Enzimi e microorganismi per la valorizzazione di scarti agro-industriali: istruzioni per l’uso

By Cinzia Pezzella– Jun 09, 2021

Scuola di Agraria e Medicina Veterinaria dell’Università degli Studi di Napoli Federico II

Meet and greet

My Study and Research path

PhD IN INDUSTRIAL BIOTECHNOLOGY

Department of Chemical Sciences, Federico II University, Naples, Italy
Thesis: **“Development of oxidative bio-systems for the treatment of industrial coloured wastewaters”**
Supervisor: Prof. Giovanni Sannia

NATIONAL ACADEMIC QUALIFICATION AS ASSOCIATE PROFESSOR

SSD CHIM/11 Chemical and fermentation technology

MASTER DEGREE IN INDUSTRIAL BIOTECHNOLOGY

Department of Chemical Sciences, Federico II University, Naples
Thesis: **“The secretion of psychrophilic α-amylase in Gram-negative bacteria: molecular evidence of new secretion systems”** Supervisor: Prof. Maria Luisa Tutino

Post-Doc

Department of Chemical Sciences, Federico II University, Naples, Italy
Involvement in National and International Research projects

BioPox
Biotech solutions for industry

FIXED TIME RESEARCHER RTDb

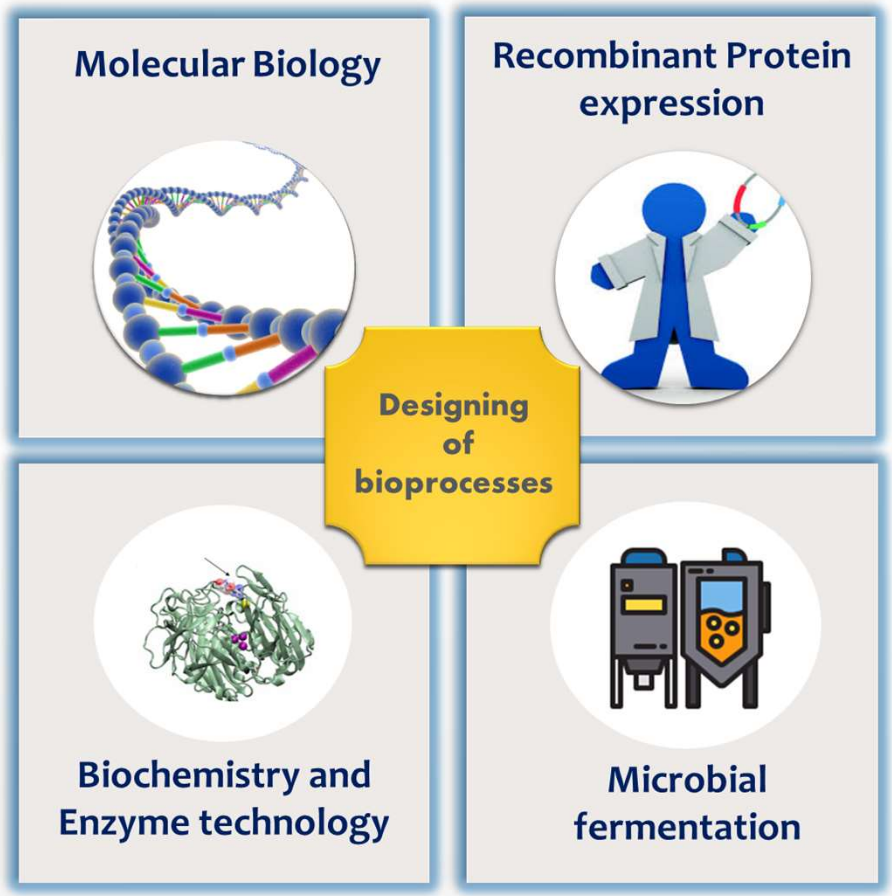
Department of Agricultural Sciences, Federico II University, Portici, Italy
SSD CHIM/11 Chemical and fermentation technology

My Background

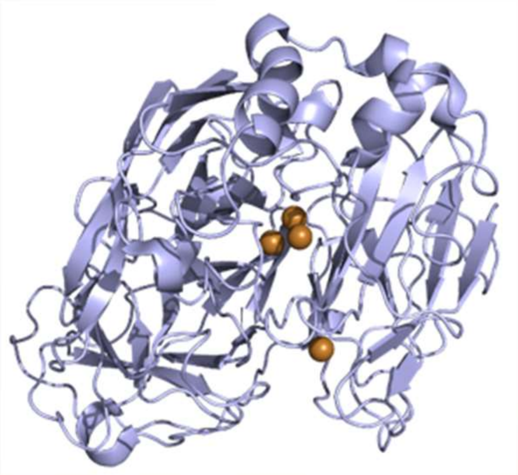
A “pure” biotechnologist

What is a Biotechnologist?

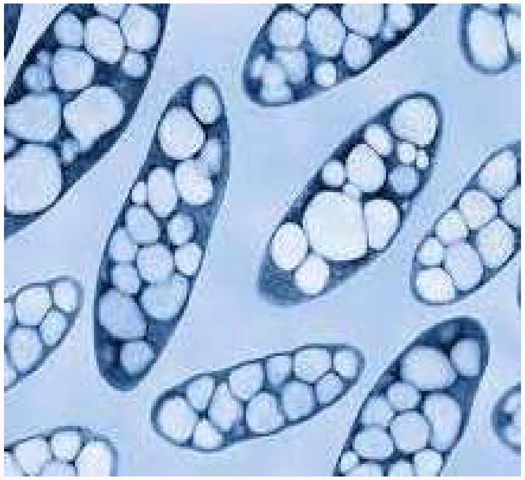
A biotechnologist uses biological processes to their advantage in industrial and other application fields (agriculture, cosmetics, pharmaceuticals, food).



Which are the biosystems of interest?



Fungal Laccases



Polyhydroxyalkanoates (PHA) producing bacteria

Laccases

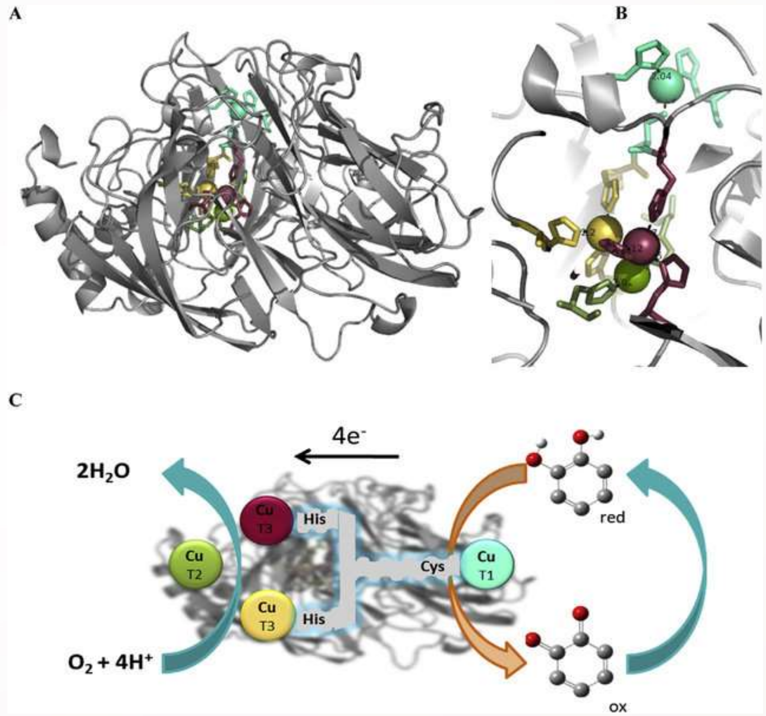
- **Multi-copper-containing enzymes** catalysing the **oxidation** of a wide spectrum of **aromatic compounds**, primarily phenols and anilines, along with reducing molecular oxygen to water.

