Applicazioni biotecnologiche degli enzimi: le lipasi

Le lipasi

- Catalizzano l'idrolisi e la sintesi di acilgliceroli
- Sono stabili in solventi organici
- Non richiedono cofattori
- Hanno bassa specificità di substrato
- Hanno elevata **enantioselettività**

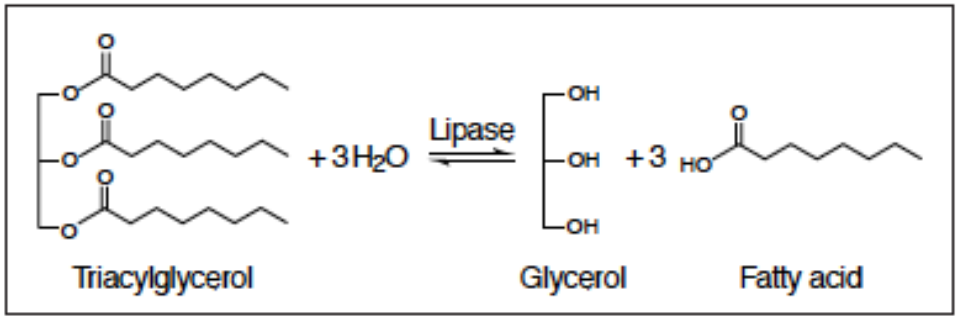


Figure 1

The catalytic action of lipases. A triglyceride can be hydrolysed to form glycerol and fatty acids, or the reverse (synthesis) reaction can combine glycerol and fatty acids to form the triglyceride.

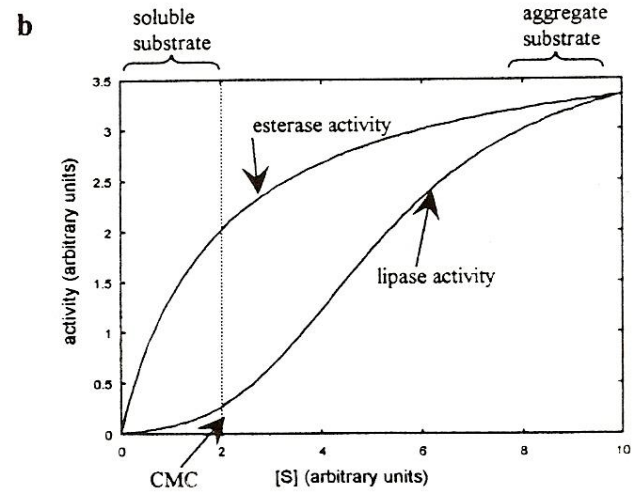


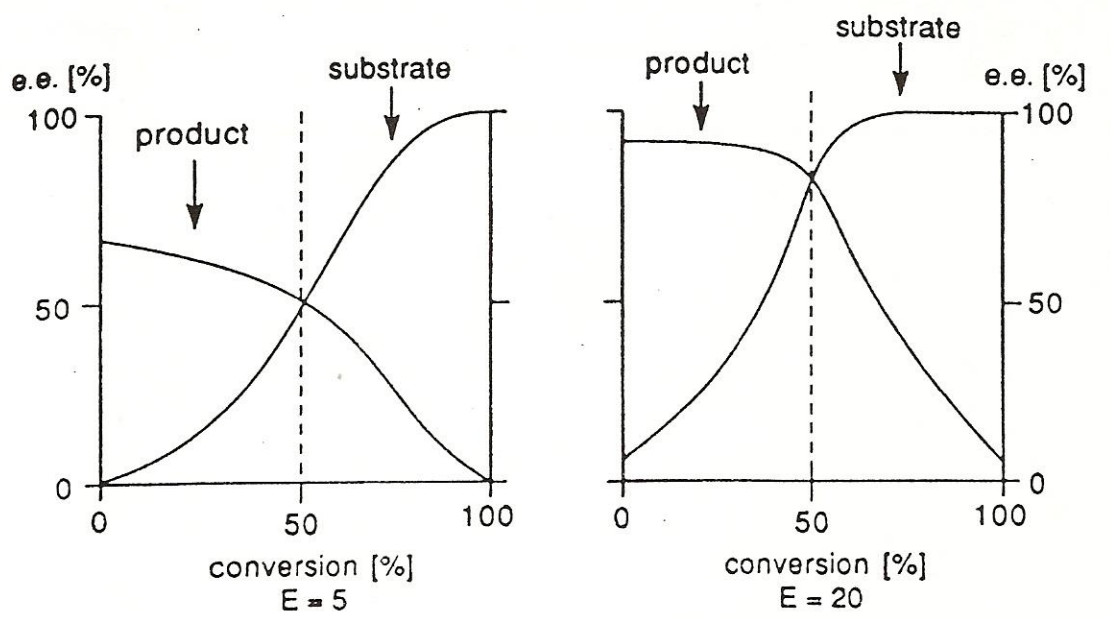
Figure 1 (a) The mechanism of action of lipases by interfacial activation at the oil–water interface. S is the substrate and P is the product. (b) The activity of esterases and lipases in aqueous solution. [S] is the substrate concentration and CMC is the critical micellar concentration of the substrate

Vantaggi dell'uso di solventi organici

TABLE 2: Advantages of organic solvents over aqueous media.

(i) Better solubility of substrates and product.
(ii) Shifting of thermodynamic equilibria (synthesis takes place instead of hydrolysis).
(iii) Simpler removal of solvent (most organic solvents have lower boiling point than water).
(iv) Reduction in water-dependent side reactions such as hydrolysis of acid anhydrides or polymerization of quinines.
(v) Removal of enzyme after reaction since it is not dissolved.
(vi) Better thermal stability of enzymes since water is required to inactivate enzymes at high temperatures.
(vii) Elimination of microbial contamination.
(viii) Potential of enzymes to be used directly within a chemical process.

Valutazione della purezza chirale di prodotti e substrati per reazioni catalizzate da un enzima enantioselettivo



Enzima con E = 5

Enzima con E = 20

Rapporto enantiomerico E = $(Vmax/Km)_{veloce}/(Vmax/Km)_{lento}$

Struttura della lipasi di *Burkholderia cepacia*

Le lipasi catalizzano l'idrolisi di legami estere con un meccanismo simile a quello delle proteasi a serina.

