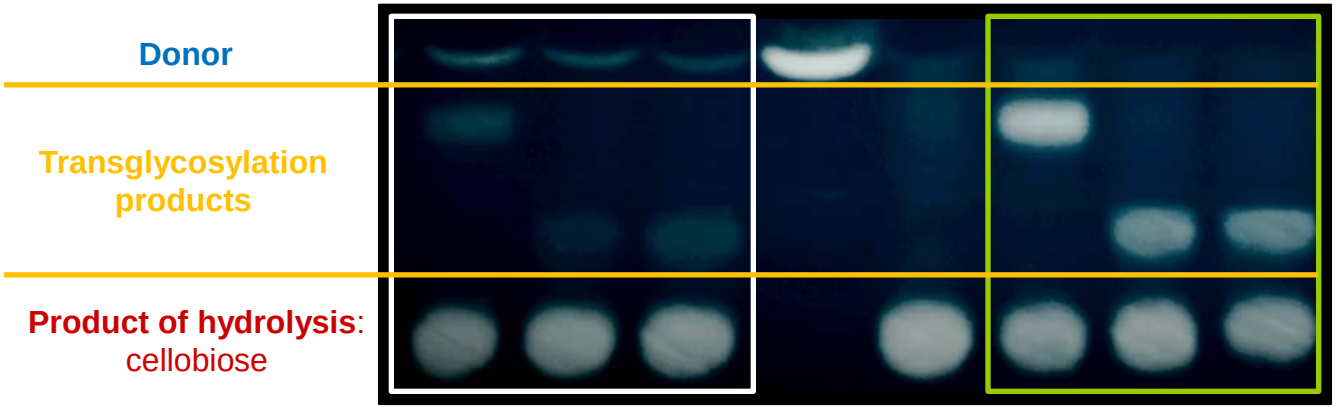
Fraunhofer IGB

WP3: Glycoceramidase-engineering for Synthesis

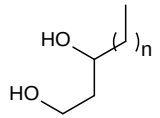
Cellobioside Donor + **Acceptor** —EGC II→ **CellobiosideLipid**

2-chloro-4-nitrophenyl β-D-cellobioside

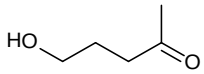




Acceptors

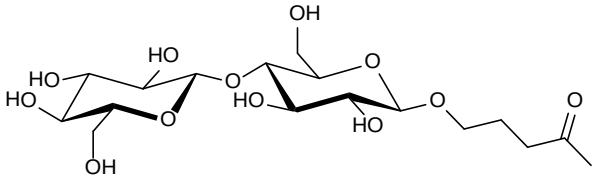


(**A**) n = 4, octane-1,3-diol
(**C**) n = 1, pentane-1,3-diol



(**B**) 5-hydroxypentan-2-one

- (**A** and **C**): mixtures of regioisomers (62% for acceptor A)
- (**B**): **cellobiosidelipid 59%**



© Fraunhofer IGB

LISBP

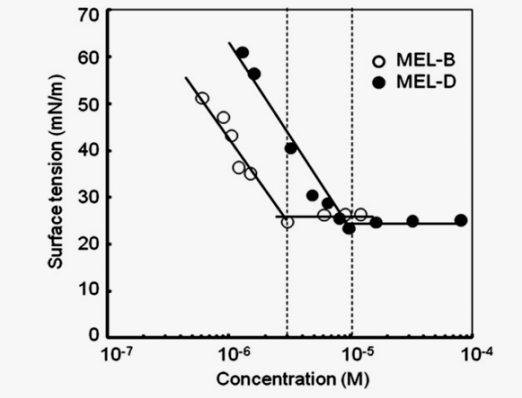
Fraunhofer IGB

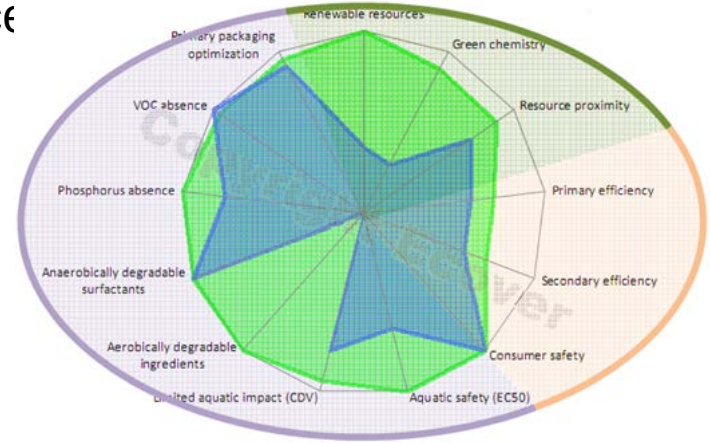
WP4: Product characterisation and selection for product development