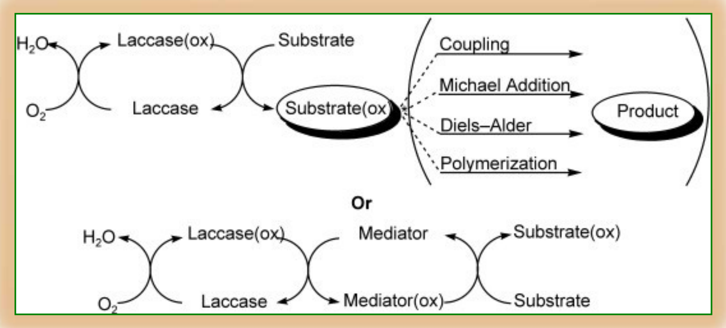
- The Cu1 is the primary electron acceptor site in laccase catalysed reaction. Four 1-electron oxidations of a reducing substrate occur at this site. The electron is then transferred, through the highly conserved His-Cys-His tripeptide, to the TNC, **where O_2 is reduced to water.**

- Found in **plants**, **fungi** and **bacteria**

- Particularly widespread in **ligninolytic basidiomycetes fungi,** where they take part to **lignin degradation**

- The spectra of oxidizable substrates can be expanded by means of low molecular weight compounds (**mediators**)





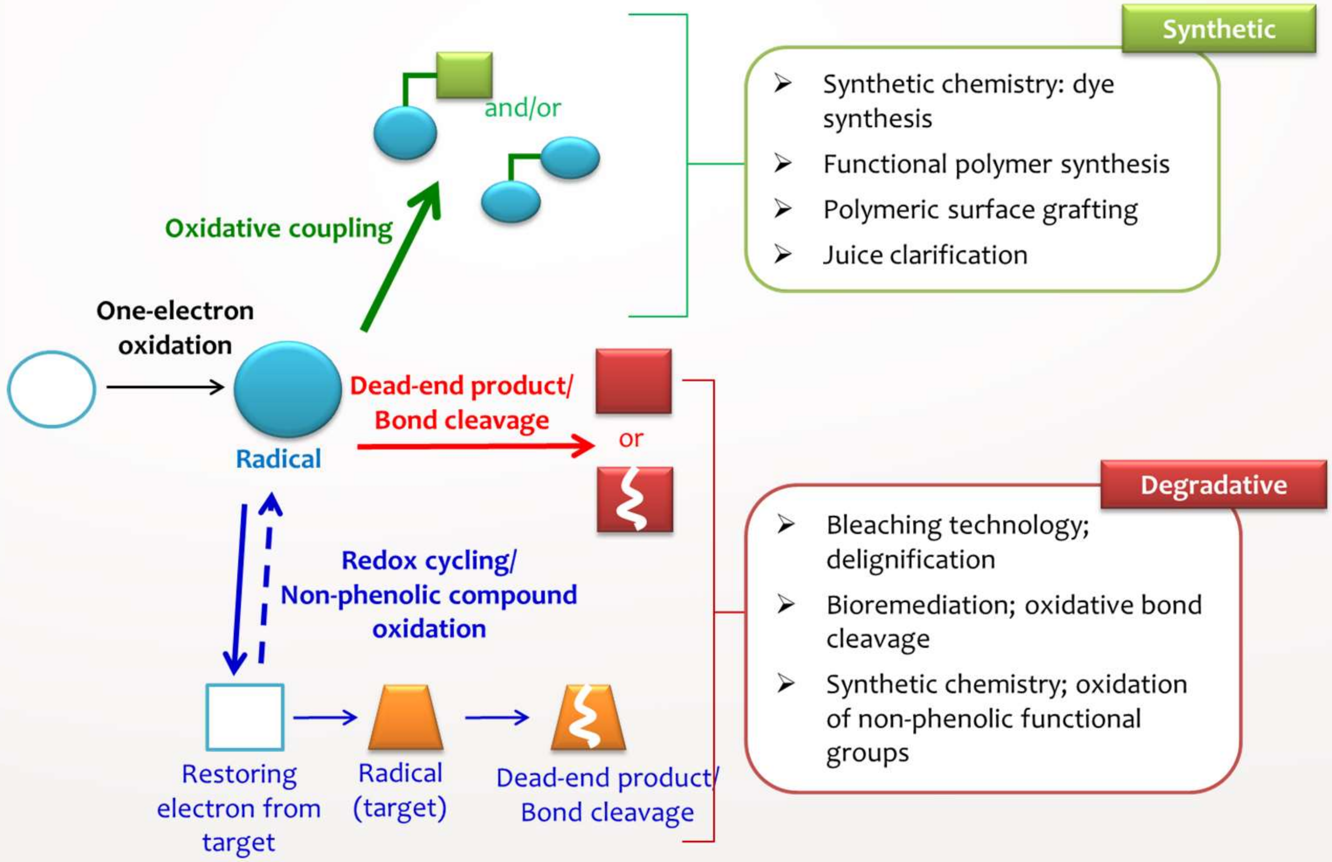
Laccases

$

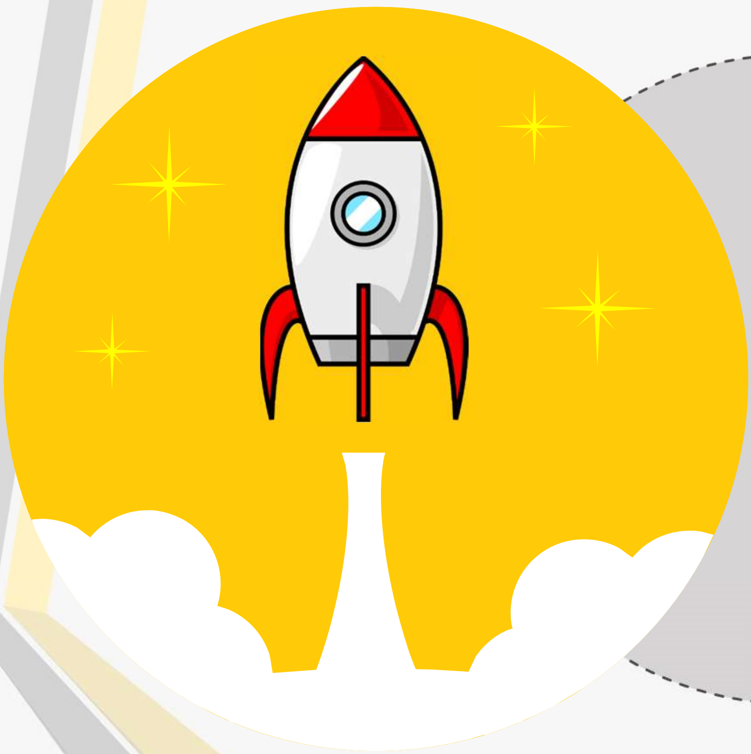
The **global Laccase market** is valued at 2965.6 million USD in 2020 is expected to reach 2850 million USD by the end of 2026, growing at a CAGR of -0.6% during 2021-2026.

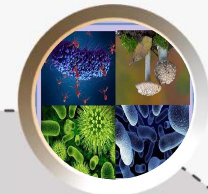
Wide industrial applicability!

Synthetic vs Degradative routes: versatile applicability of laccases



How to enjoy laccases?



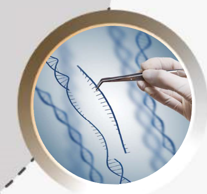


ENZYME DISCOVERY

- ✓ Enzymes from extremophilic or “niche” environments
- ✓ Metagenomic approaches

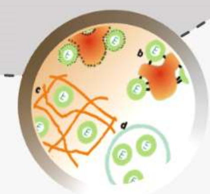
COST-EFFECTIVE PRODUCTION

- ✓ Native or recombinant hosts
- ✓ Process optimization
- ✓ Downstream process and product formulation
- ✓ Strain improvement



ENZYME ENGINEERING

- ✓ Rational designing or directed evolution
- ✓ Computer-aided approaches



ENZYME IMMOBILIZATION

- ✓ Effective reuse of the enzyme and enzyme stabilization
- ✓ Expand the applicability

“How to enjoy laccases” (Pezzella C., Guarino L., and Piscitelli A.) Cell. Mol. Life Sci. 2015 72:923-40

ENZYME DISCOVERY

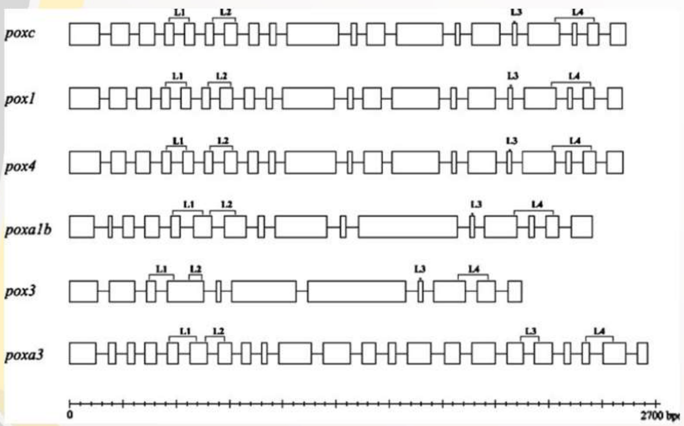
Pleurotus ostreatus laccases

Curr Genet
DOI 10.1007/s00294-008-0221-y

RESEARCH ARTICLE

The *Pleurotus ostreatus* laccase multi-gene family: isolation and heterologous expression of new family members

Cinzia Pezzella · Flavia Autore · Paola Giardina · Alessandra Piscitelli · Giovanni Sannia · Vincenza Faraco

