~ ECCO Turin 2019 ~

IMMOBILIZED *RHODOCOCCUS* BIOCATALYSTS FOR ENHANCED BIOREMEDIATION

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Introduction

- Crude oil extraction and transport are often accompanied by soil contamination with hydrocarbons and heavy metals
- Land contamination negatively impacts economical and social developments, threats human health and natural biodiversity
- Bioremediation has a great potential to restore polluted environments by using biodegradation & bioaccumulation activities of microorganisms
- Immobilized biocatalyst advantages: high cell loading, stress resistance, functional stability, repeated usage, convenient storage and transport

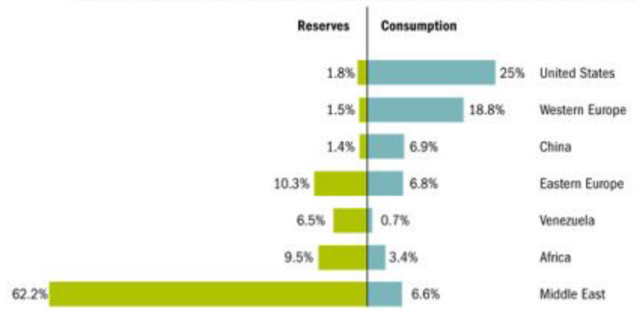
Who has the oil?

World Reserves of Oil

	Billions of Barrels	Percentage of World Reserves
Saudi Arabia	262.73	22.3%
Iran	132.46	11.2%
Iraq	115.00	9.7%
Kuwait	99.00	8.4%
United Arab Emirates	97.80	8.3%
Venezuela	77.22	6.5%
Russia	72.27	6.1%
Kazakhstan	39.62	3.4%
Libya	39.12	3.3%
Nigeria	35.25	3.0%
United States	21.37	1.8%
China	17.07	1.4%
Canada	16.80	1.4%
Qatar	15.20	1.3%

The U.S. uses the most oil

Percentage of world oil reserves vs. oil consumption



environmental action

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617-747-4404 (ph) • 617-292-8057 (fx)
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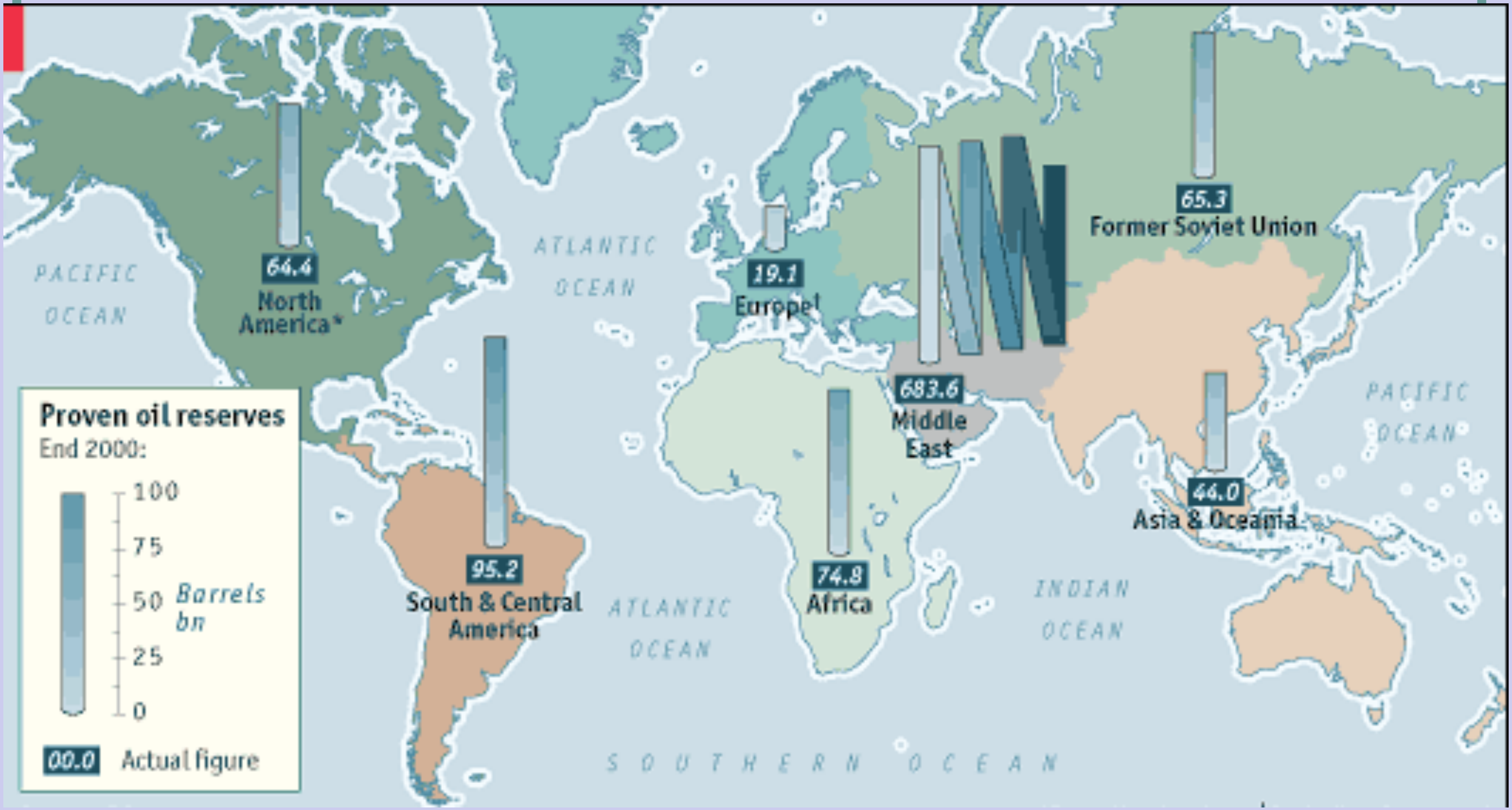
www.environmental-action.org

Each country's size is proportional to the amount of oil it contains (oil reserves);
Source: *BP Statistical Review* Year-End 2004 & Energy Information Administration

World distribution of oil (proven reserves)



Amoco Cadiz / Exxon Valdez

1978
France

6th largest oil spill:
68.7 million gallons

1989
Alaska

53rd largest oilspill:
11 million gallons

Ixtoc I blowout, Mexico 1979

World's second largest oil spill: est. 428 million gallons

Deliberate release, Kuwait

Unburned oil in the Burqan Oilfield

Burning oil wells, Kuwait

World’s largest oil spill was deliberate: estimated 1.5 billion litres

Deepwater Horizon, Mexican Gulf, 2010

Estimated 50-60 thousand barrels/day

Total release ~ 4-5 million tones

Oil industry in Perm region

- The Urals is the second largest oil-production area in Russia.
- A quarter of the industry of Perm region is oil and gas.
- There are 222 oilfields in Perm region, and unexplored oil resources estimate about 600 million tones.

Usinsk catastrophe, 1994

The worst accidental spill on land

130 000 tones of crude oil released from a ruptured pipeline

Worst oil spill on land (*Guinness Book*)

Waste oil pits

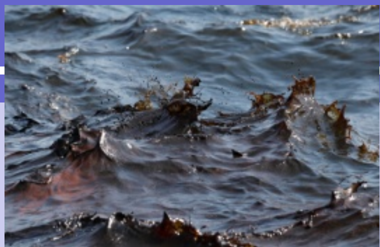
- A leftover from oil exploration and refinery on land
- Over the years light fractions evaporate, and the pits contain viscous and debris laden asphalt-like oil
- Oil wastes are harmful due (i) volatile hydrocarbon emission; (ii) penetration into soil and groundwater
- There are several oil waste pits in Perm region

Petroleum hydrocarbons – Priority Pollutants

1. Acenaphthene
2. Benzene
38. Ethyl benzene
39. Fluoranthene
55. Naphthalene
72. Benzo(a)anthracene
73. **Benzo(a)pyrene**
74. Benzo(b)fluoranthene
75. Benzo(k)fluoranthene
76. Chrysene
77. Acenaphthylene
78. Anthracene
79. Benzo(g,h,i)perylene
80. Fluorene
81. Phenanthrene
82. Dimethylbenza(a)anthracene
83. Indeno(1,2,3CD)pyrene
84. Pyrene
86. Toluene

Endocrine-disruptive action !!!

Negative impact on natural ecosystems



Oxygen limitation

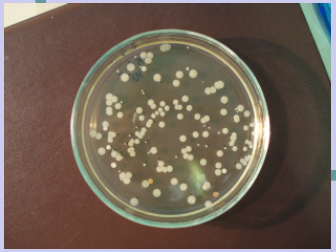
Water retention

Direct toxic action on living organisms

Organic carbon increase

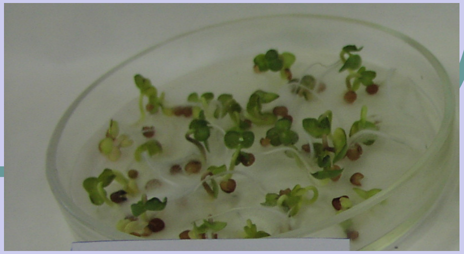
Decrease in bioavailability of organic carbon

Symbiotic interaction breakdown



Microbial population reduction

Food chain disruption

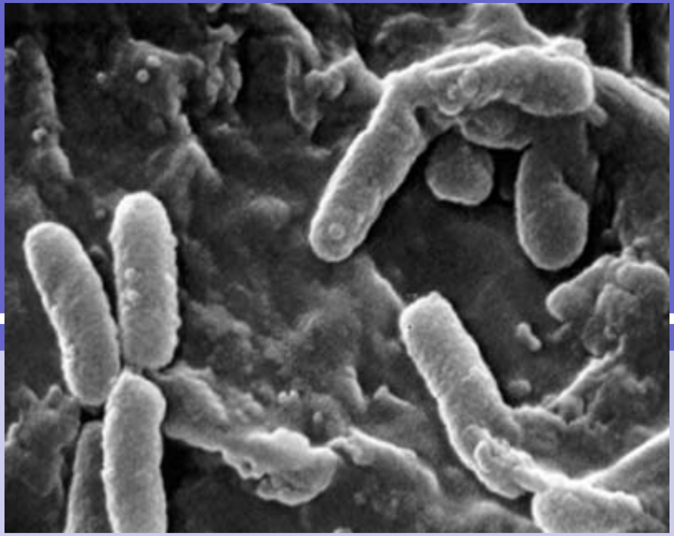


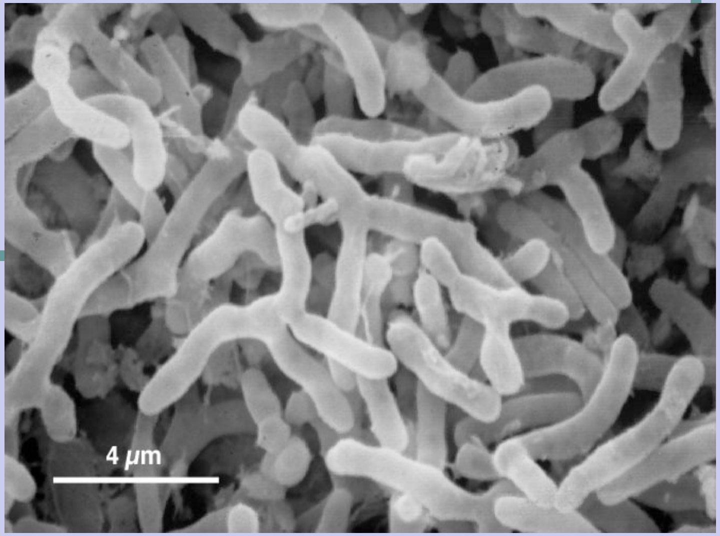
Biodiversity of oil-degrading microorganisms

Morphogroups	Total > 150 genera
Mycelial fungi	*Antrodia, Phanerochaete, Pleurotus, Chrysosporium, Cunninghamella, Stropharia, Cladosporium, Hypocrea, Graphium, Fusarium, Aspergillus, Mucor, Penicillium, Rhizopus, Trichoderma, Cladophialophora*
Yeasts	*Candida, Clavispora, Debaryomyces, Leucosporidium, Lodderomyces,Yarrowia, Rhodosporidium, Rhodotorula, Trichosporon,Sporidiobolus, Sporobolomyces, Stephanoascus*
Bacteria	*Aeromonas, Arthrobacter, Bacillus, Burkholderia, Sphingomonas, Rhodococcus, Mycobacterium, Acinetobacter, Alteromonas, Moraxella, Micrococcus, Flavobacterium, Pseudomonas, Cyanobacteria*
Algae	*Chlorella, Phaeodactylum, Haematococcus, Emiliania, Dunaliella, Umbellularia, Cinnamomum, Nannochloropsis*

Mostly studied hydrocarbon-oxidizing bacteria

Pseudomonas ***Rhodococcus***

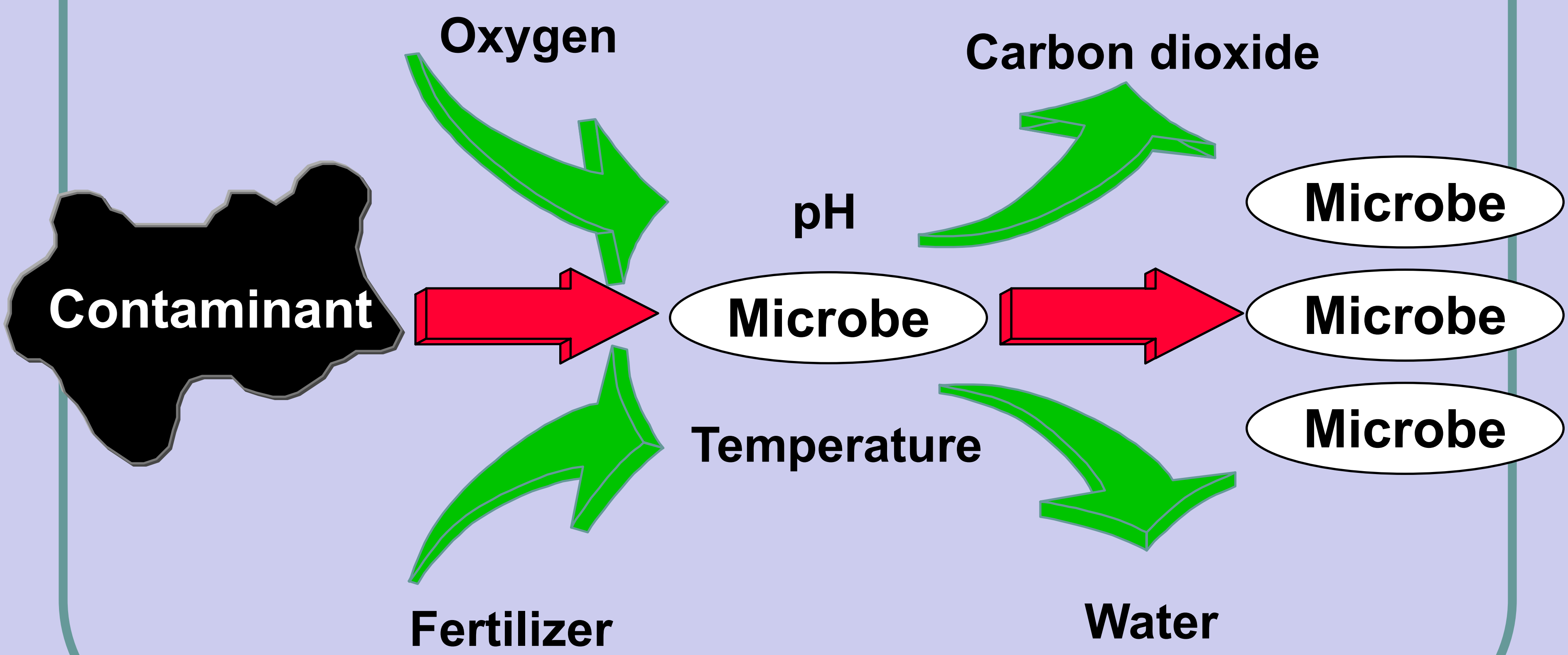




What is bioremediation ?