I substrati delle lipasi sono in genere poco solubili in acqua.

L'attività catalitica aumenta all'interfaccia acqua/lipide

La maggior parte delle lipasi utilizzate nell'industria deriva da microrganismi.

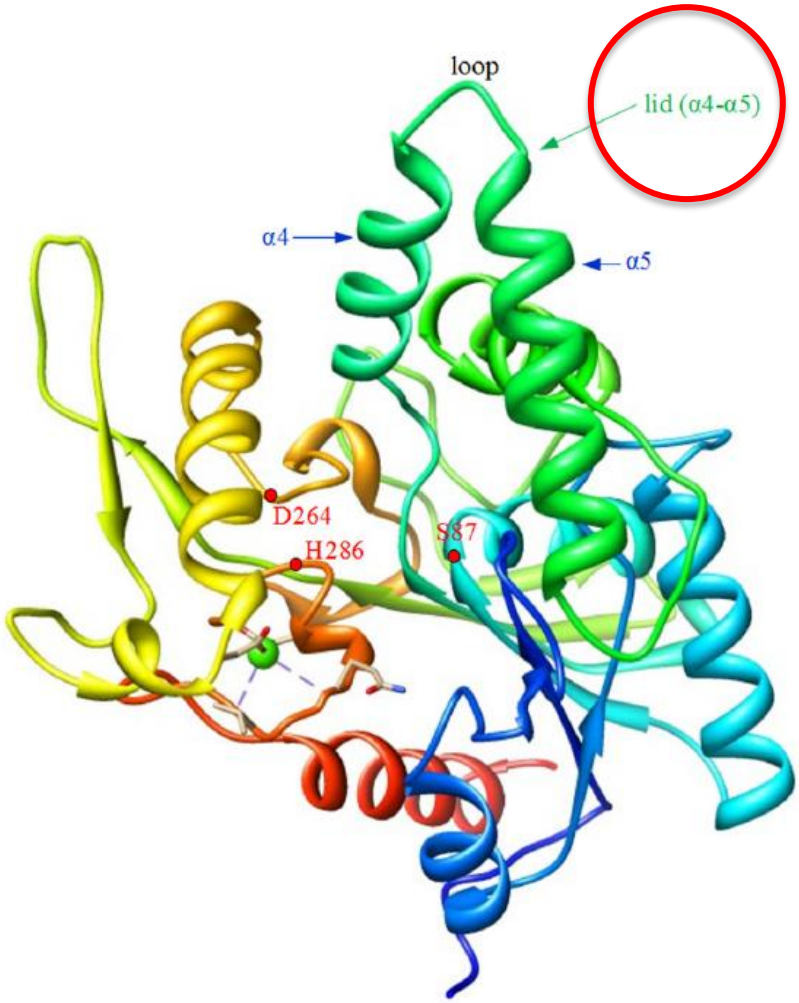


Figure 2. The structure of *Burkholderia cepacia* lipase: The lid region 118–159 (α4-loop-α5) and the catalytic triad (S87, D264, and H286) by red spheres are shown (Schrag et al., 1997).