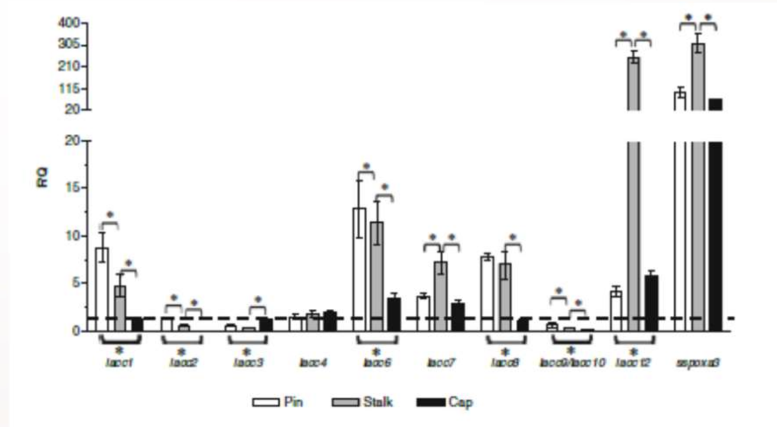


Appl Microbiol Biotechnol
DOI 10.1007/s00253-012-3980-9

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Transcriptional analysis of *Pleurotus ostreatus* laccase genes

Cinzia Pezzella · Vincenzo Lettera · Alessandra Piscitelli · Paola Giardina · Giovanni Sannia

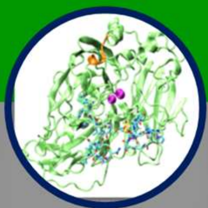


This study allowed enlarging the assortment of *P. ostreatus* laccases laying the basis for the selection of the most suitable biocatalysts for specific industrial application

Reported results depicted a complex picture of the laccase expression profile, allowing to speculate on the isoform role *in vivo*.

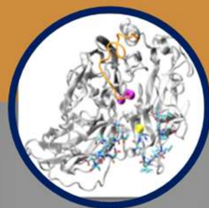
ENZYME DISCOVERY

POXC laccase

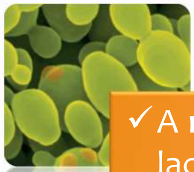


- Acidic laccase
- High redox potential (+0.74V vs NHE)
- Dye synthesis
- **High Native production**
- No recombinant expression

POXA1b laccase

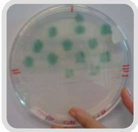


- Neutral laccase
- High redox potential (+0.65 V vs NHE)
- Stable at alkaline pH
- Low Native production
- **High recombinant expression**



✓ A **new expression system** based on constitutive laccase production in ***Pichia pastoris*** has been developed

✓ **Production levels** have been optimized



✓ **Purification protocols** have been set up

At all the scales the glycerol-based fermentation is more economic than the methanol-based one. The price forecast for rPOXA1b production is 0.34 € k U^{-1} for glycerol-based process, and is very competitive with the current price of commercial laccase.

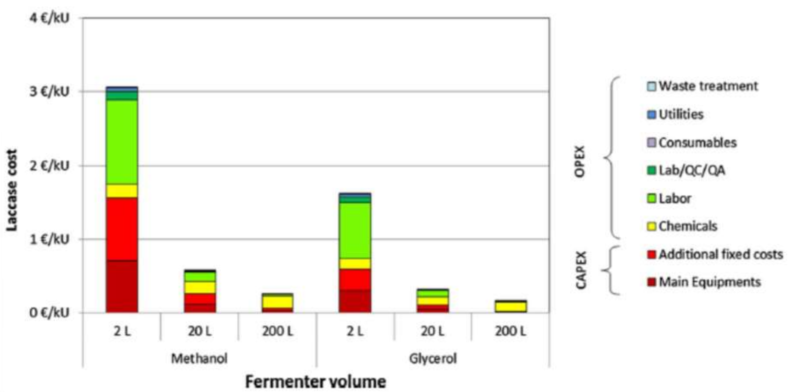
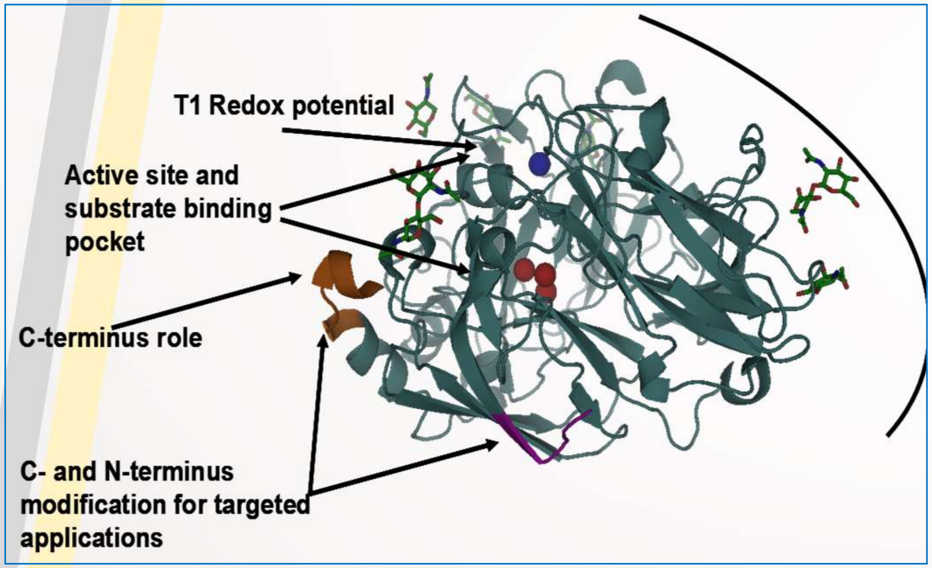


Fig. 4. Cost breakdown for methanol and glycerol based process at different scales.

"A step forward in laccase exploitation: Recombinant production and evaluation of techno-economic feasibility of the process" (Pezzella C., Giacobelli V.G., Lettera V., Olivieri G., Cicatiello P., Sannia G., Piscitelli A) (2017), Journal of Biotechnology

ENZYME ENGINEERING

- ✓ **Functional evolution** of **POXA1b** laccase has been performed combining rounds of **random** and **rational mutagenesis**
- ✓ **Three generations of libraries** (3,300 variants) have been screened using **different criteria**, and **several variants** endowed with improved features have been **selected**



Laccase engineering allows to "create" **tailored enzyme** for specific industrial applications

Evolved laccases

Mutants	Activity	Stability				
		pH3	pH5	pH7	pH10	60°C
1M9B	1.5 x	-	-	-	-	-
1L2B	2.5 x	=	+	-	=	=
1M10B	2.5 x	=	+	=	++	=
3M7C	3 x	+	++	++	=	+
2L4A	2.7 x	-	++	=	=	=
3L7H	2.7 x	-	++	-	-	=
1L9A	3 x	-	+	=	-	=
R4	2.5 x	-	=	+++	++	+
1H6C	4.5 x	-	+++	+++	++	+
4M10G	4.5 x	-	++	++	+	=
1L10A	4.5 x	-	+++	-	+	=
3L2A	3 x	++	=	+++	++	=

1H6C variant shows a **higher redox potential (+0,77 V)** with respect to POXA1b (+0,65 V).

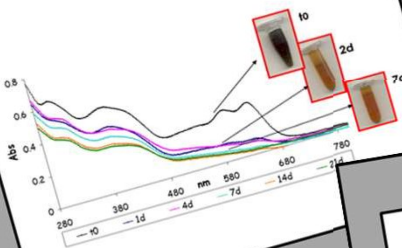
No mutations in the substrate binding pocket

Piscitelli A. *et al C.R. Biol.*, 2011 Vol. 11, 789-94
Macellaro G. *et al*, *Appl Microbiol Biotechnol.* 2014; DOI 10.1007/s00253-013-5491-8.

SNAPSHOTS

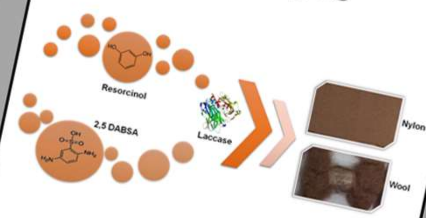
A case for laccase

Treatment of coloured wastewaters



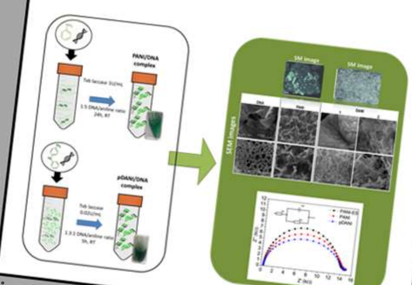
Faraco V., Pezzella C., Miele A., Giardina
G. Boodegradation 2009 20, 209–220

Synthesis of textile dyes



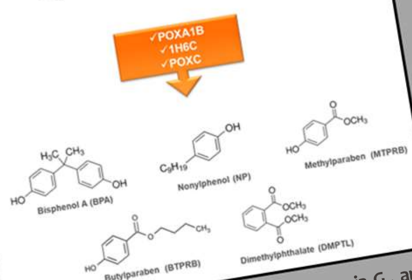
Pezzell... ...cobbe S., Giacobelli V.G., Guarino L., ...M., Sannia G and Piscitelli (2016) ... 274-279