- Biodegradation by microorganisms
  - Mineralization
    - Contaminants used as a food source and destroyed
  - Cometabolism
    - Contaminants not used as a food source, but transformed to less hazardous chemicals
- Immobilization by microorganisms or plants
  - Removal of metals by adsorption, precipitation or accumulation

Bioremediation explained



Petroleum hydrocarbons

Aliphatics

$H_3C—(CH_2)_{14}—CH_3$

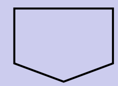
n-hexadecane

Pristane

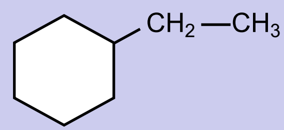
$H_2C=CH—(CH_2)_5—CH_3$

Oct-1-ene

Cycloaliphatics

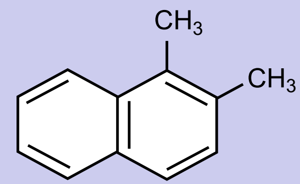


Cyclopentane

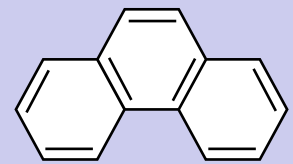


Ethyl cyclohexane

Aromatics

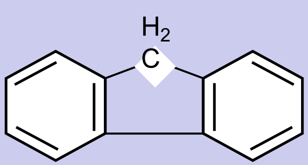


1,2-dimethyl naphthalene



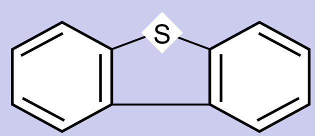
Phenanthrene

Mixed cycloaliphatics/ aromatics

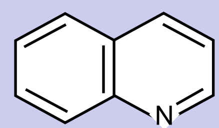


Fluorene

NSO compounds

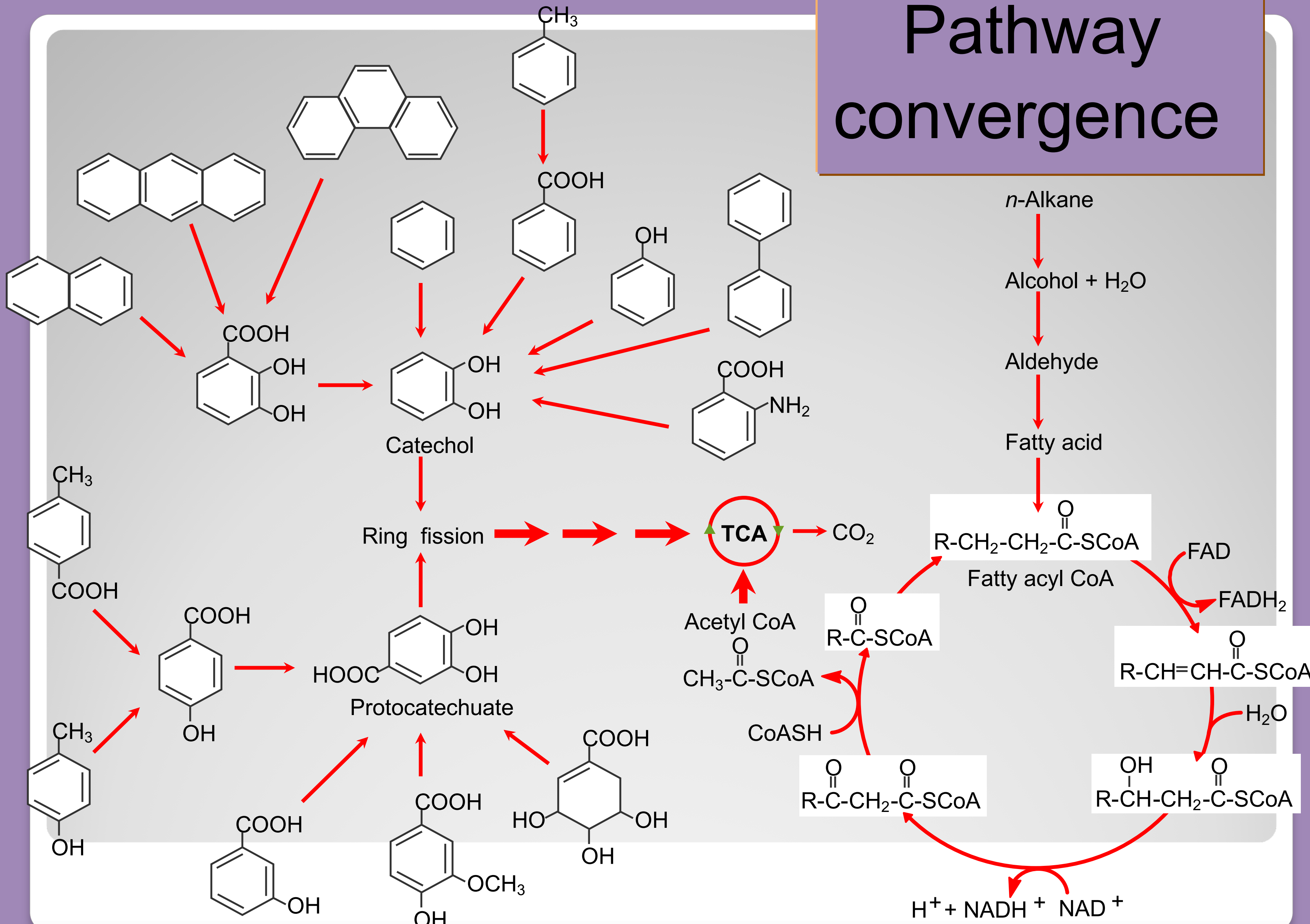


Dibenzothiophene

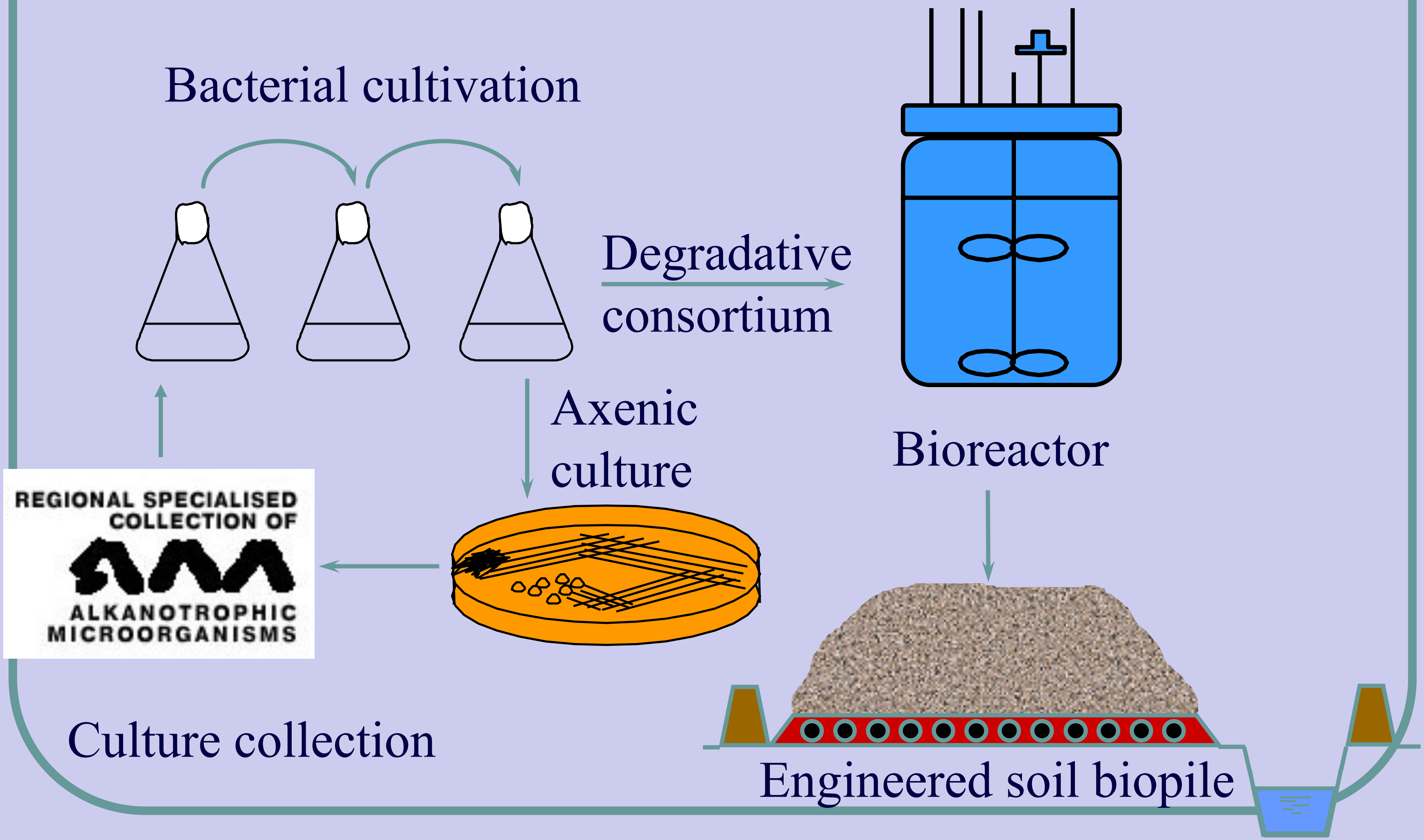