- Testing of the generated biosurfactants for basic physico-chemical properties
- Testing for antimicrobial properties (AS-HTS)
- Promising surfactants will be produced in 100 g scale for full application tests:

  Foam quality, skin compatibility (HET-CAM-Test), dishwashing and laundry performance tests

  Ecotoxicity and biodegradability of the new products





© Fraunhofer IGB

Fraunhofer IGB

WP4: Application tests performed

	CGE batch 1	GGE batch 1	MGE batch 1	CGE batch 2	MEL purified	CBL native	CBL hydrolysed
	Terpenol 1	Terpenol 2	Terpenol 3	Terpenol 4	Mannosylerythritol lipid	Cellobiose lipid	Cellobiose lipid
Solubility in water	pH adjustments needed	Water soluble, but unstable after one night at 4°C	Neutral pH	10000ppm	pH adjusted (pH=11), 5 days at 40°C	pH adjusted, 5 days at 60°C	Addition of hydrotrope (influences succeding measurements)
Min. surface tension measured [mN/m]	27	26	31	37	29	40	
Min. contact angle measured [°]	49	30	59	58	27		
CMC [ppm = mg/kg]	2000	2000	>10000	>10000	100	1500	
Foam					No foam	No foam	
Remarks					Slow		

	Partial hydrolysed SL	Complete hydrolysed SL
Solubility in water	Yes	Yes
Surface tension at CMC	38 mN/m	31 mN/m
Contact angle at CMC	39°	35°
Foam	Foam, but low capability and stability	No foam with soft or hard water

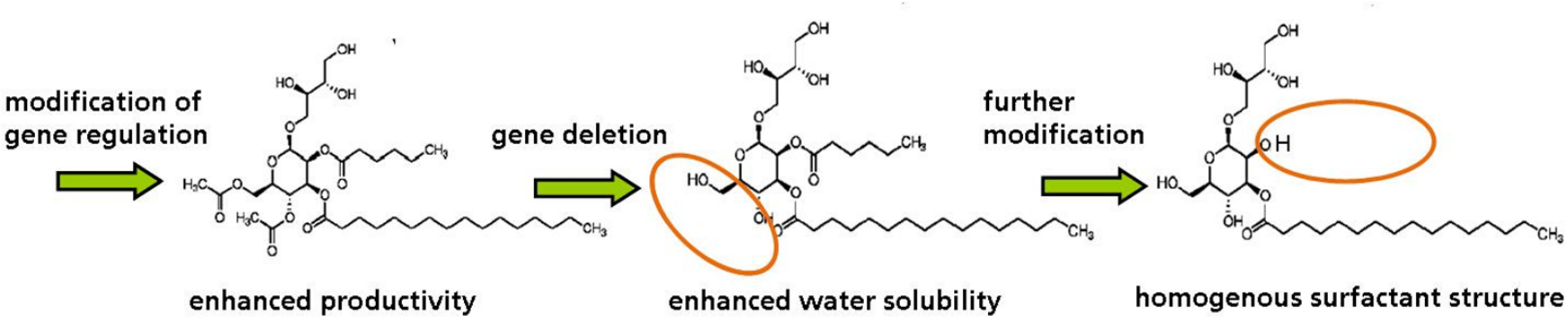
© Fraunhofer IGB

ECOVER POWERED BY NATURE

Fraunhofer IGB

WP5: Metabolic engineering: Improved strains and surfactants for industrial application