Synthesis of conductive polymers



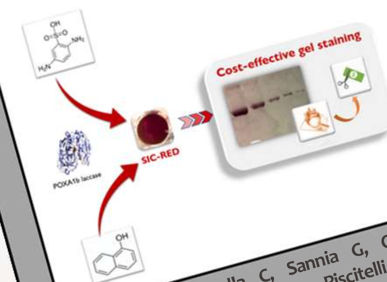
Giacobbe S, Pezzella C, Della Ventura B, Giacobelli VG, Rossi M, Fontanarosa C, Amoresano A, Sannia G, Velotta R, Piscitelli A) (2019) Engineering in Life Sciences 19(9), 631-642

Degradation of Endocrine Disrupting Chemicals (EDCs)

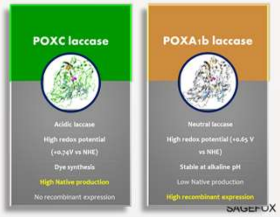


Macellaro G., Pezzella C., Cicatiello P., Sannia G., and Piscitelli A. Biomed Res. Int. (2014) DOI 10.1155/2014/614038

Synthesis of protein-dyeing product



Giacobelli VG, Pezzella C, Sannia G, Olivieri G, Fontanarosa C, Amoresano A, Piscitelli A (2018); Biocatalysis and Agricultural Biotechnology 15, pp. 270-276



Dye decolorization

- Covalent immobilization on Inorganic siliceous support
- **Very cheap**
- Good mechanical resistance
- High **Immobilization yield (70%)**
- **Improvement in enzyme stability**

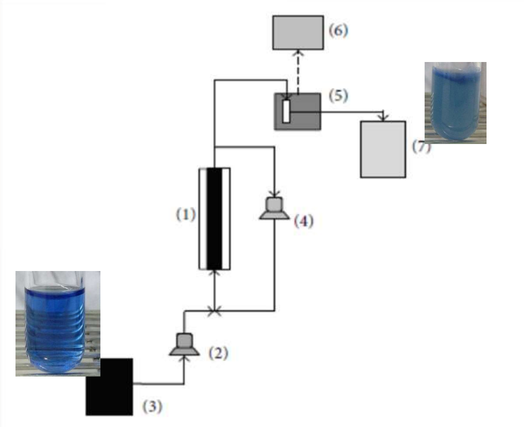


FIGURE 1: Apparatus equipped with fluidized bed reactor adopted for RBBR conversion by means of immobilized laccases. (1) Fluidized bed reactor; (2) peristaltic pump; (3) feed tank; (4) recirculation gear pump; (5) flow-cell and spectrophotometer; (6) data acquisition unit; (7) waste tank.

The applicability of immobilized laccase on a cheap support was demonstrated for the treatment of wastewaters from colour industries

Fruit Juice clarification

- Covalent immobilization on Epoxy activated poly(methacrylate) beads
- High **Immobilization yield (98%)**
- **Improvement in enzyme stability**

Polyphenols reduction %					
	Pomegranate	Cherry	Peach	Apricot	Orange
Free laccase	40	50	30	27	40
Immobilized laccase	67	60	30	48	50

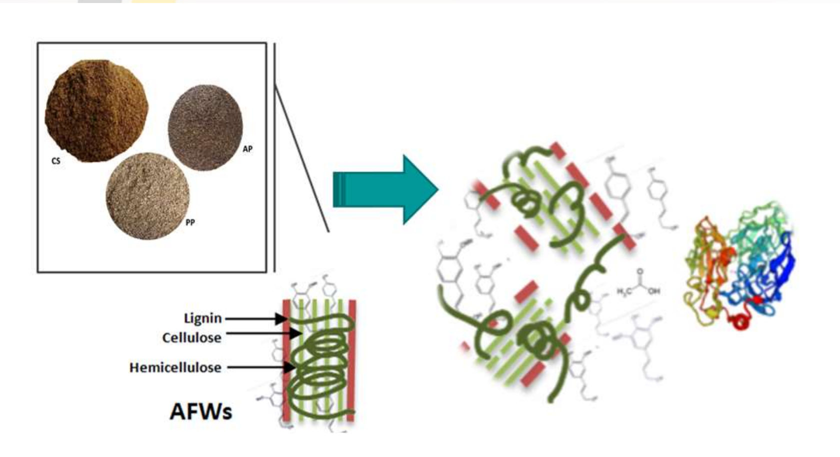
OD $_{600nm}$ Reduction %					
	Pomegranate	Cherry	Peach	Apricot	Orange
Free laccase	8	16	15	17	18
Immobilized laccase	29	16	15	17	30

A laccase-based pre-treatment of raw juice represents an effective alternative to conventional physical-chemical methods

CASE-STUDY

Agro-food waste pretreatment

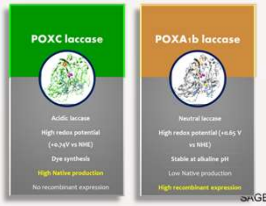
The ability of **two laccase preparations (rPOXA1b and mix$_{P.o.}$)** to **delignify** and **detoxify** three different un-pretreated AFWs (**apple pomace, AP; potato peels, PP; coffee silverskin, CS)** was evaluated with or without laccase mediator system (LMS).



AFWs	Laccase enzymes	Phenols reduction (%)	Lignin reduction (%)	Sugars conversion (%)
AP	Control			40
	rPOXA1b:mix$_{P.o.}$ 1:0 ratio	33	16	**83**
	rPOXA1b:mix$_{P.o.}$ 1:1 ratio	33	-12	n.a.
	rPOXA1b:mix$_{P.o.}$ 2:1 ratio	53	1	n.a.
	LMS			
	rPOXA1b:mix$_{P.o.}$ 1:0 ratio	30	15	n.a.
	rPOXA1b:mix$_{P.o.}$ 2:1 ratio	30	11	n.a.
CS	Control			27
	rPOXA1b:mix$_{P.o.}$ 1:0 ratio	50	15	n.a.
	rPOXA1b:mix$_{P.o.}$ 0:1 ratio	20	-5	n.a.
	LMS			
	rPOXA1b:mix$_{P.o.}$ 1:0 ratio	69	48	**73**
	rPOXA1b:mix$_{P.o.}$ 0:1 ratio	36	-8	n.a.
PP	Control			18
	rPOXA1b:mix$_{P.o.}$ 1:0 ratio	32	35	n.a.
	rPOXA1b:mix$_{P.o.}$ 1:1 ratio	49	36	n.a.
	rPOXA1b:mix$_{P.o.}$ 2:1 ratio	48	50	**60**
	LMS			
	rPOXA1b:mix$_{P.o.}$ 1:1 ratio	49	49	47