Meccanismo d'azione delle lipasi

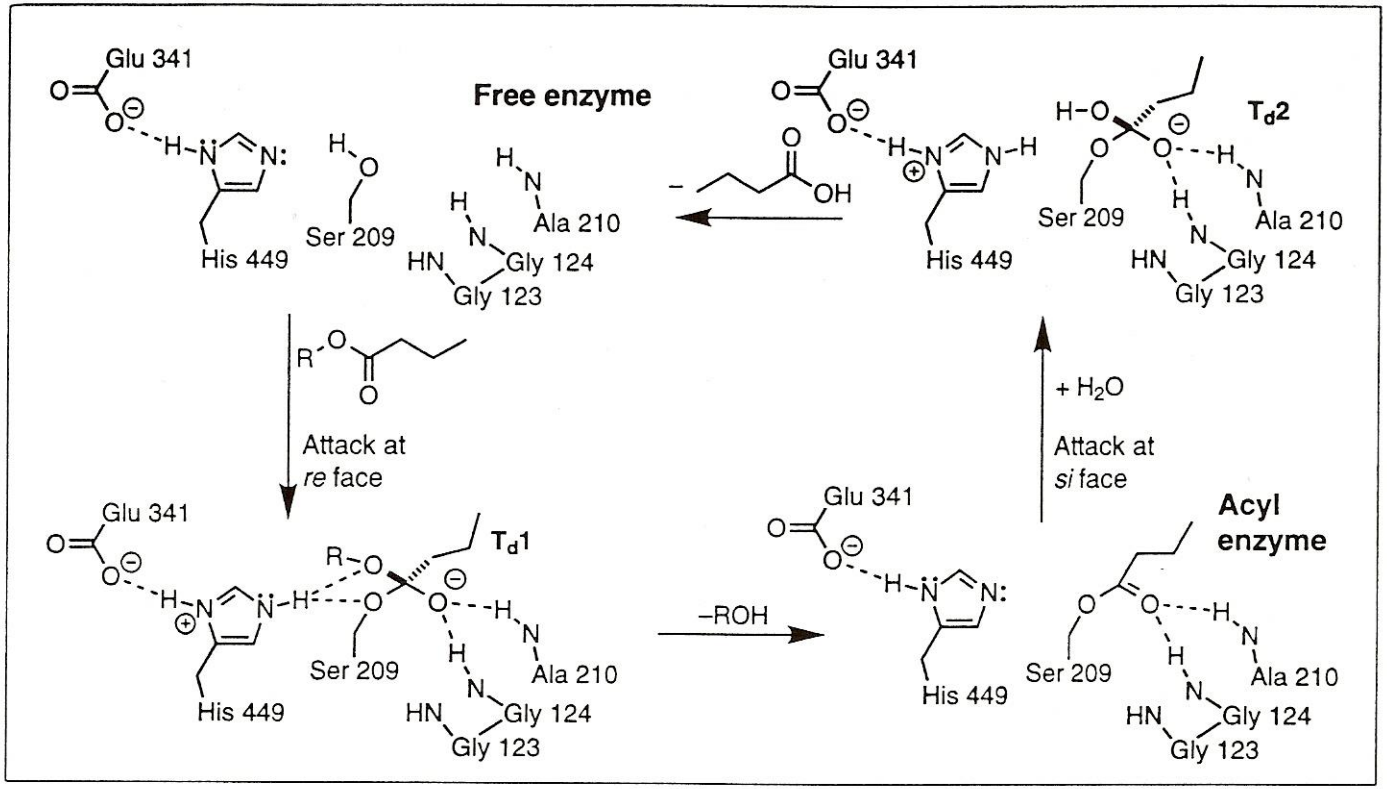


Figure 3

Hydrolysis of a butyrate ester catalyzed by lipase involves an acyl enzyme and two different tetrahedral intermediates. The transition state for the reaction resembles the first tetrahedral intermediate, T_d1, when acylation limits the rate, and resembles the second tetrahedral intermediate, T_d2, when deacylation limits the rate. The amino acid numbering corresponds to the active site of lipase from *Candida rugosa*, CRL. Crystal structures of the transition-state analogs suggest that during the formation of T_d1, Ser209 attacks the ester at the *re* face (from the bottom in the orientation shown); however, during the formation of T_d2, water probably attacks at the *si* face of the acyl enzyme (from the top in the orientation shown).

Reazioni catalizzate dalle lipasi: idrolisi e sintesi (transesterificazione)

(i) *Hydrolysis*

$$RCOOR' + H_2O \longrightarrow RCOOH + R'OH \quad (1)$$

(ii) *Synthesis.* Reactions under this category can be further separated into the following categories.

(a) *Esterification*

$$RCOOH + R'OH \longrightarrow RCOOR' + H_2O \quad (2)$$

(b) *Interesterification*

$$RCOOR' + R''COOR^* \longrightarrow RCOOR^* + R''COOR' \quad (3)$$

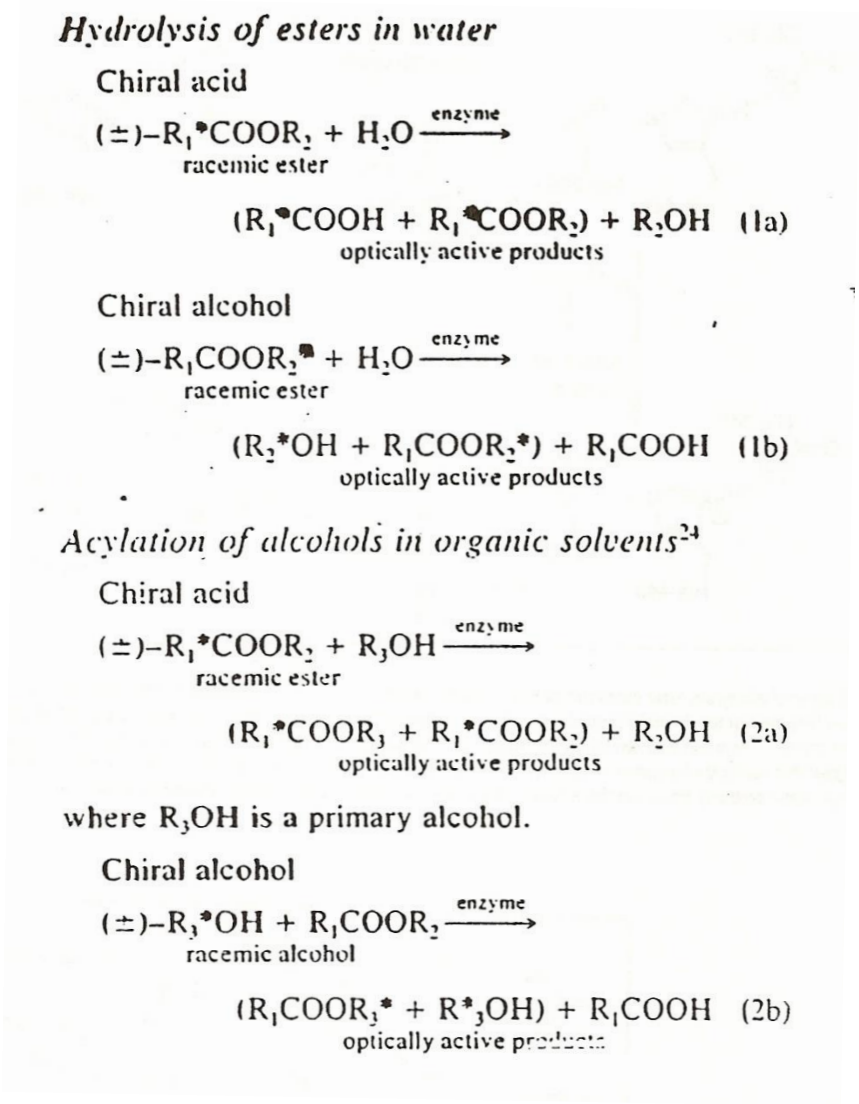
(c) *Alcoholysis*

$$RCOOR' + R''OH \longrightarrow RCOOR'' + R'OH \quad (4)$$

(d) *Acidolysis*

$$RCOOR' + R''COOH \longrightarrow R''COOR' + RCOOH \quad (5)$$

Reazioni catalizzate dalle lipasi: substrati e prodotti chirali



Hydrolysis of esters in water

Chiral acid

$(\pm)\text{-}R_1{}^*COOR_2 + H_2O \xrightarrow{\text{enzyme}}$
racemic ester

$(R_1{}^*COOH + R_1{}^*COOR_2) + R_2OH$ (1a)
optically active products

Chiral alcohol

$(\pm)\text{-}R_1COOR_2{}^* + H_2O \xrightarrow{\text{enzyme}}$
racemic ester

$(R_2{}^*OH + R_1COOR_2{}^*) + R_1COOH$ (1b)
optically active products

Acylation of alcohols in organic solvents[24]

Chiral acid

$(\pm)\text{-}R_1{}^*COOR_2 + R_3OH \xrightarrow{\text{enzyme}}$
racemic ester

$(R_1{}^*COOR_3 + R_1{}^*COOR_2) + R_2OH$ (2a)
optically active products

where R_3OH is a primary alcohol.