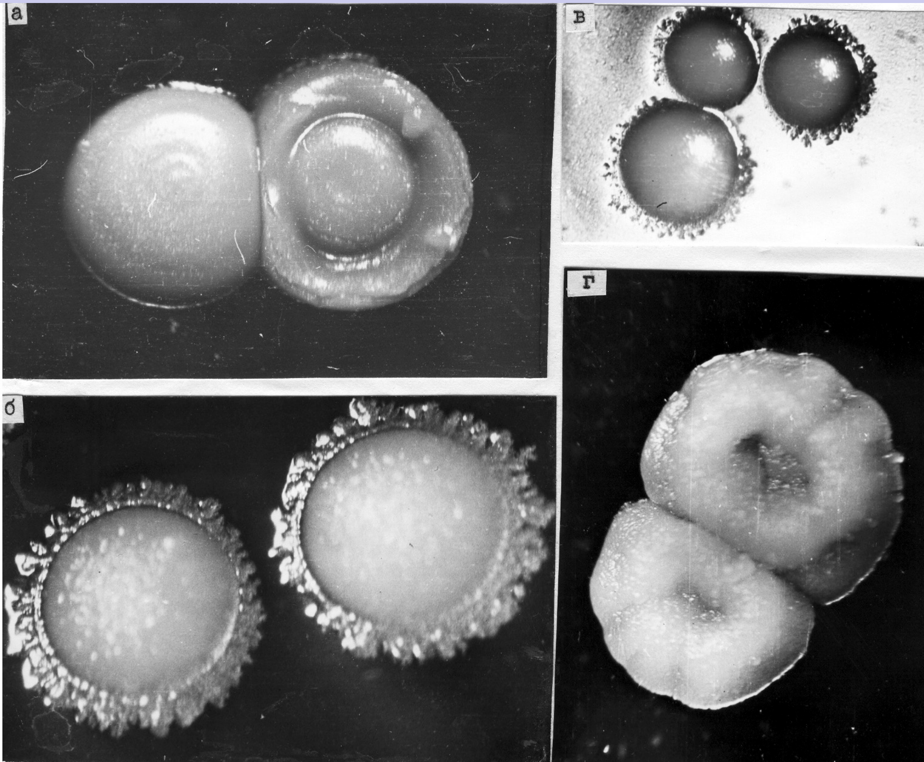
Quinoline

Pathway convergence



Bioaugmentation as bioremediation strategy

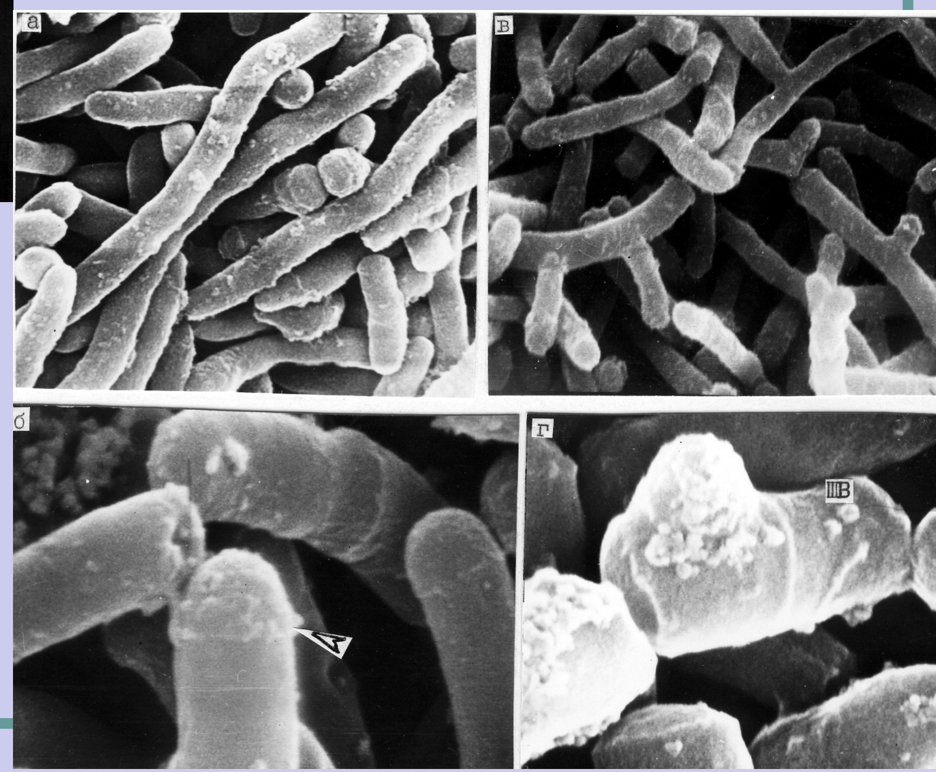




The IEGM Collection of Alkanotrophic Microorganisms

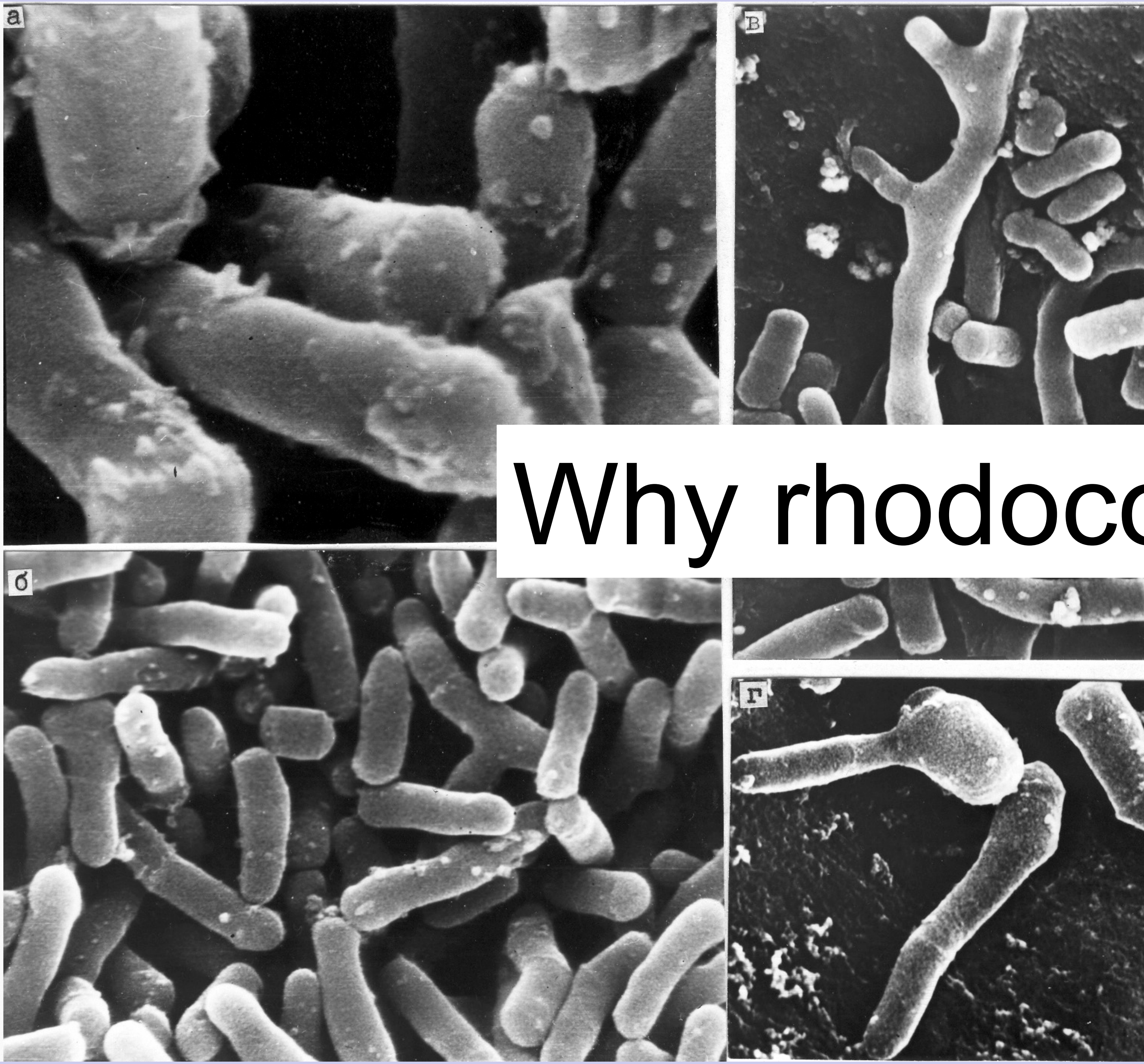
More than **3000** non-pathogenic and aerobic bacterial cultures isolated from **contrasting** climatic regions.

www. iegmcol.ru



- 86 species of 19 bacterial genera
- Cultures of the genus ***Rhodococcus*** comprise the major portion of the Collection
- Strains – **biodestructors** of organic pollutants, **producers** of amino acids, **enzymes** and **biosurfactants**

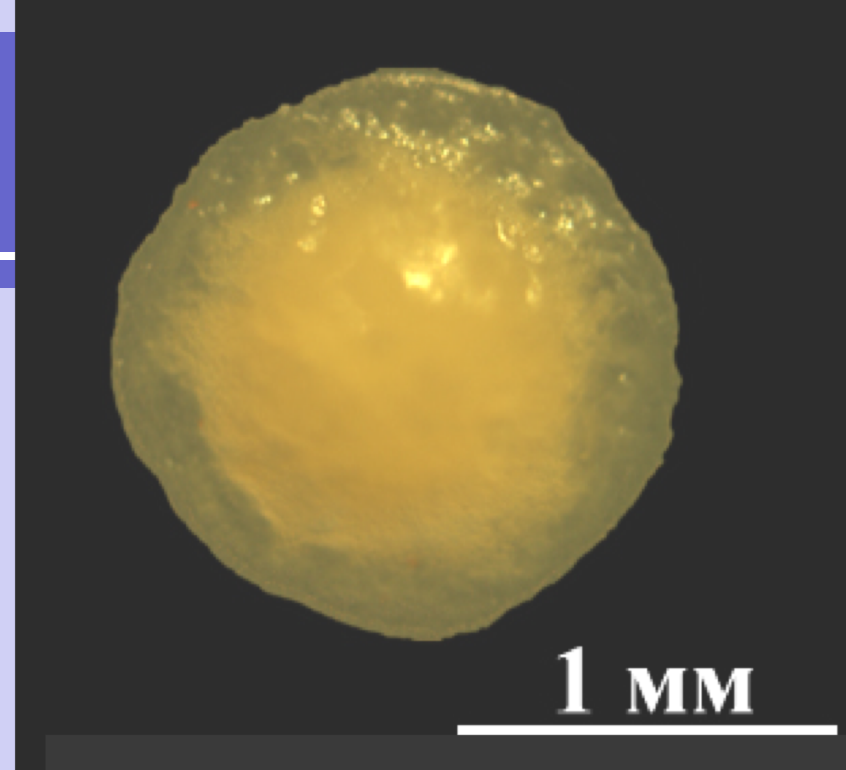
WDCM # 768 http://www.wfcc.info/datacenter.html



Why rhodococci ?

REGIONAL SPECIALISED COLLECTION OF ALKANOTROPHIC MICROORGANISMS


1 мм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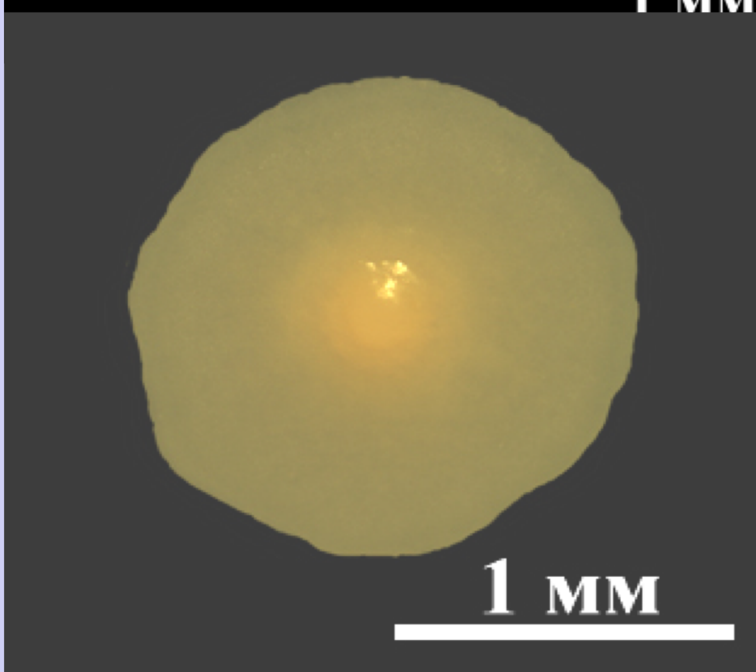
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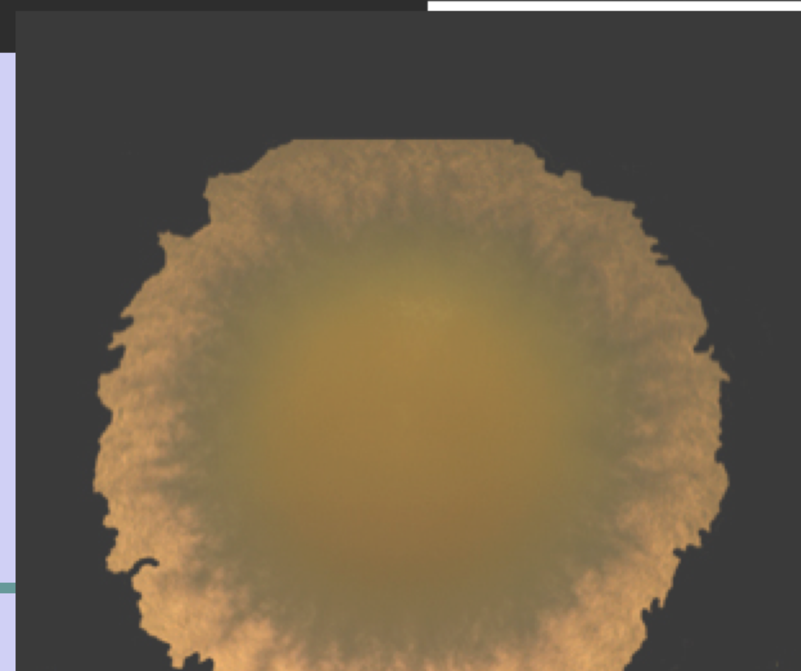
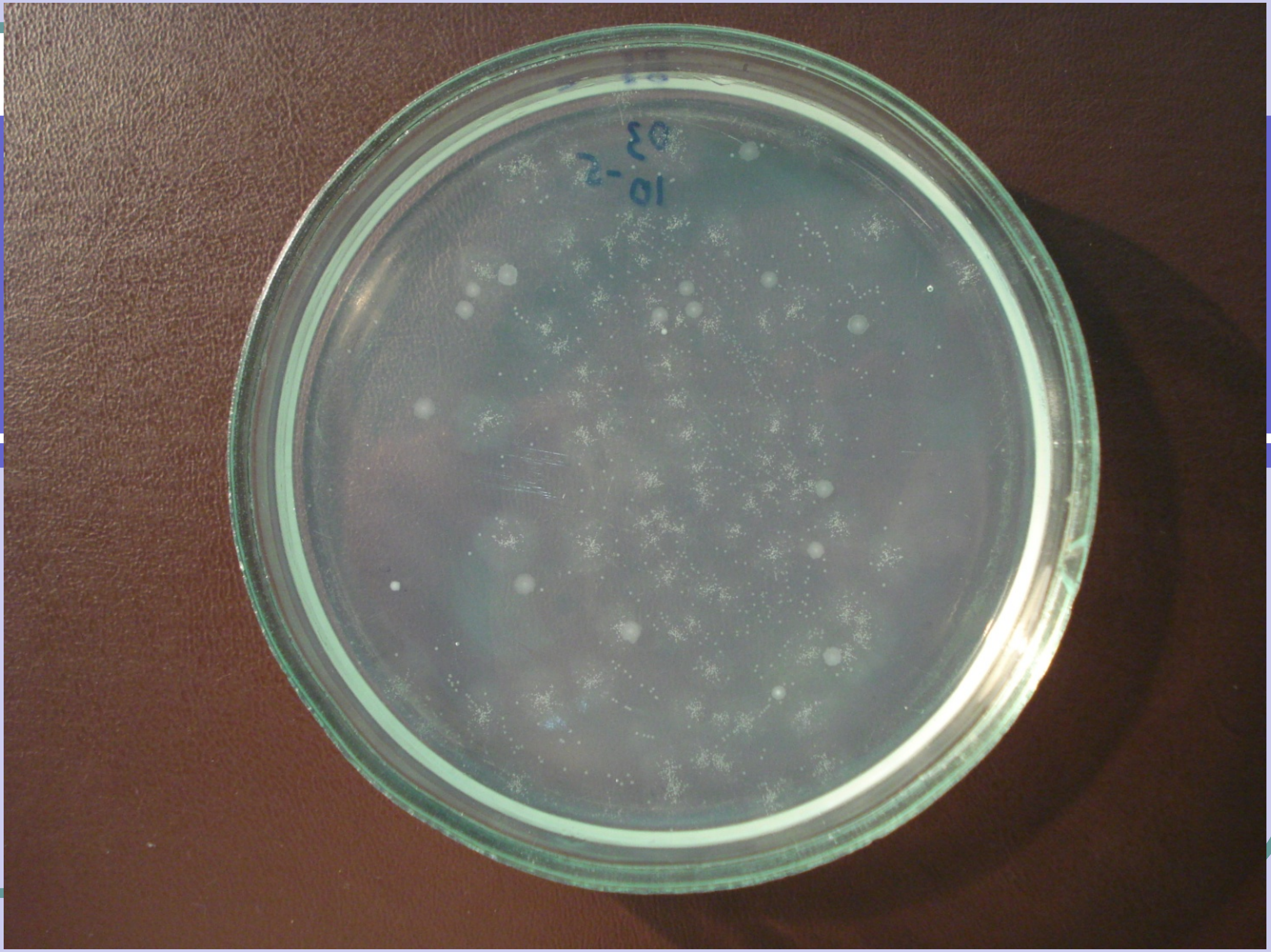