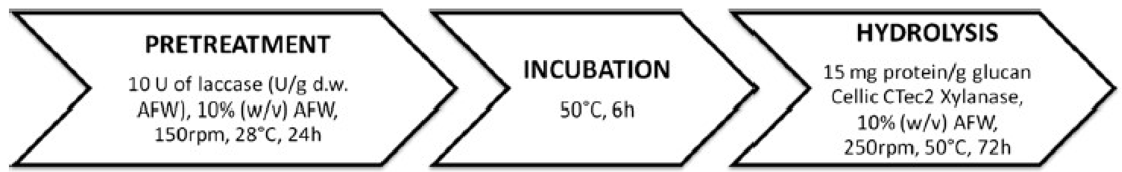
Detoxification

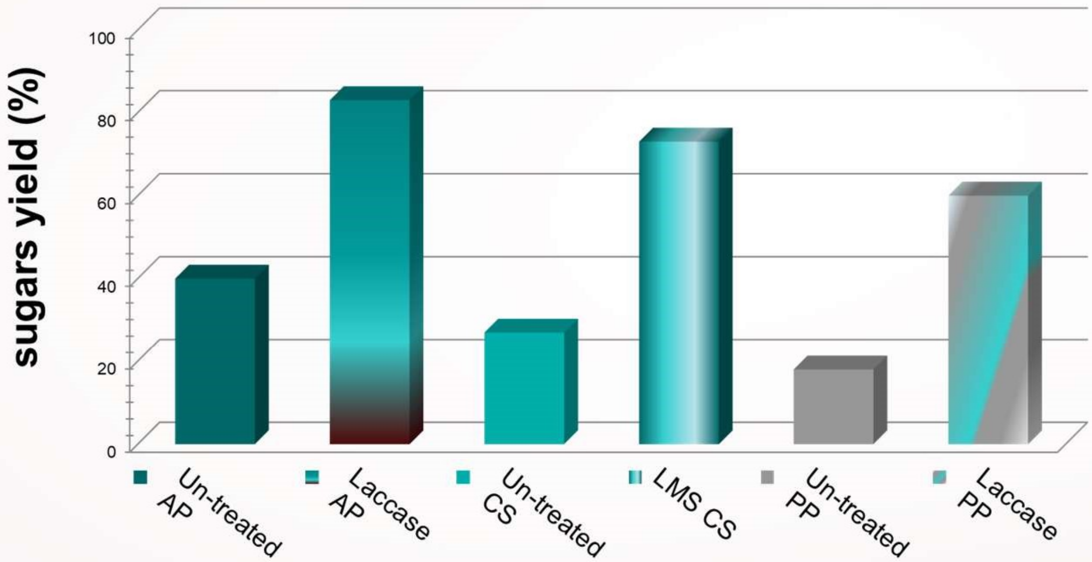
Delignification



CASE-STUDY

Agro-food waste pretreatment



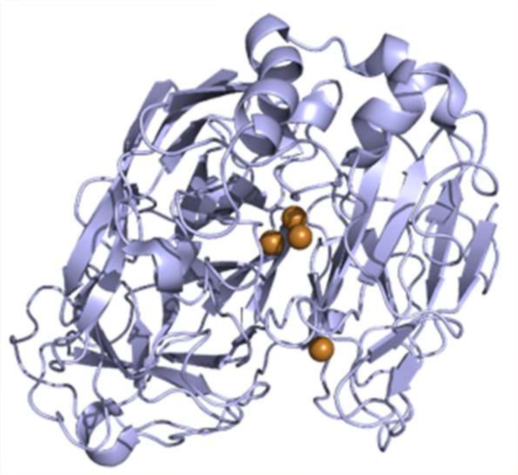


The sequential protocol, without filtration and washing steps between pretreatment and enzymatic hydrolysis, was performed: before the enzymatic hydrolysis, laccase pretreated AFWs were incubated for 6 h at 50°C in order to deactivate laccases and avoid their interference with cellulase enzymes.

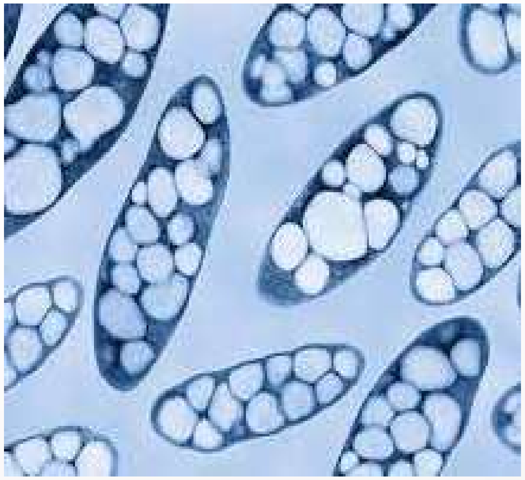
"Laccase pretreatment for agrofood wastes valorization" (Giacobbe S, Pezzella C, Lettera V, Sannia G, Piscitelli A) (2018) Bioresource Technology 265, pp. 59-65

"Butanol production from laccase-pretreated brewer's spent grain" (Giacobbe S, Piscitelli A, Raganati F, Lettera V, Sannia G, Marzocchella A, Pezzella C.) (2019); Biotechnology for Biofuels; 12:47

Which are the biosystems of interest?



Fungal Laccases



Polyhydroxyalkanoates (PHA) producing bacteria

PHA
A dream come true?

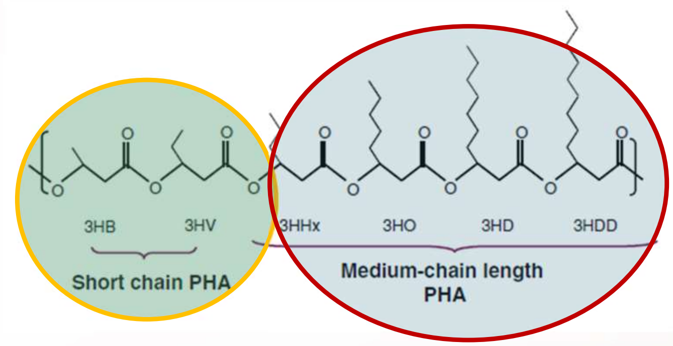
- Polyhydroxyalkanoates (PHAs) are **biodegradable** and **naturally synthesized polyesters**, accumulated by various microorganisms as carbon, energy, and redox storage material

They are attracting extensive interest as "green" polymers due to their peculiar properties:

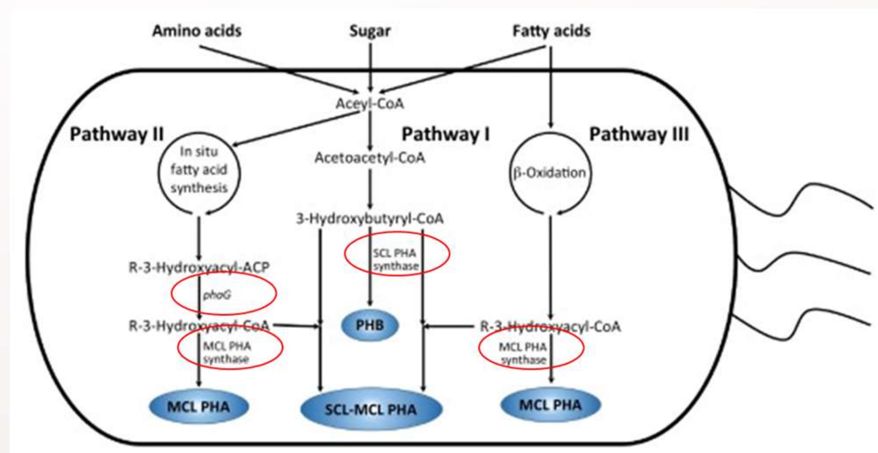
- ✓ **Substitution potential for industrial thermoplastics** such as PP, PE, PVC, PET
- ✓ **Biodegradability** in aerobic and anaerobic conditions including aquatic environments
- ✓ **Bio-based, renewable** origin
- ✓ **Biocompatibility** with cells and tissues
- ✓ **Structural diversity**

Wide applicative potential!

Their monomer size: **Short chain length PHA (scl-PHA)** with C4-C5 monomers, or **Medium chain length PHAs (mcl-PHAs)** with C6-C14 monomers



PHA synthesis mechanisms predominantly comprise three pathways



How to exploit PHA potential?

The challenge for the new polymers produced by microorganism is to ***retain the physical-chemical characteristics*** *of traditional petrochemically-derived plastics, but benefit from* ***biocompatibility*** *and* ***biodegradability****.*

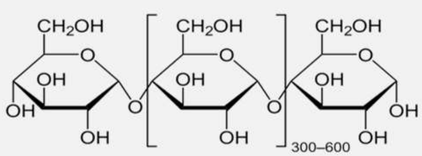
In spite of the worldwide efforts committed to biopolymer research, PHAs are still not actually competitive to petrochemical plastics mainly considering production costs and to a certain extent concerning their material properties.

Tailoring of PHA properties

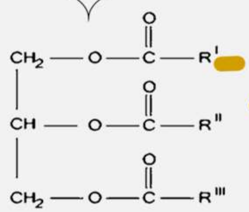
In vivo **and** ***Post-synthesis*** **strategies**

Sustainable PHA production

PHA from wastes

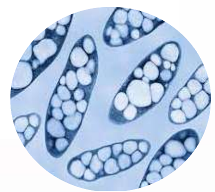


Sugar



Triglyceride

Food Wastes	Main examples	Composition
Fruits and vegetables	Pulp fruit, vinasse,	Sugars, carbohydrates
Waste oils	Palm oil, rapeseed oil, waste frying oil	High content of lipis, free fatty acids and triacyilgrylcerol
Animal by products	Animal Fats	
Organic crops	Lignocellulosic wastes, wheat bran	sugars, lipids, carbohydrates, and mineral acids