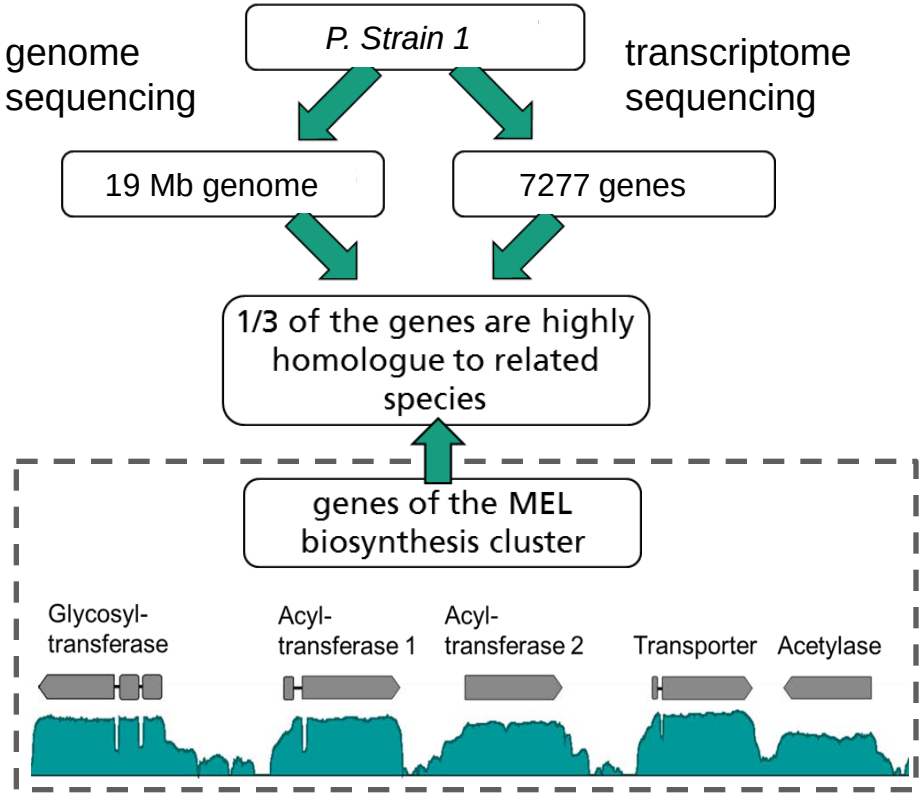
- Identification of biosurfactant pathways
- Identification of regulatory mechanisms
- Engineering of biosynthetic pathways to obtain higher production yields and taylor surfactant structures

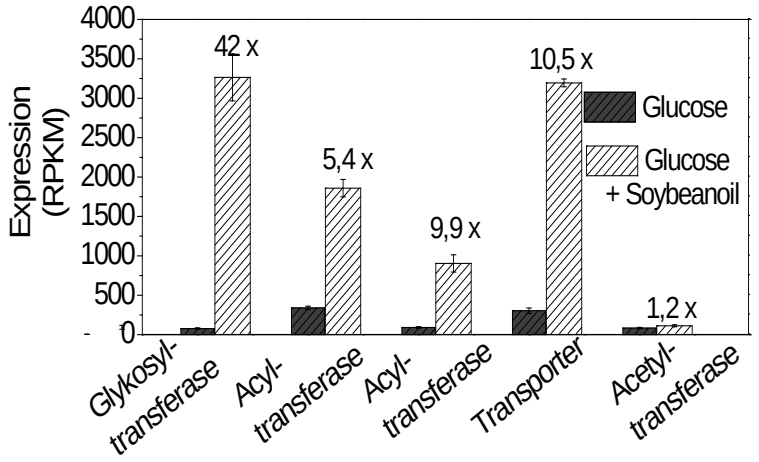


© Fraunhofer IGB

Fraunhofer IGB

WP5: Investigation of the MEL-metabolism in *P. strain1* on genome and transcriptome level

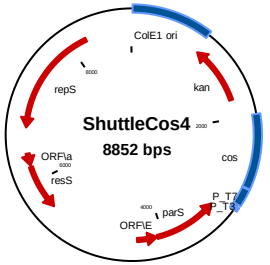




Differential expression analysis with and without a hydrophobic c-source reveales 13 upregulated metabolic groups including the MEL pathway