Chiral alcohol

$(\pm)\text{-}R_3{}^*OH + R_1COOR_2 \xrightarrow{\text{enzyme}}$
racemic alcohol

$(R_1COOR_3{}^* + R^*{}_3OH) + R_1COOH$ (2b)
optically active products

Per rendere la reazione di transesterificazione irreversibile si possono utilizzare come gruppi acilanti degli esteri che diano origine a prodotti che non sono più substrato della lipasi.

$R_1COOCH{=}CH_2 + R_2OH$
$\rightarrow R_1COOR_2 + HOCH{=}CH_2$

$HOCH{=}CH_2$ è instabile e si trasforma in CH_3CHO

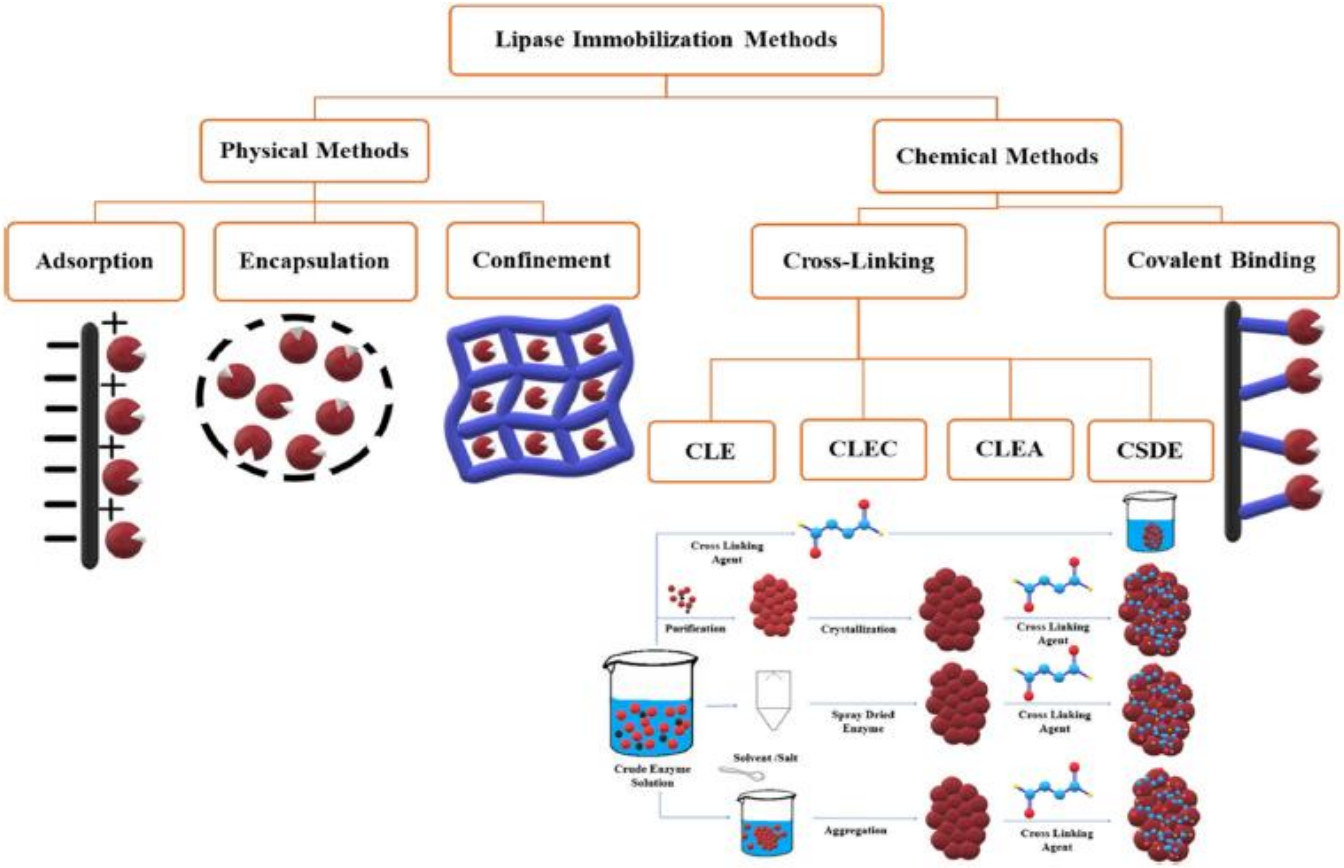
Applicazioni biotecnologiche delle lipasi

Reazioni di idrolisi
- additivi nei detersivi

Reazioni di idrolisi/transesterificazione
- produzione di ingredienti nei cibi
- produzione di ingredienti nei cosmetici
- produzione di biodiesel
- produzione di farmaci

Risoluzione cinetica di miscele racemiche
- risoluzione cinetica dinamica di miscele racemiche per ottenere la conversione completa del substrato

Tecniche di immobilizzazione delle lipasi



Tecniche di immobilizzazione delle lipasi

- Cristalli reticolati CLEC (cross-linked enzyme crystals) con glutaraldeide. I cristalli reticolati sono insolubili, stabili in acqua e in solventi organici, sono altamente porosi, permettono la diffusione del substrato e possono essere facilmente recuperati al termine della reazione.
- Aggregati reticolati CLEA (cross-linked enzyme aggregates) con glutaraldeide.
- Intrappolamento in gel di silice modificato con gruppi alchilici $CH_3Si(OCH_3)_3$ e $Si(OCH_3)_4$ per creare un microambiente **idrofobico** e mantenere attività catalitica
- Legame su nanoparticelle di silice attivate