Rhodococcus advantages

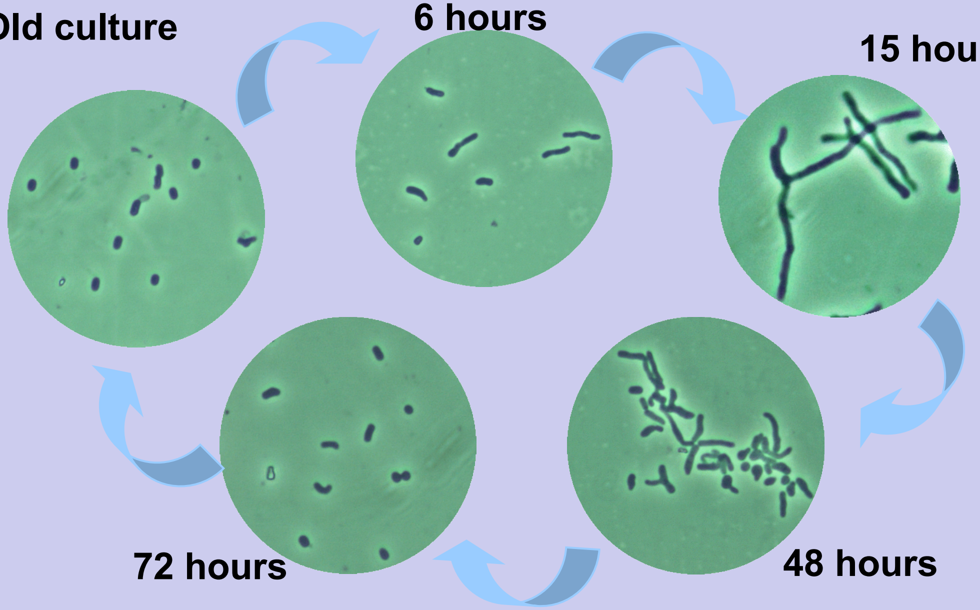
- Typically bacterial type of growth
- Non-spore forming and non-motile
- Oligocarbo- and oligonitrophilia
- No antagonistic action and pathogenicity
- High stress resistance
- Adhesion to hydrophobic liquids & solids
- **High catabolic diversity and unique enzymatic capabilities**

Oligocarbophilic *Rhodococcus*

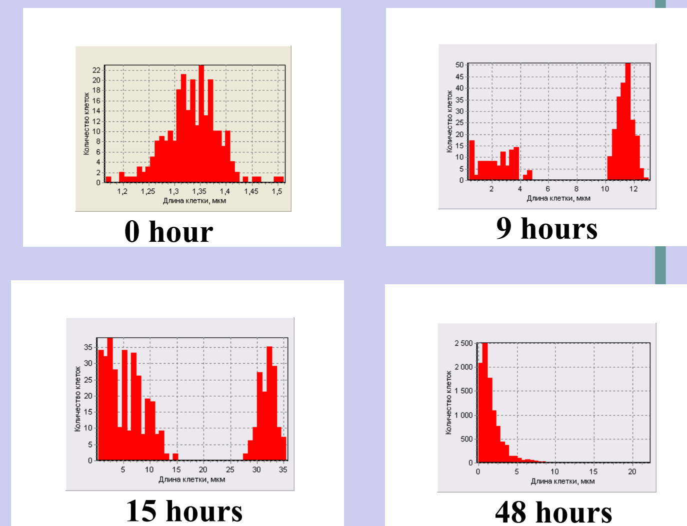


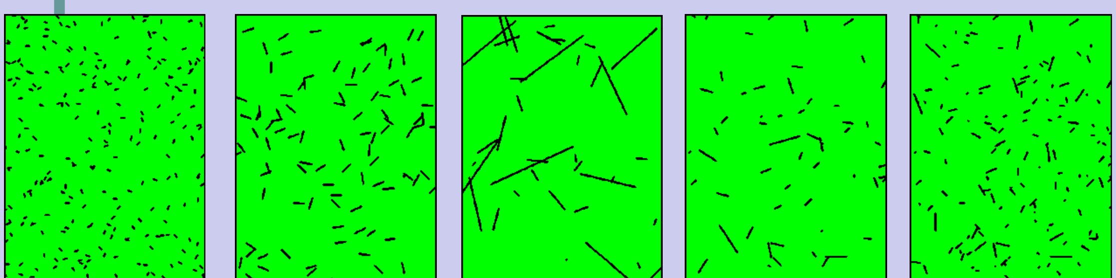
Growth of *Rhodococcus erythropolis* on “minimal” agar.
Oligotrophs – organisms able to grow at organic carbon concentration < **1 mg /l.**

Growth cycle of *Rhodococcus ruber*



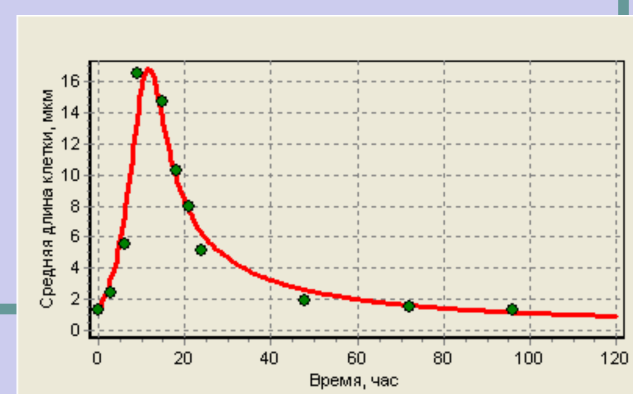
Cell number *vs.* cell length

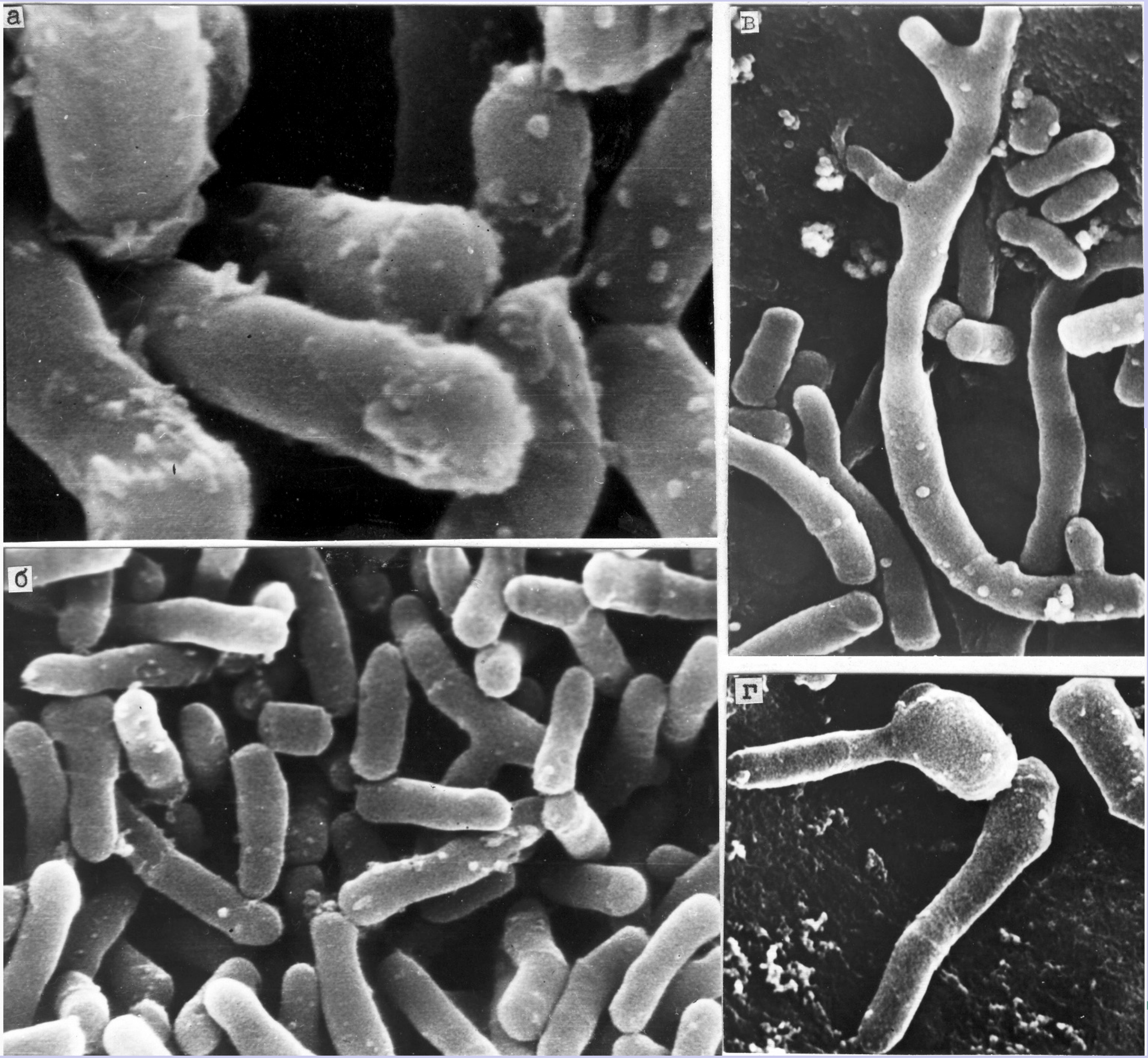




Mathematical model

Experimental and theoretical graphs of cell length *vs.* time

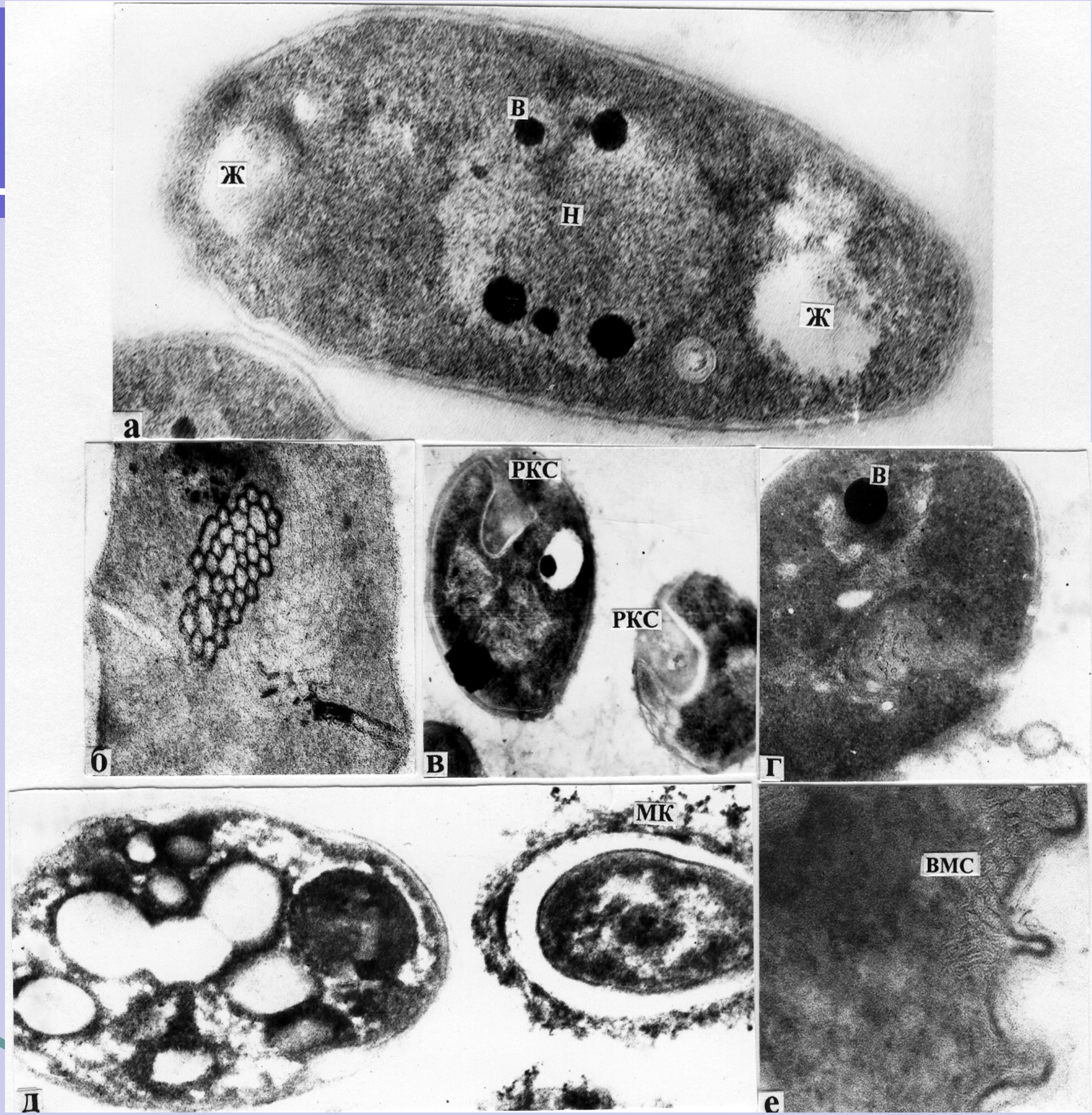




Scanning Electron Microscope (SEM) images of *R. ruber* IEGM 231 cells

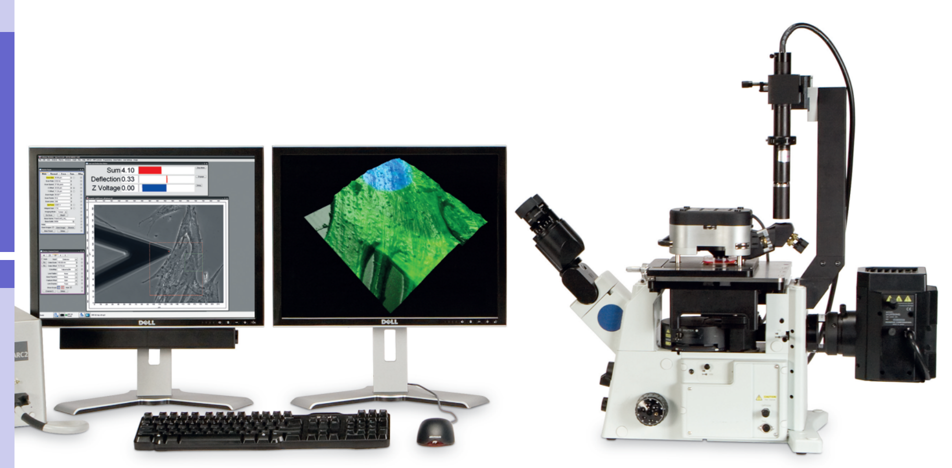
REGIONAL SPECIALISED COLLECTION OF ALKANOTROPHIC MICROORGANISMS

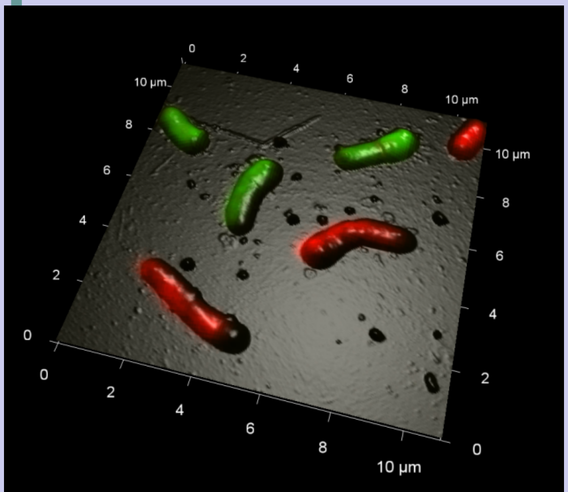
Transmission Electron Microscope (TEM) images of *R. ruber* IEGM 231 cells