WP5: Novel biosurfactant operons: Large insert libraries

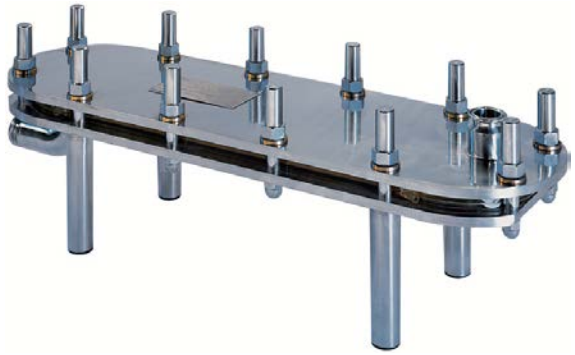
- building of of a new shuttle cosmid
  - screenings in *E. coli* and *Bacillus* sp. possible
- protocols for high efficient transformation of cosmid libraries in screening host *B. subtilis* (surfactin-deficient) established
- Control library from *B. subtilis* (surfactin producer) built
- surfactin production of B. subtilis DSM was confirmed by emulsification tests
- screening was performed in DWP with MTP assay, Anthracene solubility assay and Methylene blue assay
- no biosurfactant positive hits were found



© Fraunhofer IGB
c-LEcta enzymes and strains
Fraunhofer IGB

WP6: Bioprocess Engineering and DSP

- Scale Up/Batchwise DSP
- Membrane design for In Situ Product Recovery and set up of an integrated process

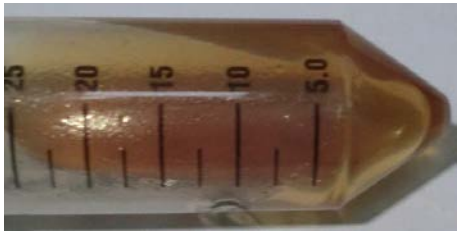


© Fraunhofer IGB

Fraunhofer IGB

WP6: Downstream processing of MEL and CL

Biosurfactant	Purity	Consistency
Mannosylerythritol Lipid (MEL)	60-80 %	Viscous Fluid
Cellobiose Lipid (CL)	60-80 %	Powder



- Recovery and concentration of the samples
- Obtain a purity >> 90 %

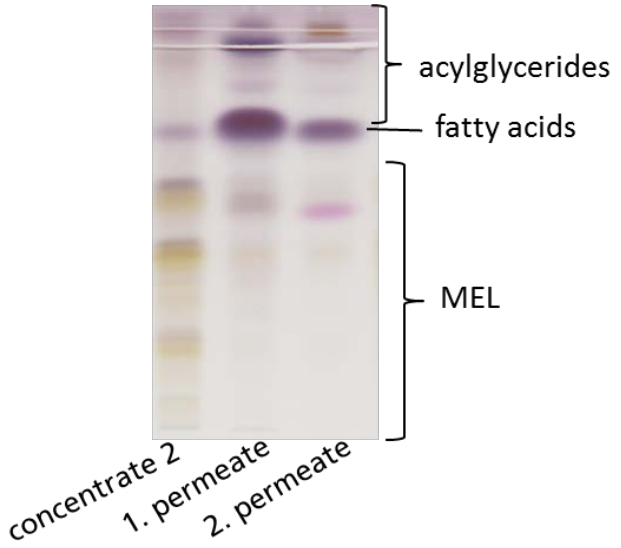
- Procedures
  - Precipitation of the biosurfactants
    (➔ not suitable, poor quality of the product)
  - Extraction with Supercritical CO_2
  - Membrane filtration
    variation of membranes, solvents and ratios of the mixture biosurfactant / solvent

DSP of MEL and CL: Membrane filtration

- Membrane filtration of MEL (63 % ➔ 90 % purity)

  ➔ Suitable membrane, solvent and sample mixture for MEL indentified

  ➔ two step filtration leads to 90 % purity

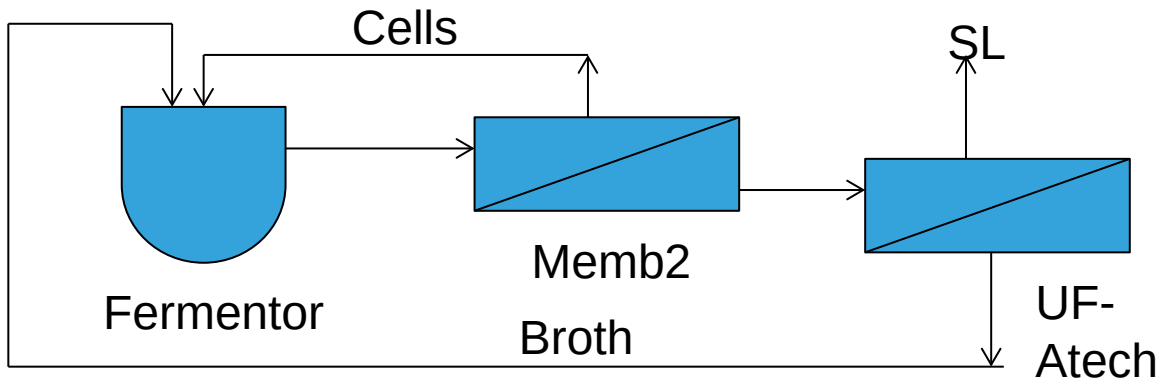


- Membrane filtration of CL (46 % ➔ 90-100 % purity)