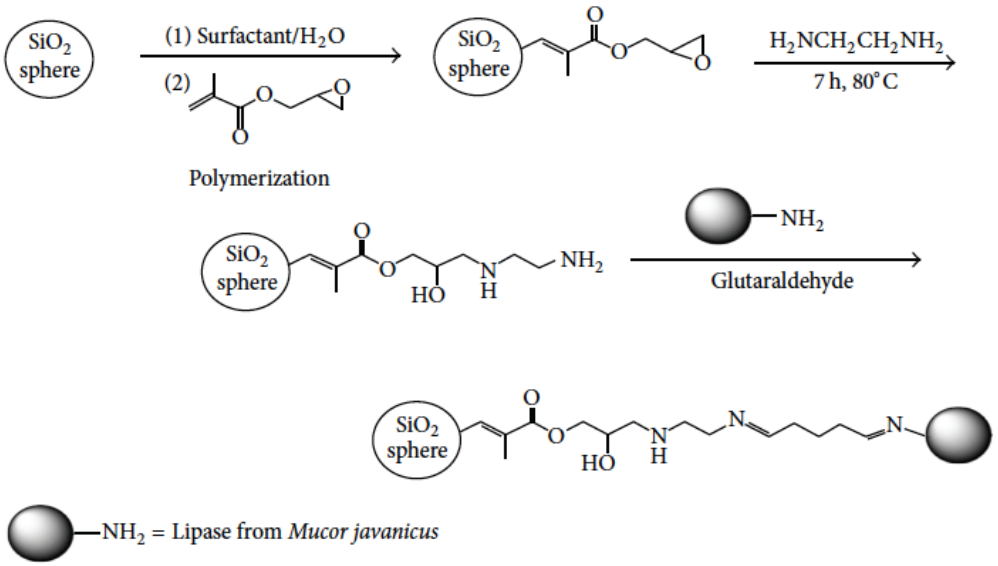


FIGURE 3: Lipase immobilization on silica nanoparticle.

Tecniche di immobilizzazione delle lipasi

- Legame su nanoparticelle magnetiche funzionalizzate

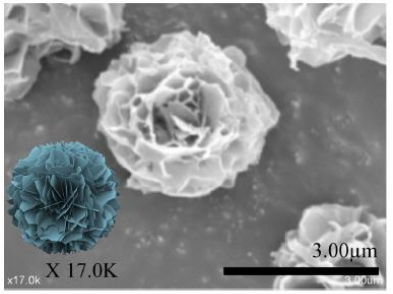


Fig. 7. Scanning electron micrographs of nanoflower.

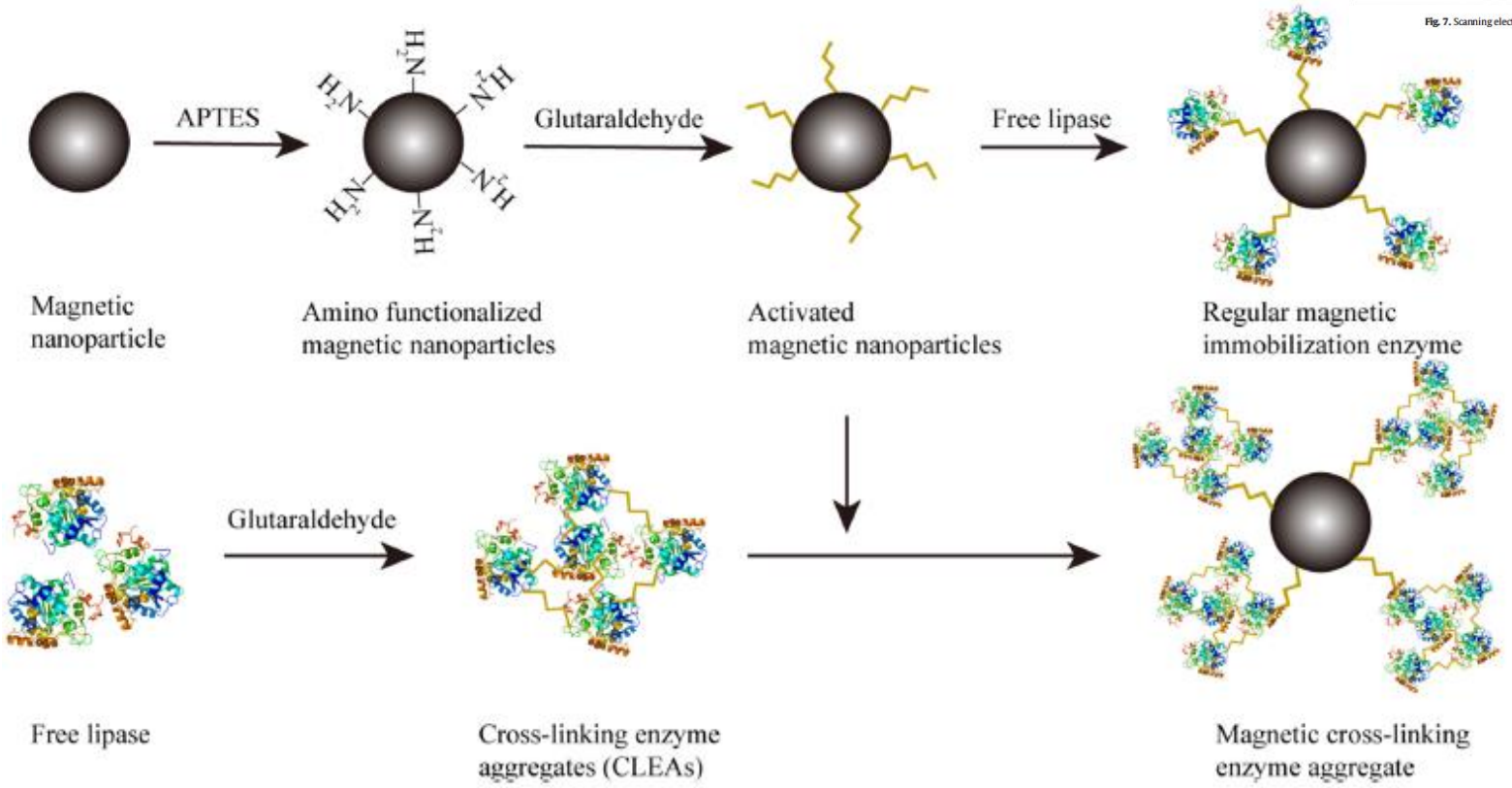


Fig. 2. Schematic representation of the synthesis of regular magnetic lipase and magnetic cross-linking enzyme aggregates.