REGIONAL SPECIALISED COLLECTION OF ALKANOTROPHIC MICROORGANISMS

Atomic force & confocal laser scanning microscopy





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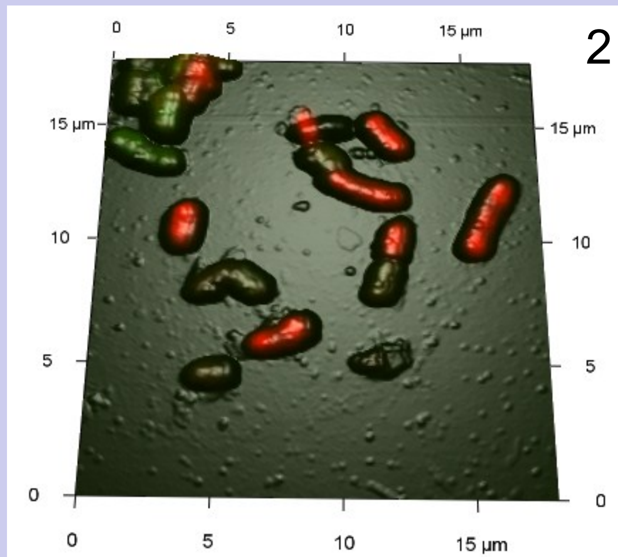

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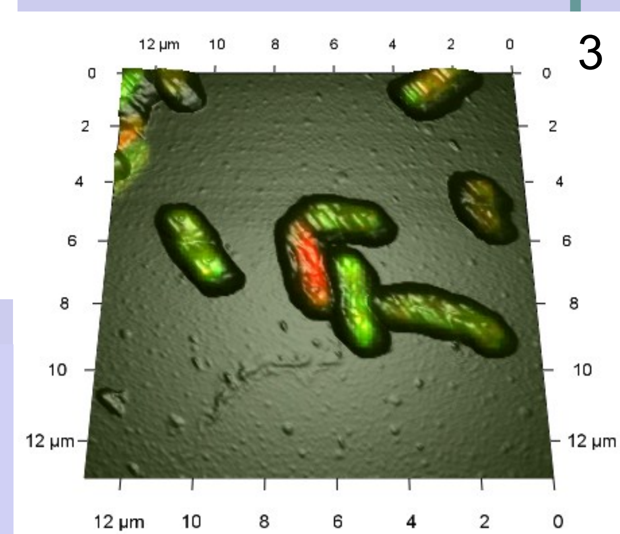

3

LIVE/DEAD® BacLight™ Bacterial Viability Kit (Invitrogen)

Combined AFM/CLSM images of *Rhodococcus* cells exposed to organic solvents: 1 – control, 2 – cyclohexane, 3 – toluene.
Viable cells – green, dead cells – red color.

Metabolized organic substances/pollutants

Saturated hydrocarbons	Gaseous: C_3-C_4. Volatile: C_5-C_{10} Liquid: C_{11}-C_{16} Solid: C_{17}-C_{20}	**Aromatic amines**	Anilines, toluidines (*o-, м-, p-*)
Aliphatic alcohols	Monohydric: ethanol, propanol-1, butanol-1, pentanol-1, octanol-1, hexanol-1, isopropanol, isobutanol	**Organic sulphides**	Thioanisole
Phthalic acid esters	Dimethylphthalate, dibutilphthalate, dimethylterephthalate, diethylhexylphthalate	**Crude oil**	Crude oils of various compositions, oil refinery products
Aromatic hydrocarbons and their derivatives	Methyl benzene, BTEX, phenols, naphthalene, PAHs	**Fats ad oils**	Cutting fluids, mineral and vegetable oils
Aromatic acids	*m*-Oxibenzoic, *p*-oxibenzoic, salycilic, terephthalic	**Surfactants**	Alkamon-D, alkyl-sulfonate, alkyl-behzenesulfonate
Isoprenoids	Dehydroabietinoic, isopimaric acids, β-sitosterol , betulin	**Antibiotics**	Oxacillin, chloramphenicol, erythromycin

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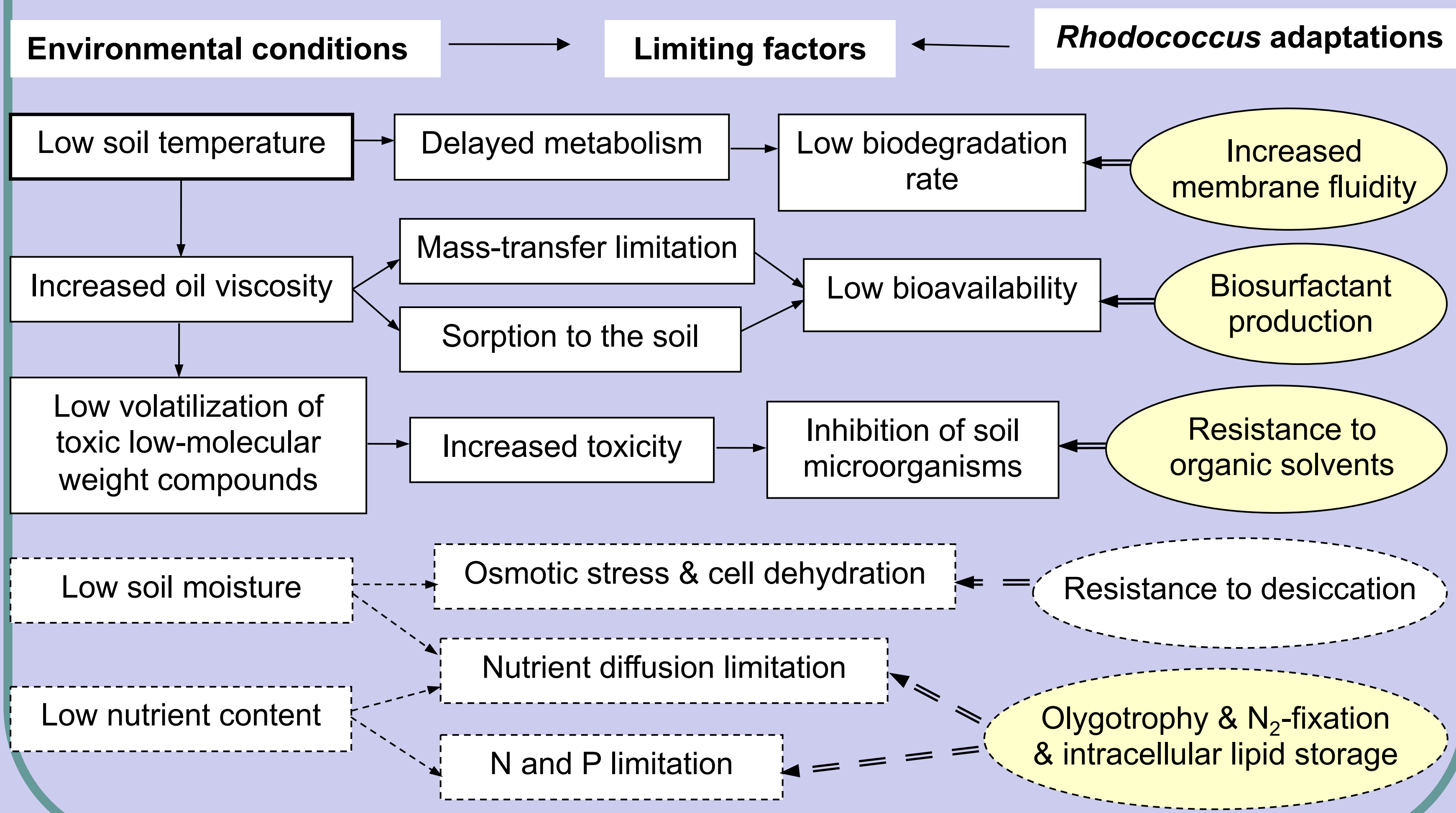
Rhodococcus adaptations to harsh soil conditions



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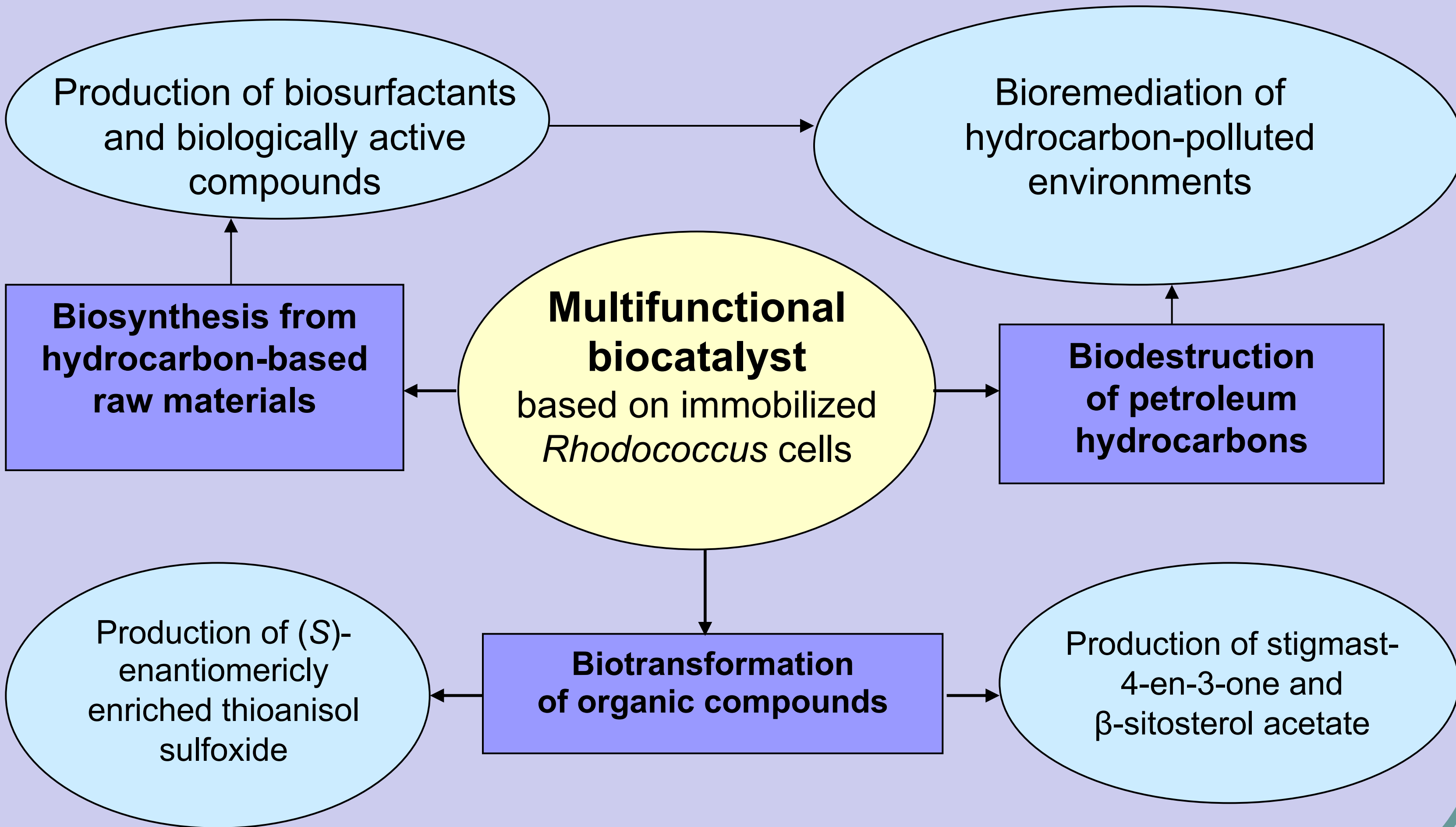
Potential biosafety risks for mycolata group

Genus (number of valid species)	Number of species (% from total number)			
	Non-pathogenic (risk group 1)*	Opportunistic (risk group 2)*	Pathogenic (risk group 3)*	No data on pathogenicity
Corynebacterium (66)	13 (20)	43 (65)	1 (2)	9 (14)
Dietzia (4)	2 (50)	1 (25)	0	1 (25)
Gordonia (19)	10 (53)	3 (16)	0	6 (32)
Mycobacterium (110)	43 (39)	54 (49)	7 (6)	6 (6)
Nocardia (61)	12 (20)	33 (54)	2 (3)	14 (23)
***Rhodococcus* (46)**	**42 (91)**	**2 (4)**	**0**	**2 (4)**
Tsukamurella (7)	1 (14)	3 (43)	1 (14)	2 (29)

*Risk group classification (prokaryotes): European Community classification. List of Prokaryotic Names with Standing in Nomenclature [http://www.bacterio.net]; Bacterial Nomenclature Up-to-Date [http://www.dsmz.de/microorganisms]