UV filter for organic sunscreen

Snacks packaging

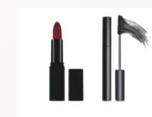
Skin exfoliant for peeling milk

Cutlery

FDM 3D filament

Drug carrier

Sunglasses



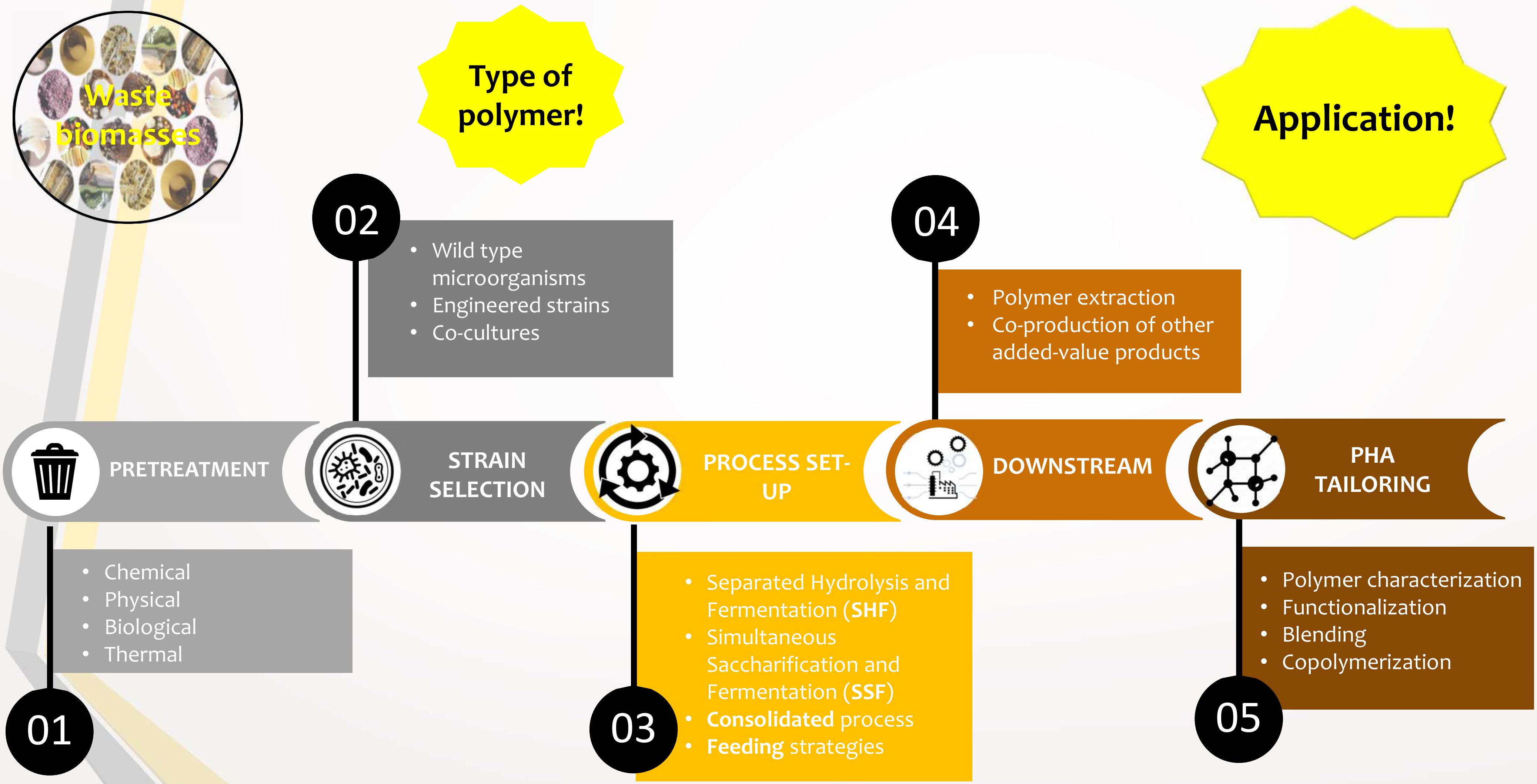
Make-up packaging

Clutch handbag

Closing Cap

Straws

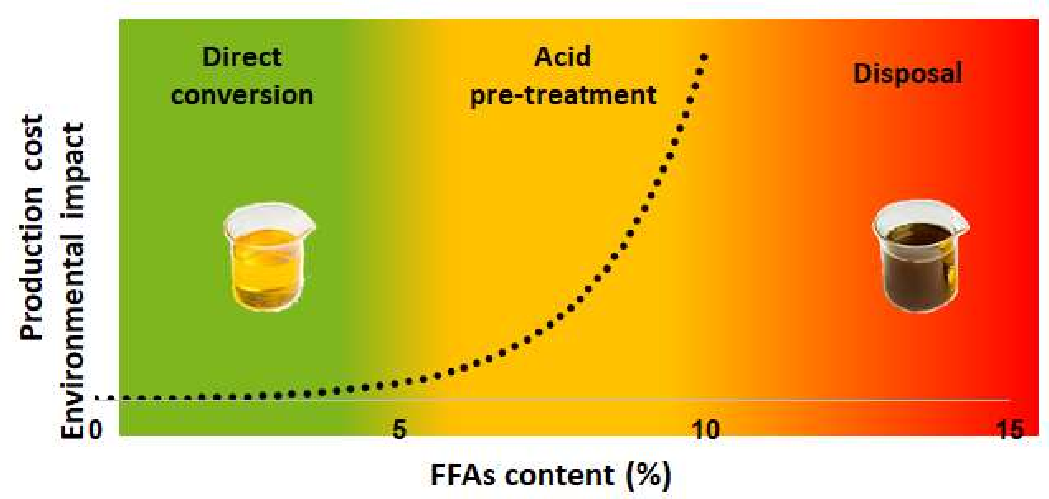
Medical sutures



"In vivo and Post-synthesis Strategies to Enhance the Properties of PHB-Based Materials: A Review" (Turco R, Santagata G, Corrado I, Pezzella C*, Di Serio M.) (2021) Front Bioeng Biotechnol., 8:619266. doi: 10.3389/fbioe.2020.619266.

PHA from wastes

Waste frying oils



Biodiesel production cost is strictly related to Free Fatty Acid (FFAs) content of feedstock.
In addition to the economical detriment, an high environmental impact is linked to the required acid pre-treatment.

A bioprocess for PHA production from waste frying oils (WFOs) with high content of FFAs was set up

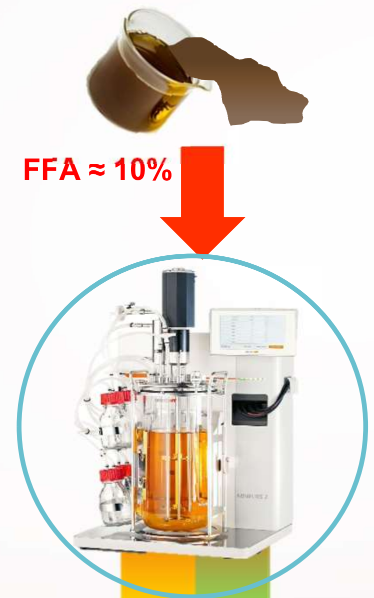
"Conversion of no/low value waste frying oils into biodiesel and polyhydroxyalkanoates" (Vastano M, Corrado I, Sannia G, Solaiman DKY, Pezzella C.) (2019) Scientific Reports 9(1):13751

PHA from wastes

Waste frying oils

Strain engineering:

***Pseudomonas resinovorans* wild type *vs* lipase free mutant**

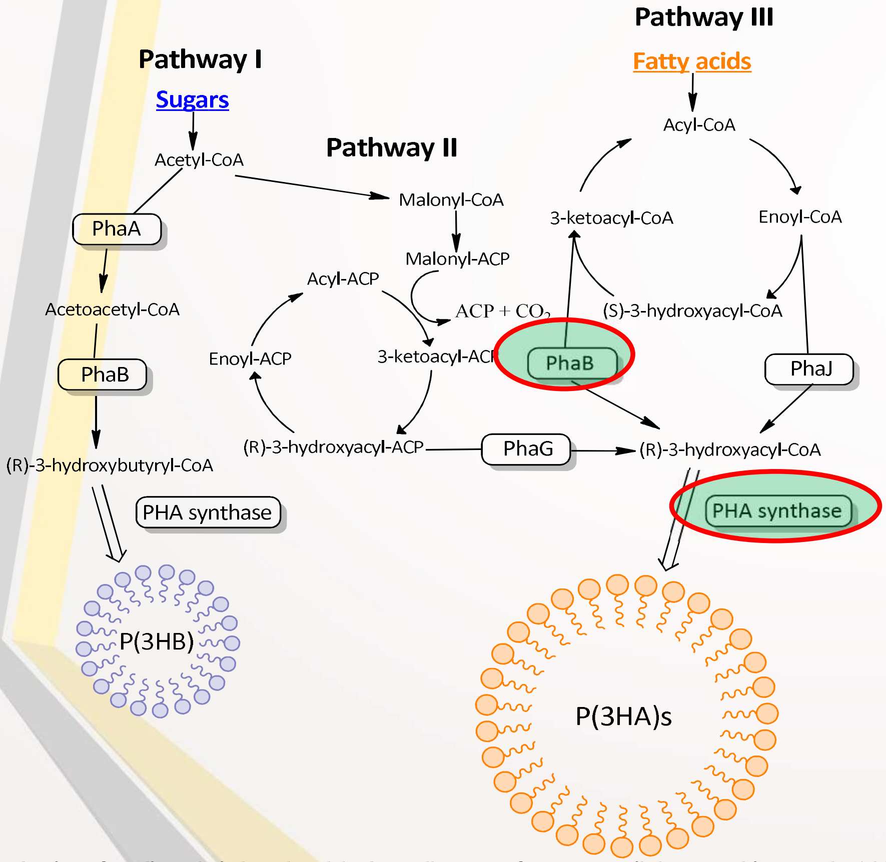


- **High yield in biodiesel conversion even with reduced amount of catalyst**
- **PHA yield (1.3 g/L)**

"Conversion of no/low value waste frying oils into biodiesel and polyhydroxyalkanoates" (Vastano M, Corrado I, Sannia G, Solaiman DKY, Pezzella C.) (2019) Scientific Reports 9(1):13751