  ➔ highly selective separation

	Concentrate	Permeate
Membrane 1 + Ethylacetate		
% CL	87	0
% other lipids	13	100
Membrane 1 + Hexane		
% CL	100	0
% other lipids	0	100

WP6: SL yield after membrane purification (ISPR)



	sediment	soluble	sediment + soluble
Membrane1	6%	63%	53%
Membrane2	30%	98%	78%

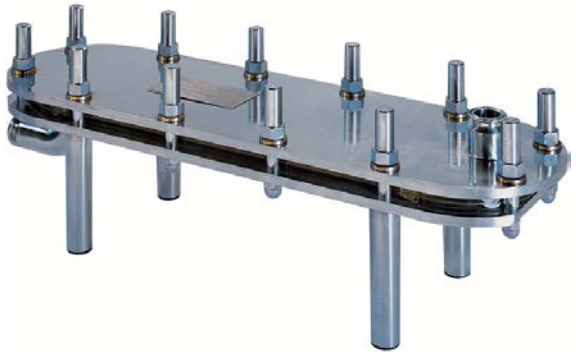
	sediment	soluble	sediment + soluble
Membrane1	2%	45%	41%
Membrane2	15%	91%	82%

© Fraunhofer IGB

WP6: DSP on sophorolipids

In Situ Product Recovery on site:

- Installation of lab stack device at pilot facilities Ecover
- First trial a few months ago
- results limited due to technical difficulties
- Second trial schedueld for end of this month



© Fraunhofer IGB

WP7: Dissemination and Exploitation

- Presentations at workshops/trade fairs
  - Forum Industrial Biotechnology @ Biotechnika (11/2011)
  - Hannover Messe (4/2012) Exhibition @ Fraunhofer House of Sustainability
  - “Grüne Woche” Berlin 1/2013 and 1/2014, Exhibition @ Fraunhofer Booth

- First International Workshop on Biosurfactants
  16.-17.5.2013 @ DECHEMA, Frankfurt

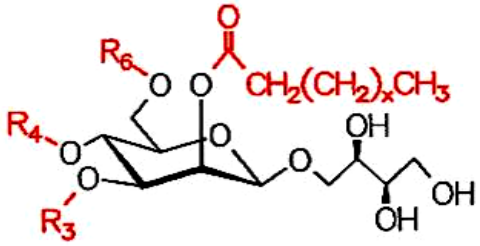
  organized by BioSurf: Christoph Syldatk, Rudolph Hausmann, Steffen Rupp
  (see http://events.dechema.de/biosurf.html)

© Fraunhofer IGB

Summary

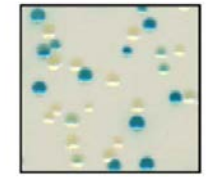
- Production of several modified bio-surfactants (SL, MEL, CL)
- Novel bio-surfactants identified and produced (Trehalose-Lipids, MEL-X)
- Novel bio-surfactants synthesized enzymatically (Terpenols, aminoglycosid lipids, cellobiose lipids)
- Identification of biosynthetic pathways for MEL production and their regulation using NGS
- Application tests and qualification of several novel bio-surfactants and their modifications (still ongoing)
- Novel DSP-Processes for SL, MEL and CL (still ongoing)
- Cost reduction in SL-production by ISPR-process

© Fraunhofer IGB

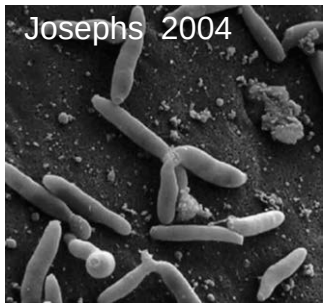


Identification and preparation

Bioprocess Engineering and DSP



Selection of mutants by **digital imaging screening**



Thank you for your attention

product development

Dissemination and Exploitation