Applicazioni biotecnologiche delle lipasi

Reazioni di idrolisi

detersivi: bassa specificità di substrato

stabilità ad alte temperature, alle proteasi e alla denaturazione chimica

optimum di pH 10-11

Reazioni di idrolisi/transesterificazione

produzione di ingredienti nei cibi: acidi grassi poli-insaturi (PUFA)

produzione di ingredienti nei cosmetici

produzione di biodiesel

produzione di farmaci

Risoluzione cinetica di miscele racemiche

risoluzione cinetica dinamica di miscele racemiche per ottenere la conversione completa del substrato ➔ farmaci

molecules

MDPI

Article

Improving the Efficiency of New Automatic Dishwashing Detergent Formulation by Addition of Thermostable Lipase, Protease and Amylase

Ashwini Naganthran [1,2], Malihe Masomian [1,2], Raja Noor Zaliha Raja Abd. Rahman [1,2,*], Mohd Shukuri Mohamad Ali [1,3] and Hisham Mohd Nooh [1,4]

Table 1. Stability of enzymes in 0.2% (*v*/*v*) or (*w*/*v*) of various surfactants, bleach, dispersing agent, builders and alkalinity agents.

Parameter	Components	Types of Enzymes (Relative Activity (%))		
		T1 Lipase	Rand Protease	Maltogenic Amylase
Control	-	100	100	100
surfactants	PEG (non-ionic)	84.58 ± 0.04	94.96 ± 0.07	113.5 ± 0.01
	G600 (non-ionic)	108.57 ± 0.07	99.33 ± 0.05	101 ± 0.07
	Tween 80 (non-ionic)	98.8 ± 0.04	115.51 ± 0.06	93.39 ± 0.07
	SDS (anionic)	14 ± 0.03	10 ± 0.001	1.08 ± 0.08
Bleach	Sodium percarbonate	5.44 ± 0.06	5.2 ± 0.05	21.81 ± 0.60
	Sodium perborate	6.40 ± 0.07	24.32 ± 0.19	1.2 ± 0.50
Dispersing agent	Sodium polyacrylate	54 ± 0.18	48 ± 0.13	71.9 ± 0.03
Builders	Sodium citrate	48 ± 0.03	44.74 ± 0.04	96 ± 0.05
	Sodium metasillicate	7.55 ± 0.06	16.65 ± 0.02	0.3 ± 0.05
	Sodium silicate	20.8 ± 0.30	16.43 ± 0.04	0.68 ± 0.04
Control	Glycine-NaOH, pH 9.0	100	100	100
Alkalinity agents	Phosphate, pH 7.0	88.4 ± 0.09	100.3 ± 0.01	125 ± 0.02
	Tris-HCl, pH 7.0	42 ± 0.04	106 ± 0.10	64.4 ± 0.21
	Sodium citrate, pH 8.3	48 ± 0.03	54.74 ± 0.04	96 ± 0.05
	Sodium bicarbonate (SB), pH 8.6	80.7 ± 0.04	83.3 ± 0.27	129 ± 0.06
	Sodium carbonate (SC): glycine (30:70), pH 9.25	120 ± 0.17	92 ± 0.01	119.1 ± 0.2
	SC:SB (30:70), pH 9.5	5 ± 0.05	67.9 ± 0.02	70 ± 0.08

Note: Data are means ± standard deviation of three determinations.

Enzimi incapsulati in gomma arabica 3-6% e maltodestrine 6-12%

Table 2. Enzymatic activity performance of encapsulated enzymes.

Enzymes		Encapsulated Enzyme	Powdered Free Enzyme	Control (Liquid Free Enzyme)
T1 lipase	Total activity (U)	1048.3	420	1098
	Activity retained (%)	95.5	38.25	100
Rand protease	Total activity (U)	10289	5032.5	11250
	Activity retained (%)	91.4	44.73	100
Maltogenic amylase	Total activity (U)	744.4	31.26	990
	Activity retained (%)	75.2	3.2	100

Detersivo A: enzimi liberi

B: enzimi incapsulati

Table 3. Effect of detergent concentration on soil removal.

Detergent Concentration (%)	Percentage of Soil Removal	
	Detergent A	Detergent B
0	8.3 ± 1.2^{p1}	8.3 ± 1.2^{q1}
1.5	41.4 ± 1.8^{p2}	49.6 ± 0.3^{q2}
2	44.0 ± 1.7^{p2}	51.0 ± 1.4^{q2}
2.5	44.6 ± 1.8^{p2}	51.3 ± 1.4^{q2}

Note: Superscripts p1 and p2 (test using detergent A) and q1 and q2 (test using detergent B) indicated groups that showed a significant difference between the groups when different detergent concentrations were used. All superscripts were obtained using post-hoc tests as shown in Table S5. Data are means ± standard deviation of three determinations.