Rhodococcus biocatalyst fields of application



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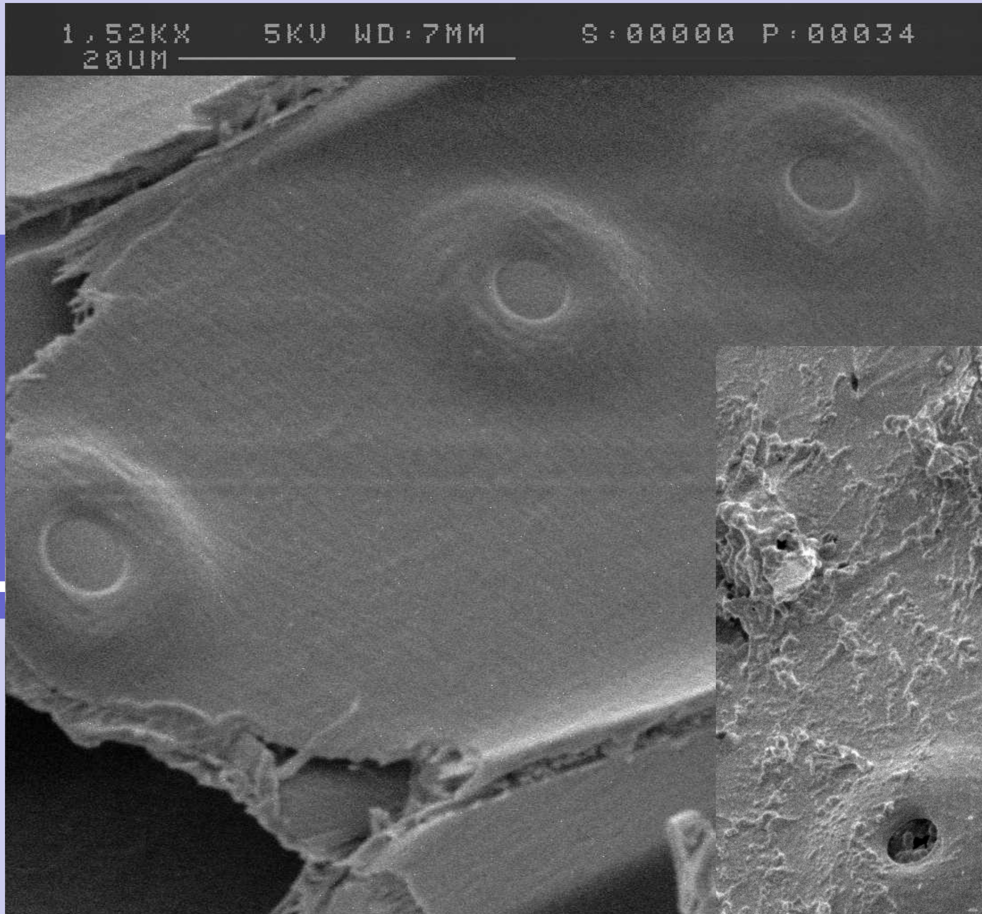
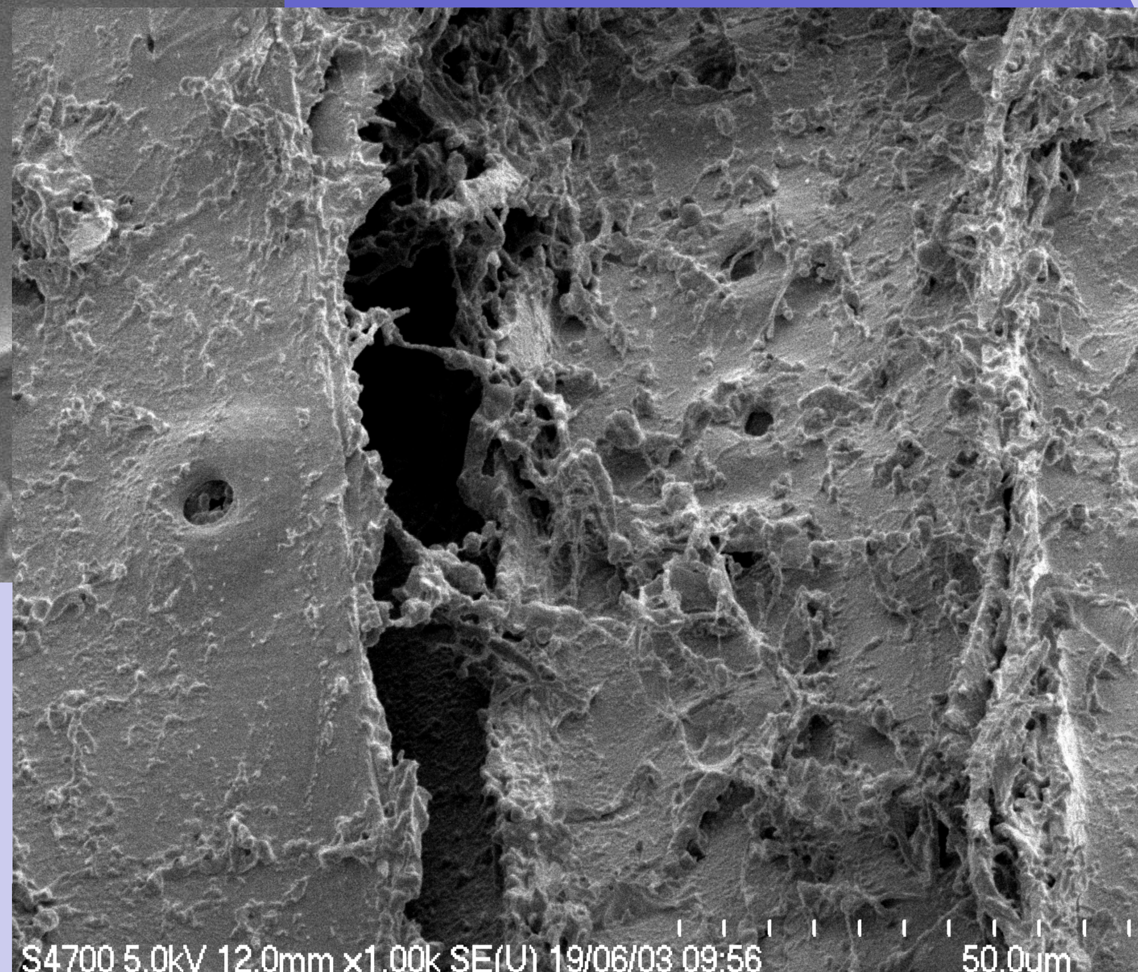
Immobilization of *Rhodococcus* cells on different matrices

Immobilization matrix		Water-absorbing capacity, g H_2O/g	Bacterial adsorption, mg of dried cells/g	Hexadecane degradation rate, mg/g·h
Base material	**Treated with hydrophobizing agent**			
Sunflower husks	None	2.03 ± 0.18	9.0 ± 3.0	53.0 ± 4.0
	Linseed oil varnish (“Olifa”) (1:2, v/v)	1.52 ± 0.08	2.0 ± 0.5	42.0 ± 6.0
Sawdust	None	2.55 ± 0.15	39.0 ± 5.0	71.0 ± 7.0
	“Olifa” (1:2, v/v)	0.39 ± 0.02	15.5 ± 1.5	46.0 ± 6.0
	“Olifa” (1:0.1, v/v)	**1.24 ± 0.09**	**46.5 ± 1.0**	**107.0 ± 9.0**
	Si-organic emulsion	1.93 ± 0.10	46.0 ± 3.0	65.0 ± 2.5
	Biosurfactant	**1.54 ± 0.05**	**40.0 ± 4.5**	**72.0 ± 4.5**
	n-Hexadecane vapour	1.68 ± 0.12	41.0 ± 4.0	42.5 ± 5.0
Chicken feathers	None	1.65 ± 0.10	6,0±1,0	43.0 ± 7.0
	“Olifa” (1:0.1, v/v)	1.48 ± 0.12	56.0 ± 6.5	61.0 ± 4.0
	Si-organic emulsion	**1.60 ± 0.04**	**69.0 ± 5.6**	**83.0 ± 8.0**

RU Patent 2298033; Podorozhko et al. (2008) *Biores. Technol*. 99:2001-2008; Kuyukina et al. (2009) *Int. Biodeter. Biodegrad*. 63:427–432; Krivoruchko et al. (2019) *Catalysts* 9(3):236

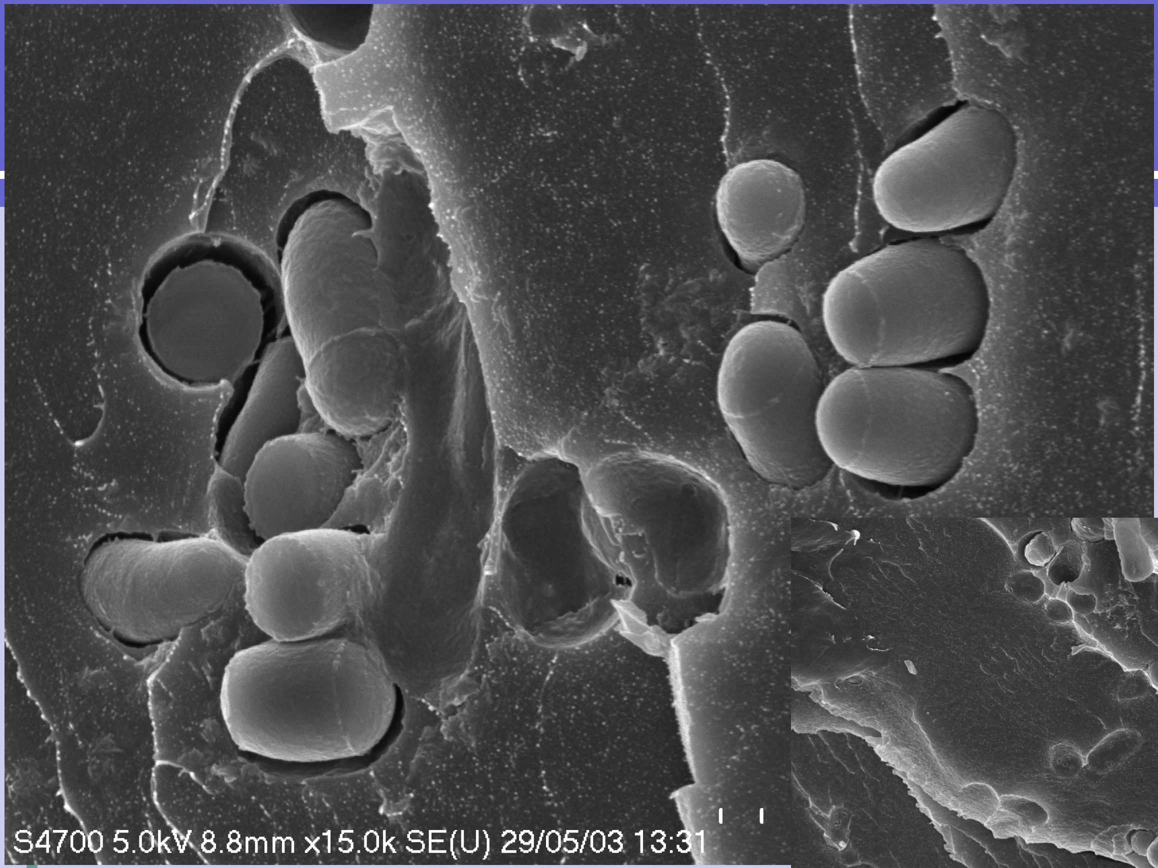


Unmodified sawdust
x 1000



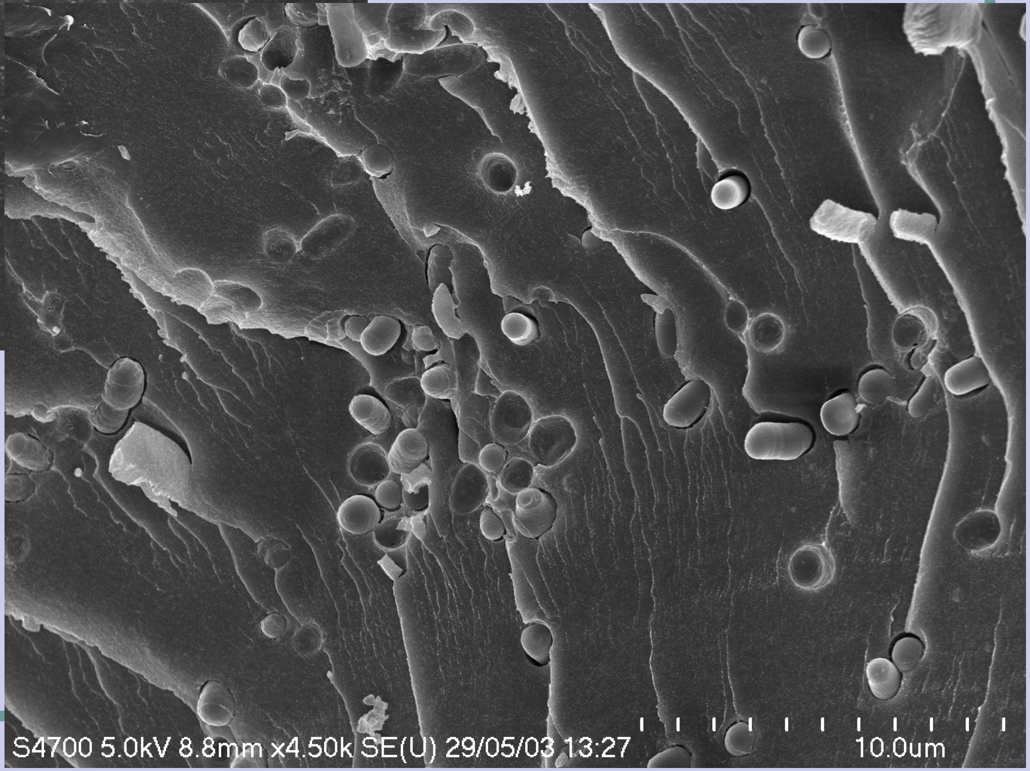
Hydrophobized sawdust with immobilised *Rhodococcus* cells
x 1000