PHA from wastes

Waste frying oils



Strain engineering:

Recombinant *E. coli* strain endowed with a heterologous pathway (*B. cereus* 6E/2 phaRBC operon) for PHA production from lipidic carbon sources

✓The system is designed to improve the fraction of **mcl-monomers** in the synthesized PHA

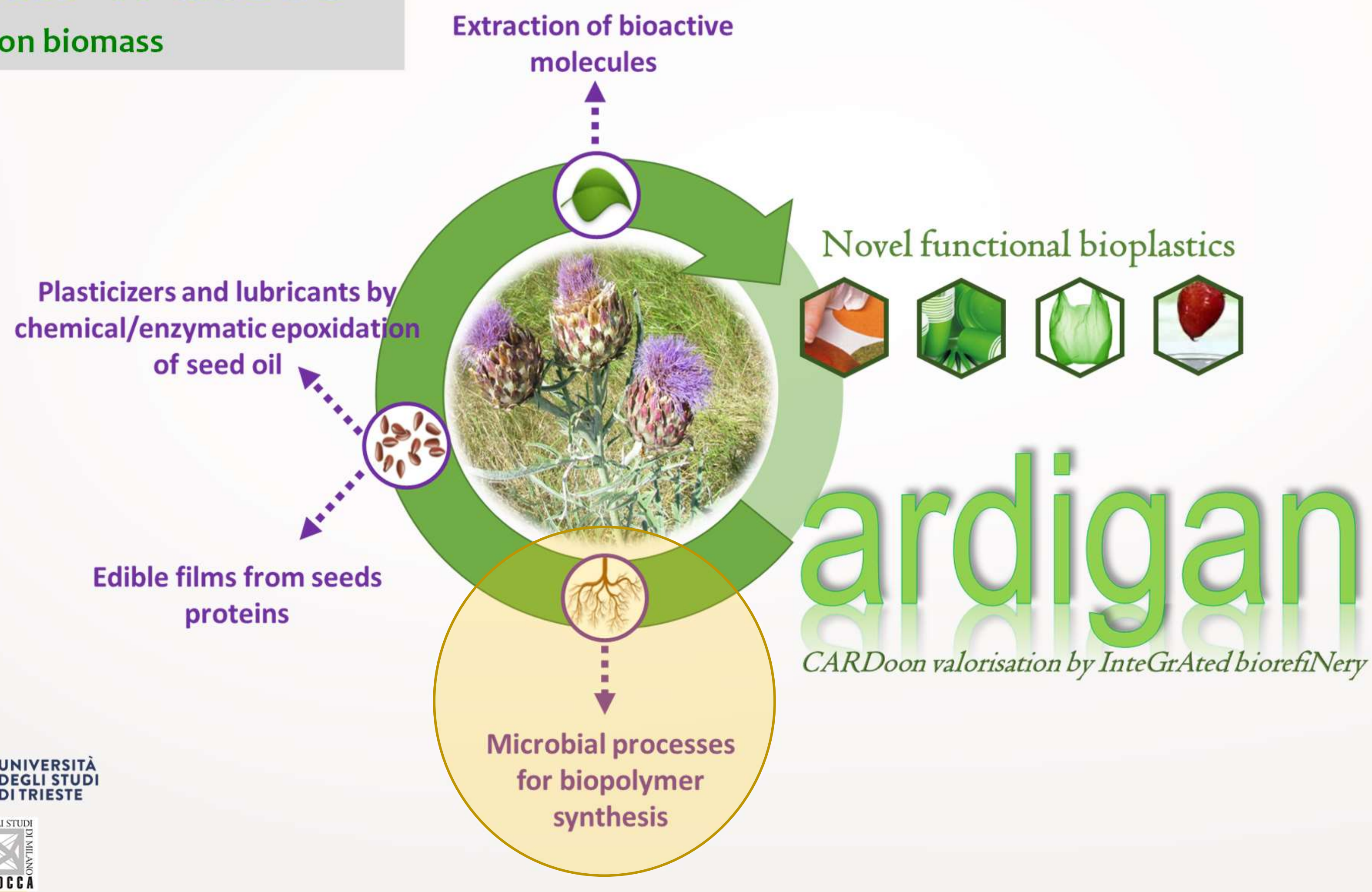
✓The strain has been exploited for the production of PHA from **Waste fried oils (WFOs)**

➢ **The system is able to produce scl-mcl copolymers (PH3B-PHHx), by driving the incorporation of 3-hydroxyhexanoate monomers (>40%) whatever is the supplied fatty acid.**

"Production of medium-chain-length polyhydroxyalkanoates from waste oils by recombinant *Escherichia coli*" (Vastano M, Casillo A, Corsaro MM, Sannia G, and Pezzella C.) Eng in Life Sci, 2015, 15, 700–709.

PHA from wastes

Cardoon biomass



UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

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IPCB ISTITUTO PER I POLIMERI COMPOSITI E BIOMATERIALI

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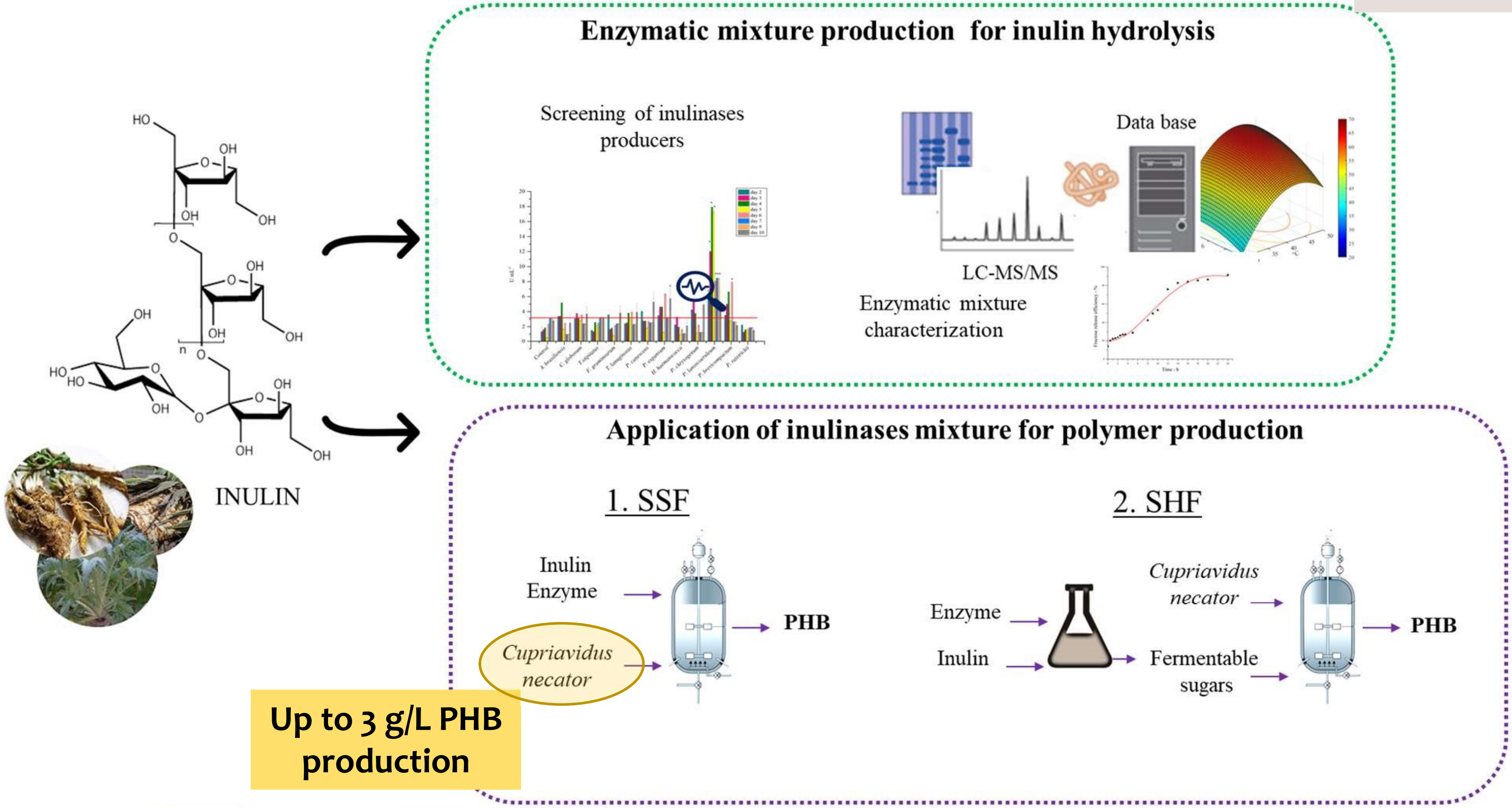
PHA from wastes

Cardoon biomass

Native producer:

Cupriavidus necator **+ enzymatic pre-treatment**



Up to 3 g/L PHB production

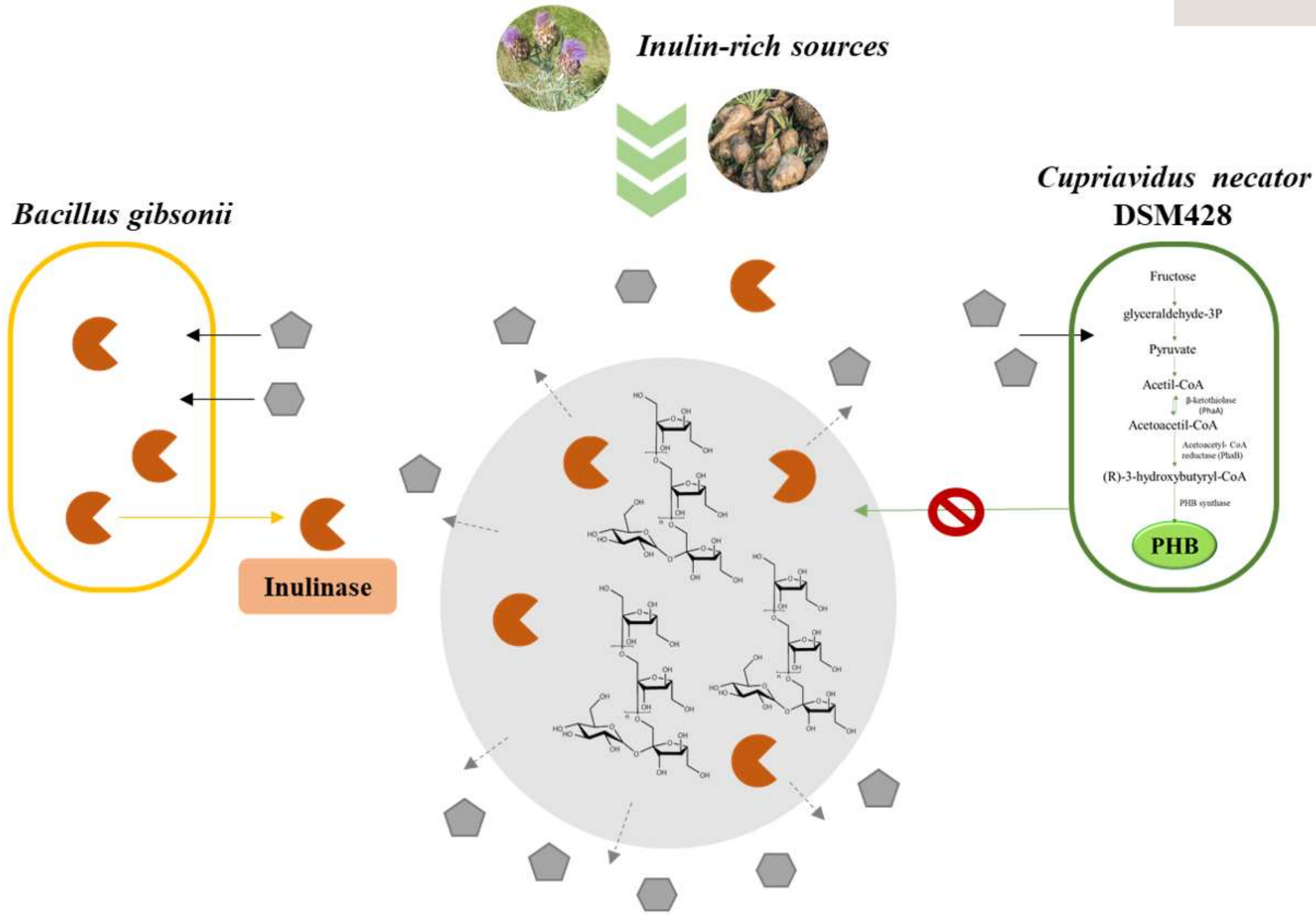
"Optimization of Inulin Hydrolysis by Penicillium lanosocoeruleum Inulinases and Efficient Conversion Into Polyhydroxyalkanoates" (Corrado I, Cascelli N, Ntasi G, Birolo L, Sannia G, Pezzella C*) (2021) Front Bioeng Biotechnol., 9, 108; doi10.3389/fbioe.2021.616908

PHA from wastes

Cardoon biomass

Artificial consortium:

PHA producer: *C. necator* + Inulinase producer: *Bacillus gibsonii*

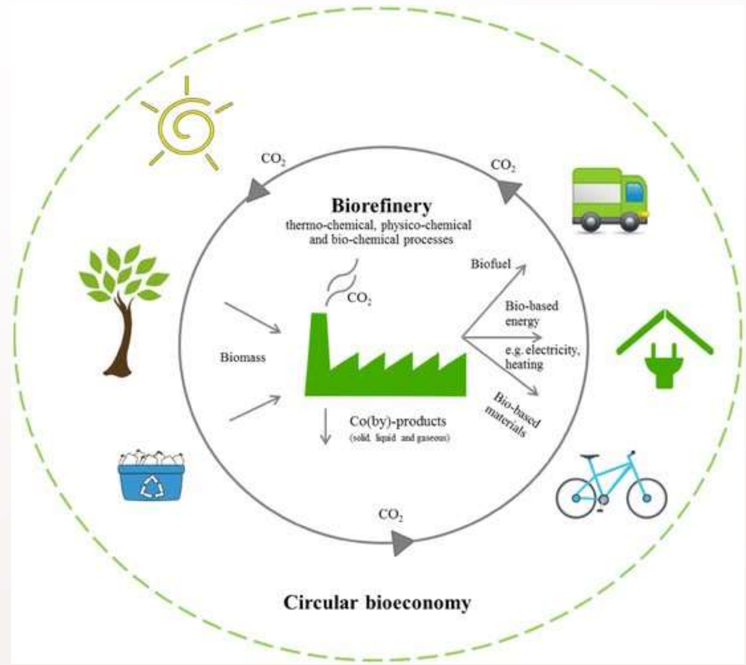


Up to 2 g/L PHB in a one-step process

"The power of two: an artificial microbial consortium for the conversion of inulin into Polyhydroxyalkanoates" (Corrado I, Petrillo C, Isticato R, Casillo A, Corsaro MM, Sannia G, Pezzella C*) (2021) Submitted to International Journal of Biological Macromolecules

- Enzyme and microorganisms are **powerful tools** for the valorization of waste materials
- **"Nature inspired"** and/or **artificially created biosystems** can be designed to address specific process needs
- The **integration of the bioprocesses** lay the basis for the development of **biorefinery systems,** allowing highly efficient and cost-effective processing of biological feedstock to a **range of biobased products**

Integration is the key!



Bibliography

- "How to enjoy laccases" (Pezzella C., Guarino L., and Piscitelli A.) Cell. Mol. Life Sci. 2015 72:923-40
- "Optimization of Inulin Hydrolysis by Penicillium lanosocoeruleum Inulinases and Efficient Conversion Into Polyhydroxyalkanoates" (Corrado I, Cascelli N, Ntasi G, Birolo L, Sannia G, Pezzella C*) (2021) Front Bioeng Biotechnol., 9, 108; doi10.3389/fbioe.2021.616908
- "In vivo and Post-synthesis Strategies to Enhance the Properties of PHB-Based Materials: A Review" (Turco R, Santagata G, Corrado I, Pezzella C*, Di Serio M.) (2021) Front Bioeng Biotechnol., 8:619266. doi: 10.3389/fbioe.2020.619266.
- "Design and characterization of poly (3- hydroxybutyrate-co-hydroxyhexanoate) nanoparticles and their grafting in whey protein-based nanocomposites". (Corrado, I., Abdalrazeq, M., Pezzella, C.*, Di Girolamo, R., Porta, R., Sannia, G., Giosafatto, C.V.L.) (2021) Food Hydrocolloid., 110, 106167.
- "Beyond natural laccases: extension of their potential applications by protein engineering" (Stanzione I, Pezzella C, Giardina P, Sannia G, Piscitelli A) (2019) Applied Microbiology and Biotechnology Volume 104, Issue 3, 1 February 2020, Pages 915-924
- "Conversion of no/low value waste frying oils into biodiesel and polyhydroxyalkanoates" (Vastano M, Corrado I, Sannia G, Solaiman DKY, Pezzella C.) (2019) Scientific Reports 9(1):13751
- "Laccase pretreatment for agrofood wastes valorization" (Giacobbe S, Pezzella C, Lettera V, Sannia G, Piscitelli A) (2018) Bioresource Technology 265, pp. 59-65
- "Butanol production from laccase-pretreated brewer's spent grain" (Giacobbe S, Piscitelli A, Raganati F, Lettera V, Sannia G, Marzocchella A, Pezzella C.) (2019); Biotechnology for Biofuels; 12:47

Prossimo Caffè scientifico
Roberta Paradiso, 23 giugno 2021

Piante e colori dello spettro: fiat lux