Acidi grassi poli-insaturi

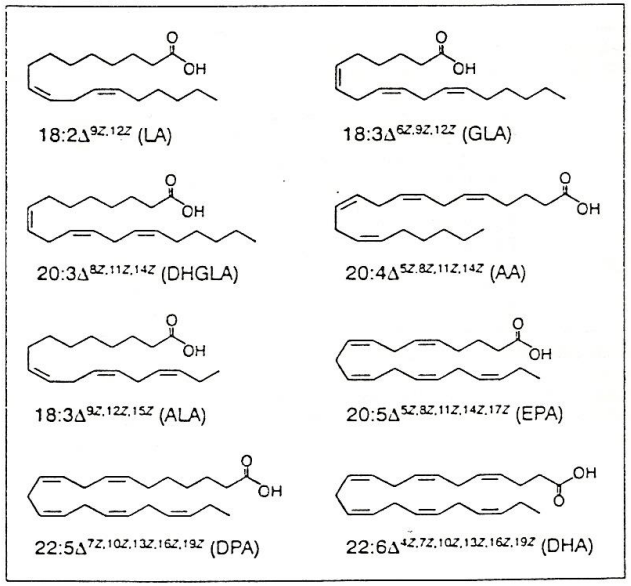
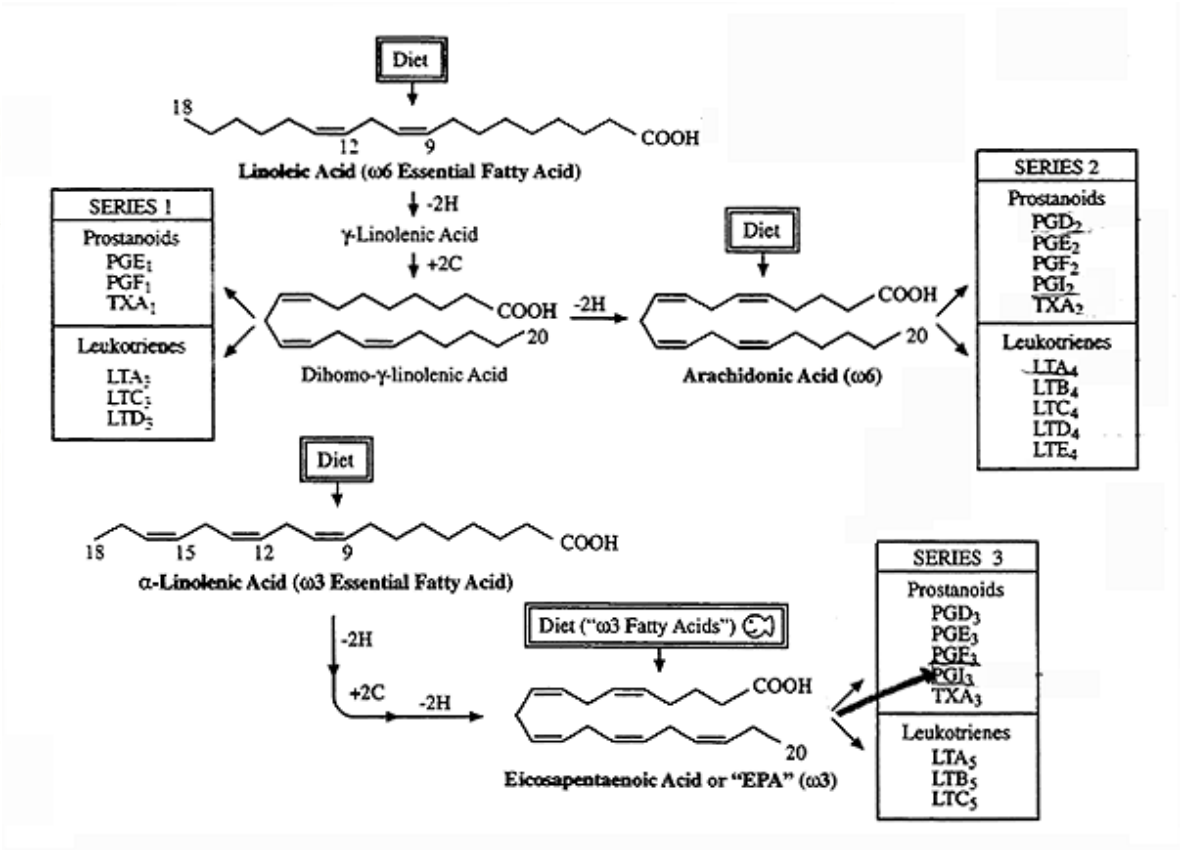
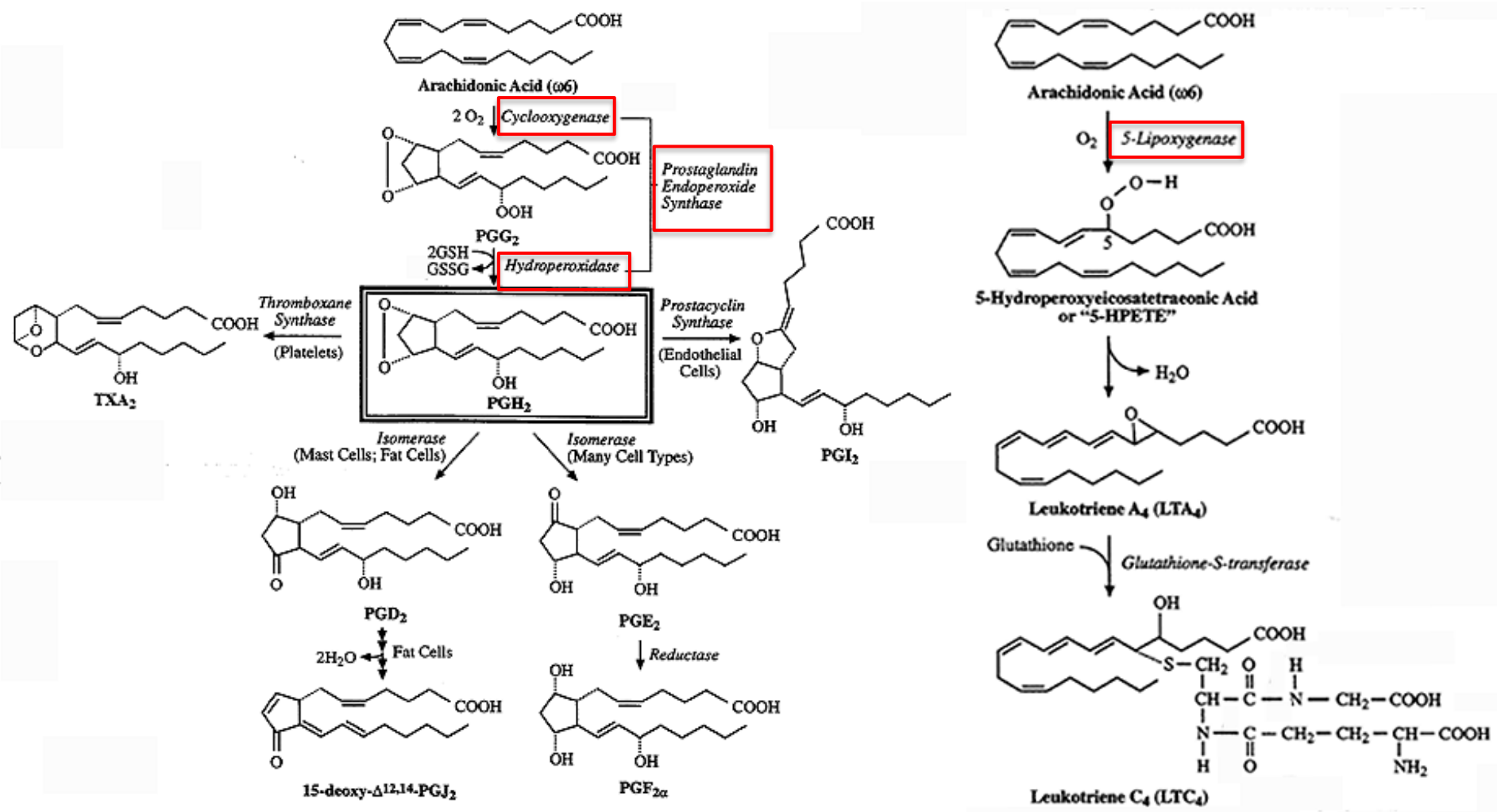


Figure 1
Representative examples of naturally occurring polyunsaturated fatty acids.