RU Patent 2298033

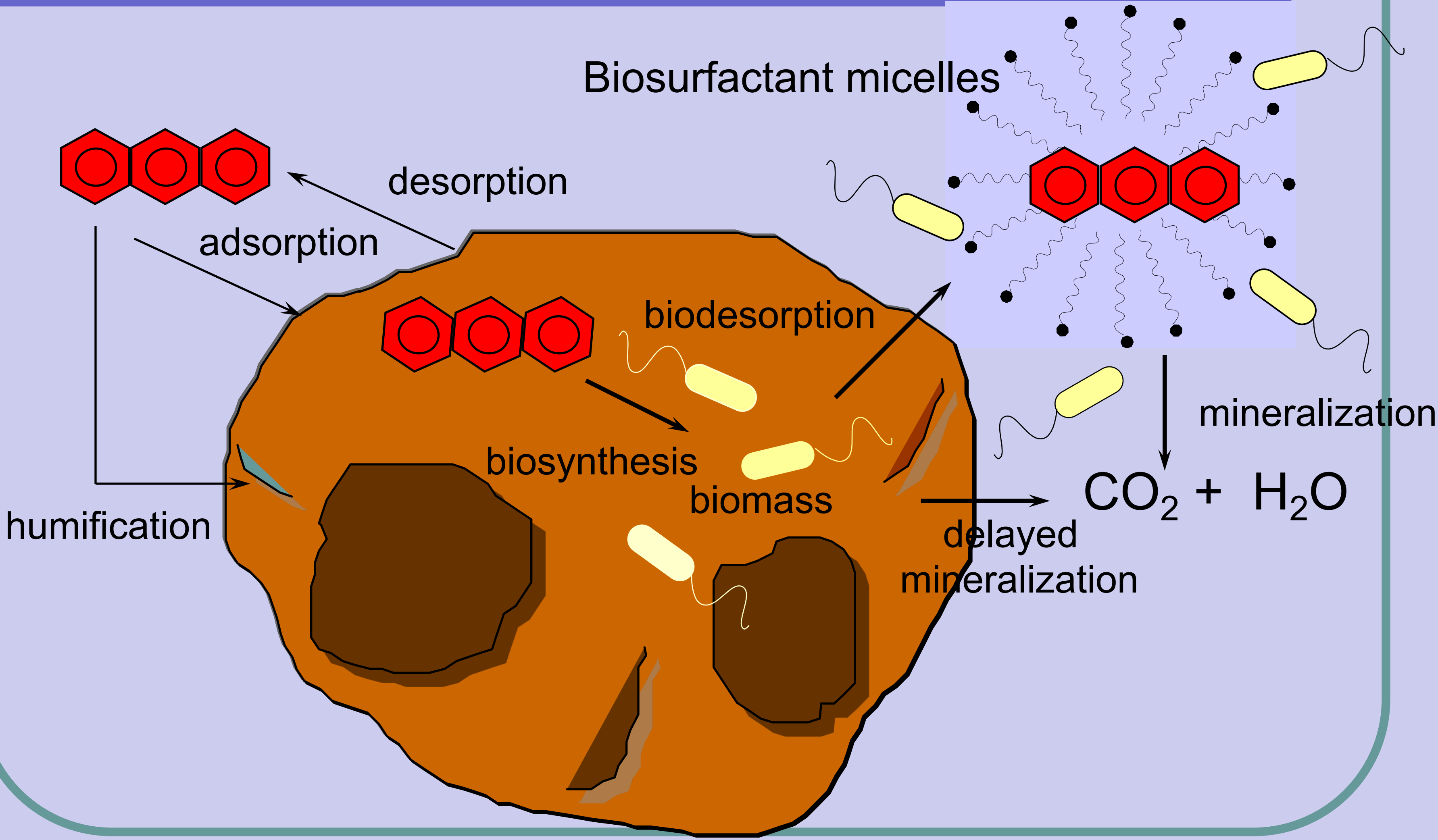


R. ruber cells entrapped in **P**oly(**V**inyl **A**lcohol) cryogel, x 15000

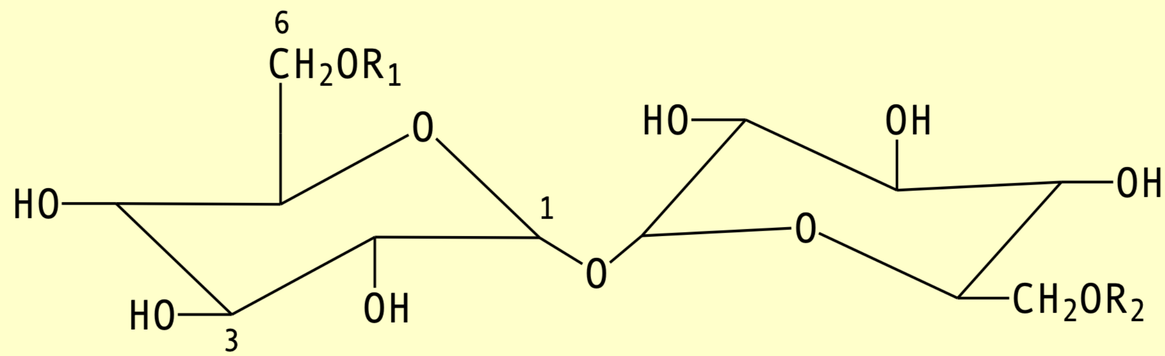
R. ruber cells entrapped in PVA cryogel, x 4500



Fate of hydrocarbon pollutants in soil



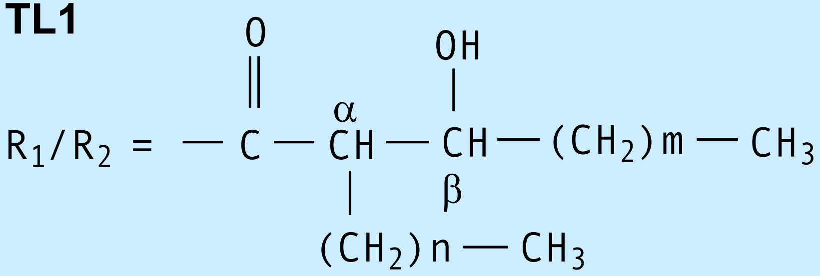
Structure of *Rhodococcus* biosurfactant



General scheme of Trehalose Lipid complex

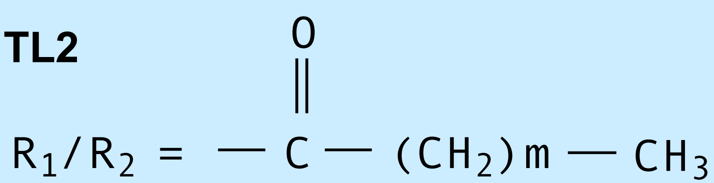
TL1, TL2 and TL3 – structural components

TL1



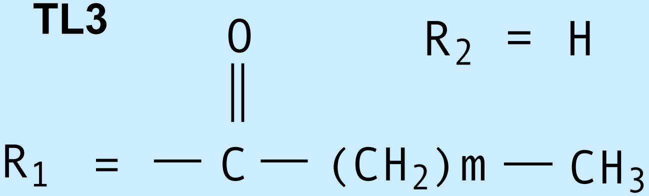
m + n = 29 – 41 (centered at 35)

TL2



m = 13 – 15 (i.e. probably 14 + 12 and 14 + 16 with main component 14 + 14)

TL3



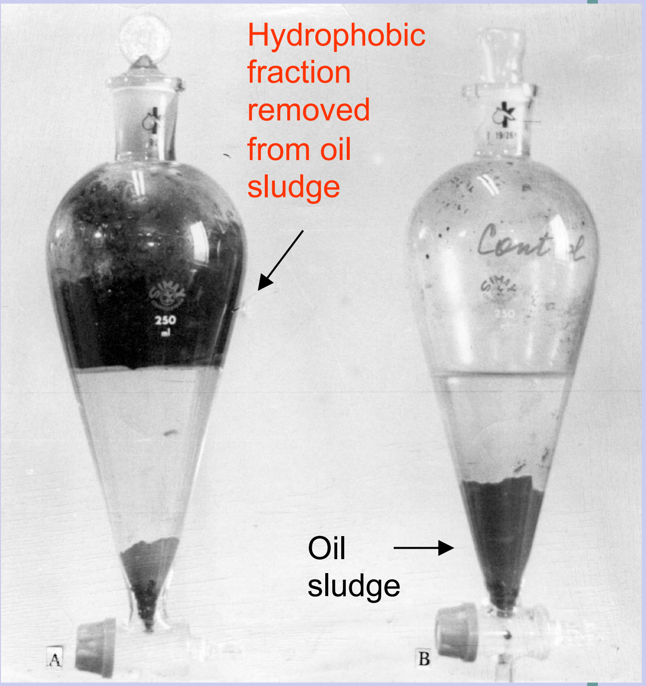
m = 10 – 14 (main component 12)

Kuyukina *et al.* (2001). *J. Microbiol. Methods* 46: 149-156

Philp *et al.* (2002). *Appl. Microbiol. Biotechnol.* 59: 318–324

Oil & PAH removal from soil using *Rhodococcus* biosurfactants

Rhodococcus species	Oil removed, % I	II	III	IV
R. erythropolis	96	77	70	63
R. opacus	87	77	22	10
R. ruber	**98**	**98**	**87**	**50**
Control (water)	31	20	5	2

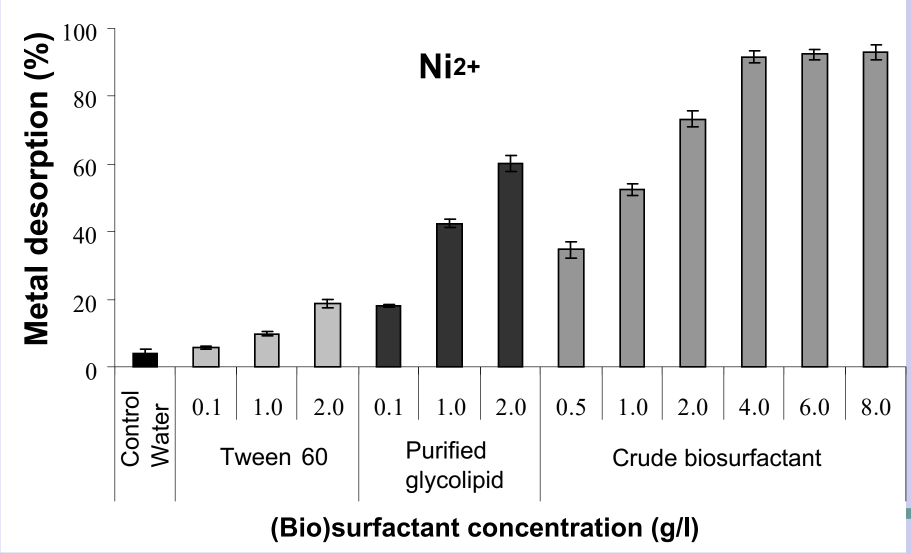


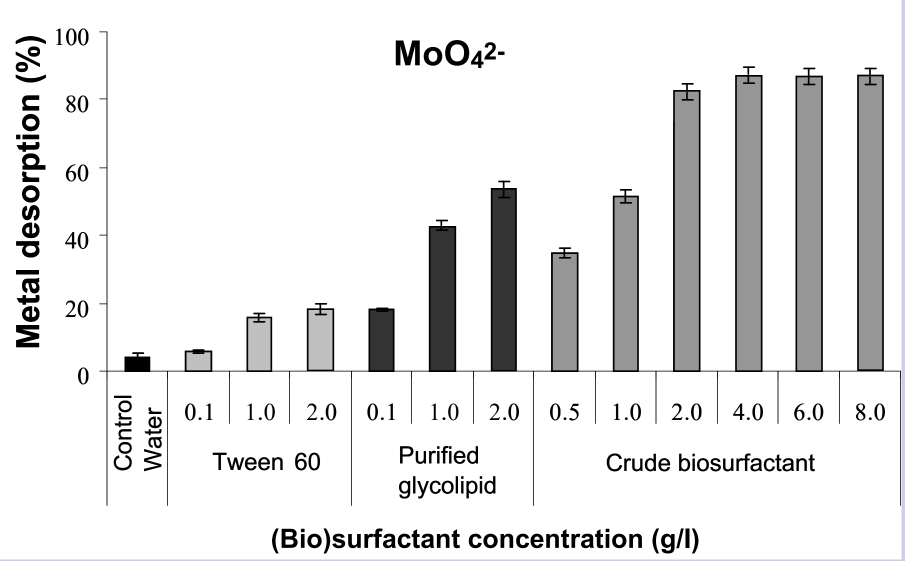
Oils have increasing asphaltenes and high molecular weight paraffins

Ivshina *et al*. (1998). *World J. Microbiol. Biotechnol.* 14: 711-717; Kuyukina *et al.* (2005). *Environ. Int.* 31: 155-161; Ivshina *et al*. (2016) *J. Hazard. Mater*. 312: 8–17

Heavy metal removal (%) from soil

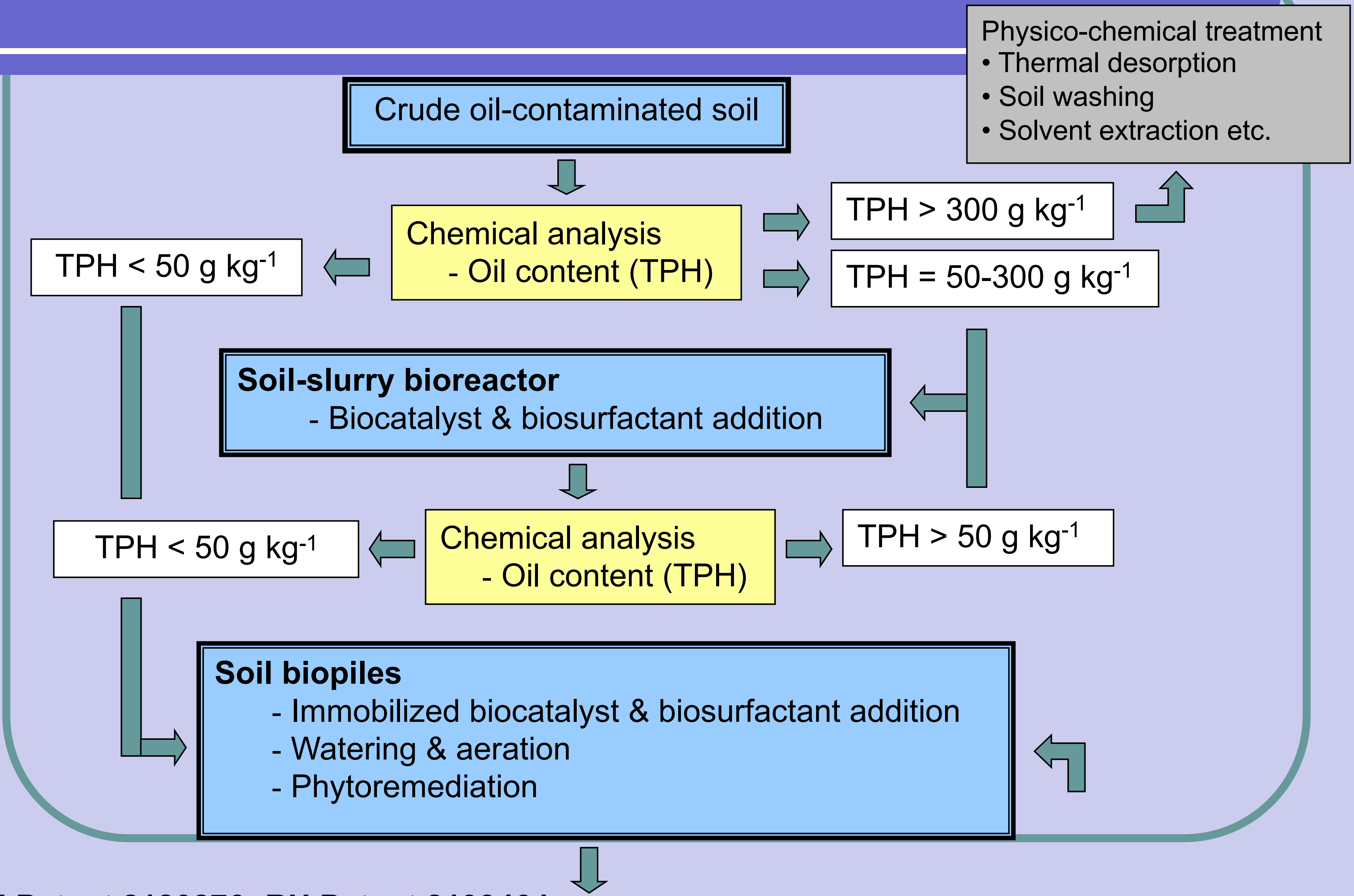
Heavy metals	*Rhodococcus* biosurfactant		Tween 60	Control (water)
	Crude	Purified		
Cd^{2+}	**82.3**	48.1	16.5	2.3
CrO_4^{2-}	**87.1**	58.0	19.3	3.9
MoO_4^{2-}	**88.3**	54.6	19.7	6.3
Ni^{2+}	**92.5**	66.7	21.1	4.8
Pb^{2+}	**68.7**	42.3	15.1	1.8





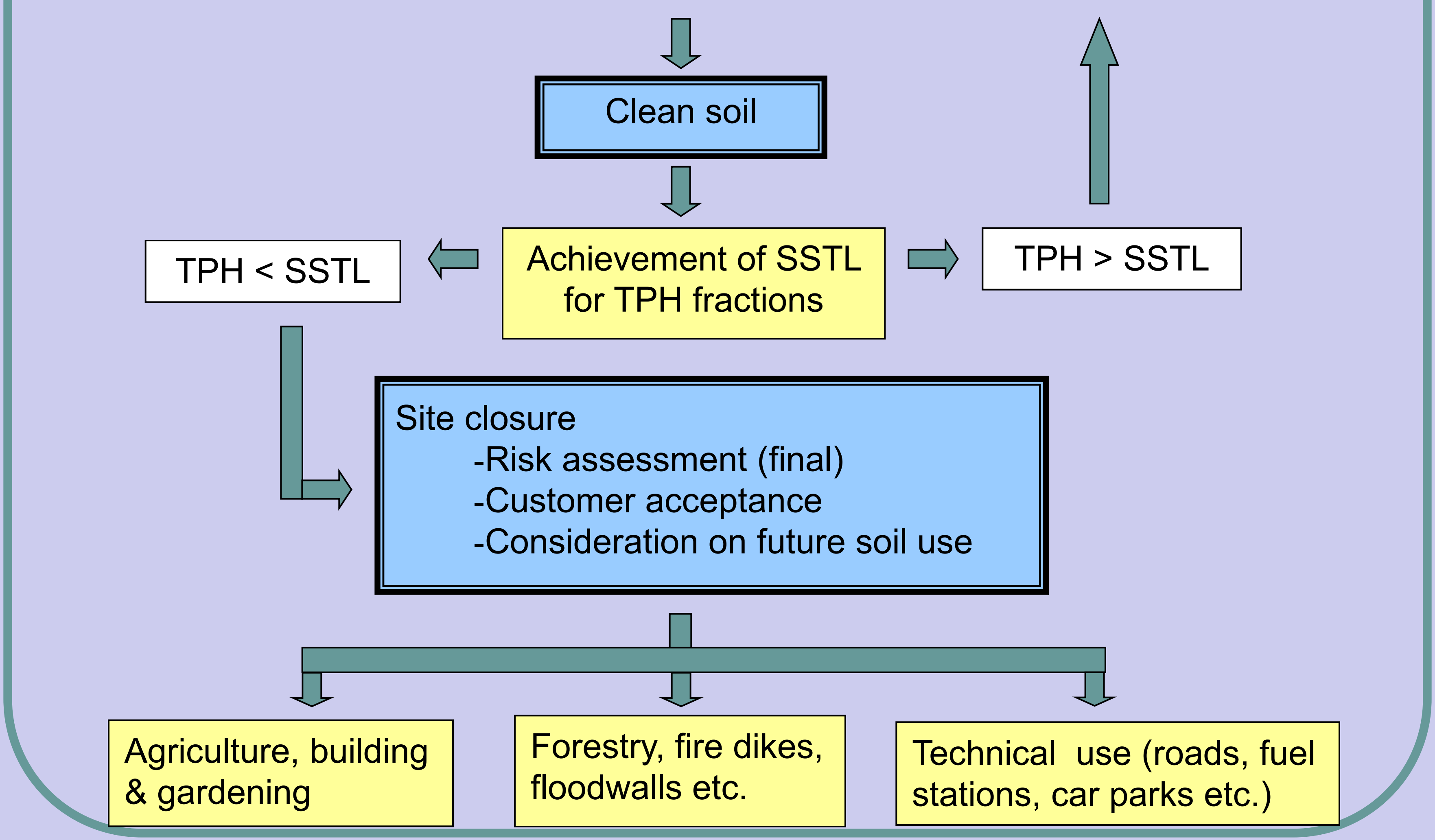
Kuyukina *et al.* (2010). *Russian J. Biomechanics.* 14, 4 (50), 34-40; Ivshina et al., (2013). *Ecology. 44, 123-130.*

Bioremediation scheme for oil-contaminated soil



RU Patent 2180276; RU Patent 2193464

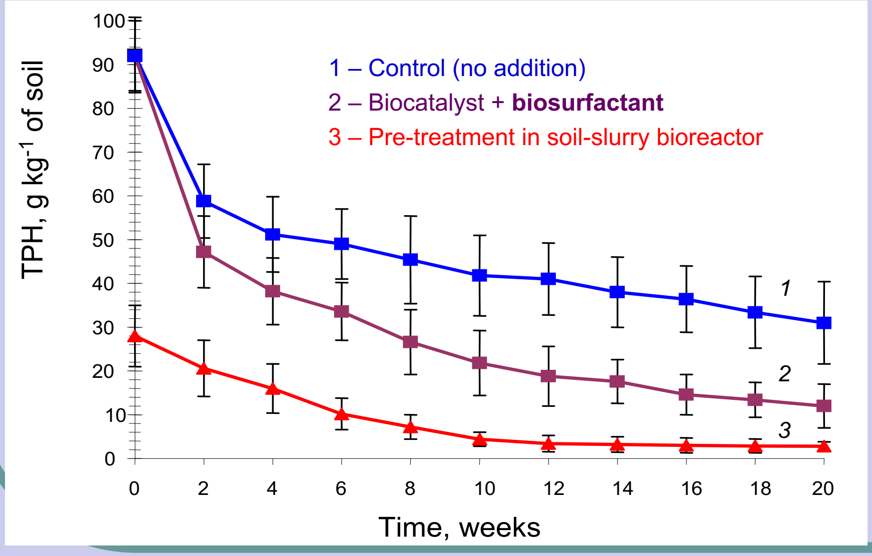
Bioremediation scheme for oil-contaminated soil



RU Patent 2180276; RU Patent 2193464

Pilot bioreactor & biopile systems

Bioreactor parameters
- Work volume – 30 m^3.
- Work regime – periodic.
- Solid phase – 30-40%.
- Air supply – 50 liter/min.
- Mixing rate – 50 rpm.
- Biocatalyst (2 kg/m^3).

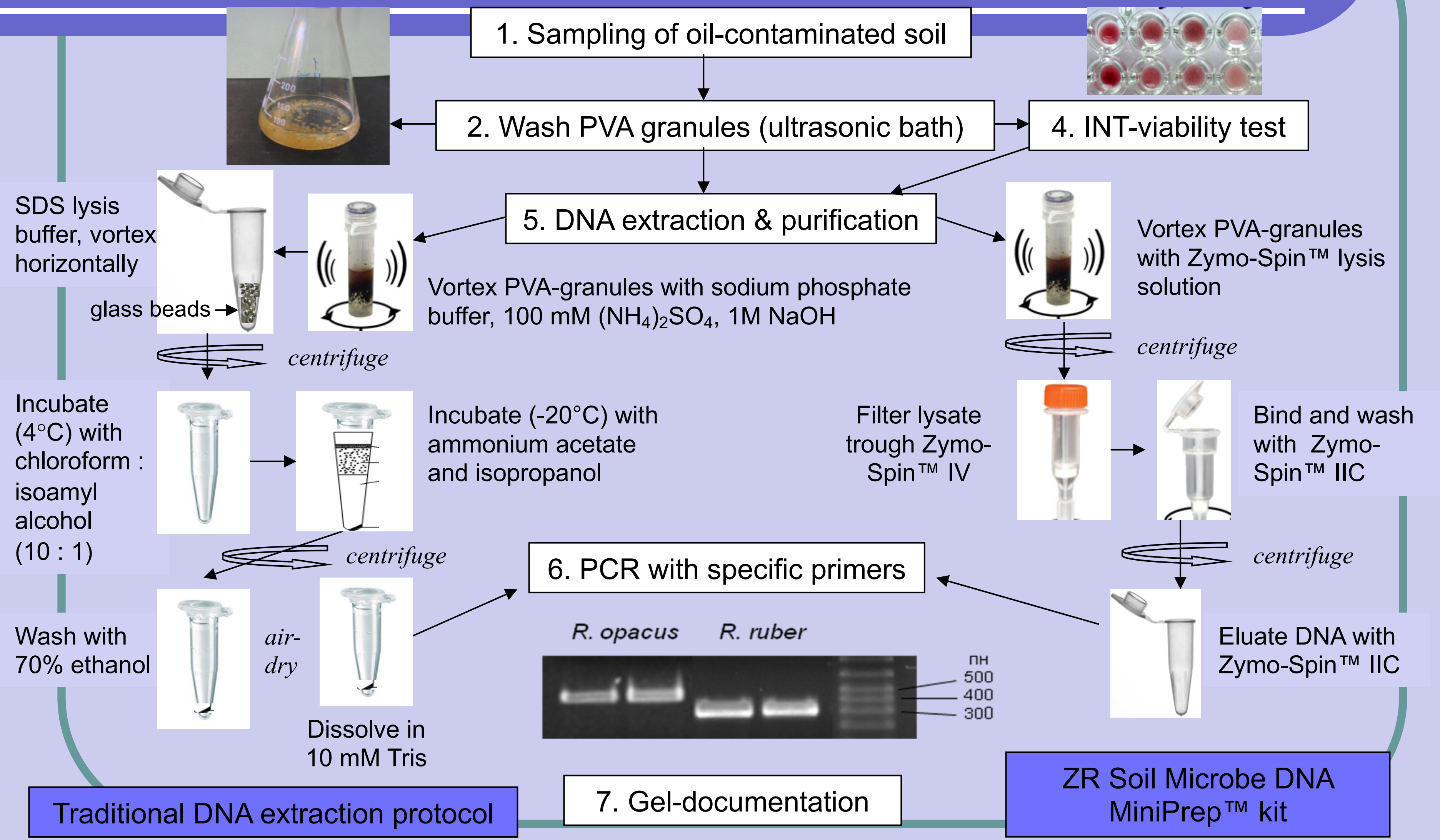


Kuyukina et al (2009) *Int. Biodeter. Biodegrad*. 63:427–432; Kuyukina et al (2017) *J. Env. Chem. Engin.* 5:1252–1260

Why slurry bioreactor ?

- Facilitates growth of hydrocarbon-oxidizing bacteria
- High contact area between oil degraders and pollutant
- Control of operating parameters (T^o, pH, O_2, biomass)
- Operation under cold conditions
- Reduction of treatment time and biocatalyst application rate

Direct PCR detection of PVA-immobilized *Rhodococcus* in soil



RU Patent 2475542; Kuyukina et al. (2013) *Int. Biodeter. Biodegrad*. 84: 118–125

Krasnodar krai (2005-2007)

Joint project with the Biotechnology Centre, Kuban State University

Bioremediation efficiency – 74,4 % after 2,5 months

Hungary, (2009-2010)

Sampling for chemical & microbiological analyses

“Priroda-Perm”, Plc. is a strategic partner

Activity fields

1. Processing and utilization of solid/liquid oily wastes.
2. Treatment and utilization of drilling mud cuttings.
3. Utilization of paraffin sediments, contaminated materials, wastewaters.
4. Emergency response to oil spills.
5. Oil storage tank cleanout.
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Processing and treatment of oil-contaminated soil (OCS) using a bioremediation technique

Unloading of OCS from oil waste storage pit using special-purpose machinery

Unloading of OCS to the technological site

Zone of liquid waste accumulation

Development of a technological site

Cleanup of the oil waste storage pit

Before

After 2 years

Conclusion

- Risk based approach to the management and bioremediation of a crude oil contaminated site is applied.
- Bioremediation techniques such as soil-slurry bioreactors, augmentation with immobilized cultures of hydrocarbon-oxidizing bacteria and biosurfactant addition were proven to be efficient in the clean-up of oil-contaminated soil in cold climate conditions.
- In a pilot scale field trial, heavily contaminated soil was cleaned-up to within risk assessment standards.
- Eco-biotechnology developed is commercialized with the Priroda-Perm company.

Acknowledgements
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Thank you for your attention !

Questions ??