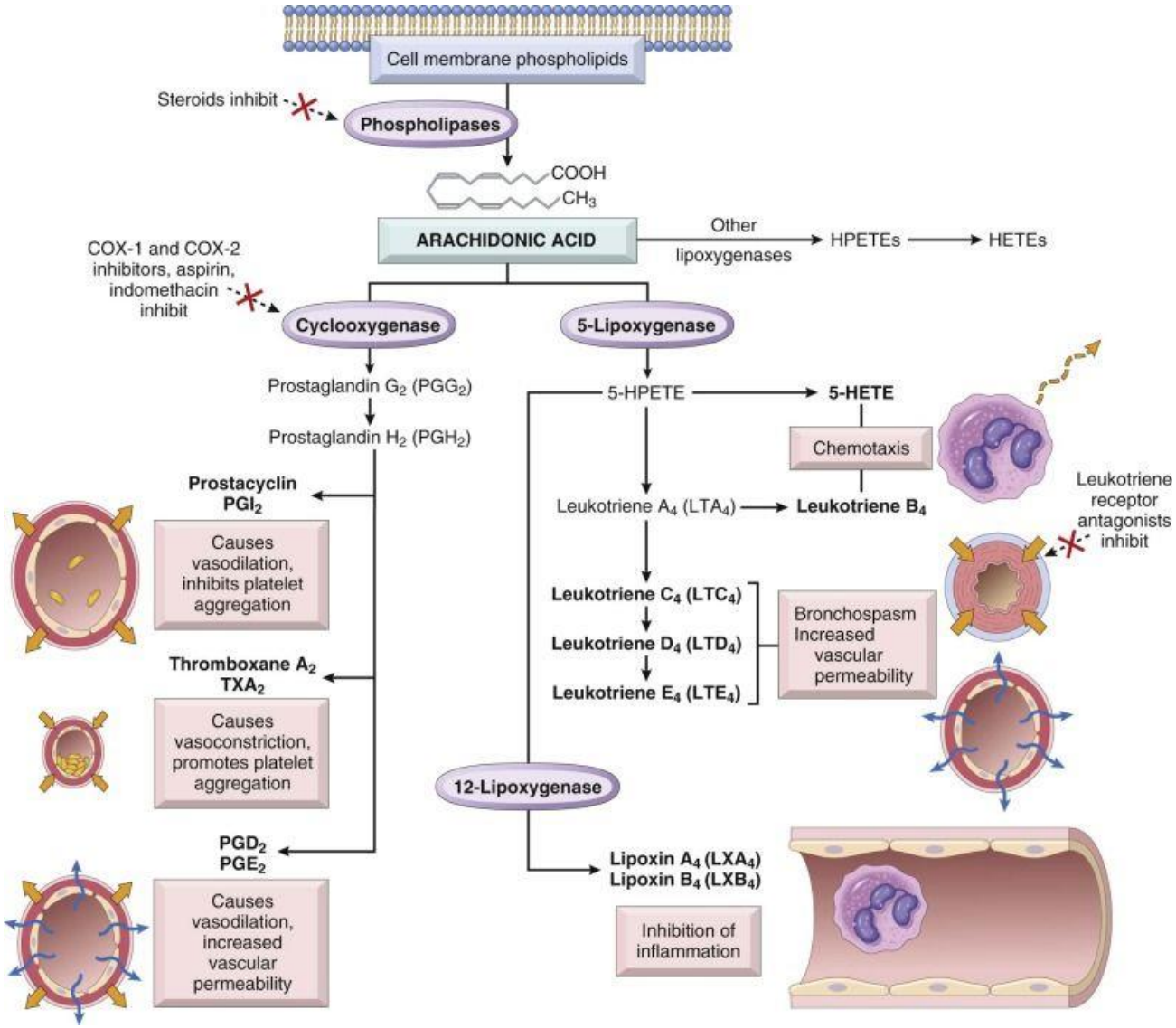


L'acido linoleico (18:2) e l'acido linolenico (18:3) sono essenziali e devono essere introdotti con la dieta. Sono i precursori degli eicosanoidi acido arachidonico (20:4) e acido eicosapentaenoico (20:5).

L'acido arachidonico è il precursore di prostaglandine, trombossani e leucotrieni



Ruolo biologico dei metaboliti dell'acido arachidonico



Applicazione di lipasi nella produzione di acidi grassi poli-insaturi (PUFA)

Table 2. Representative industrial applications of lipases to speciality PUFA lipid products.

PUFA product	Lipase catalyst	Substrate	Reaction	Applications	Ref.
FFA concentrates	*Chromobacterium viscosum*, *Pseudomonas fluorescens*	PUFA oils	Hydrolysis	Anticholesterolaemics, etc.	38
FFA concentrates	*Candida* sp.	Fish oils	Hydrolysis	Pharmaceuticals, nutraceuticals	39
DHA concentrate	*Candida* sp., *Penicillium* sp.	Fish oils	Hydrolysis	Pharmaceuticals, nutraceuticals	40
Glycerides	Thermostable lipase	PUFA esters + glycerol	Transesterification	Anti-inflammatories, etc.	41
Monoglycerides	Alkaline lipases	PUFA oils	Hydrolysis	Pharmaceuticals, nutraceuticals	42
sn-2-Diglycerides	(i) Phospholipase A_2 (ii) Phospholipase C	PUFAs + *sn*-2-lysophospholipids	(i) Esterification (ii) Hydrolysis	Anticoagulants, thrombolytics Nutraceuticals	43 43
Triglycerides	*Rhizomucor miehei*, *Rhizomucor javanicus*	PUFAs + triglycerides	Transesterification	Nutraceuticals	44
Triglycerides	Various lipases	PUFA lipids + PUFAs	Transesterification	Pharmaceuticals, nutraceuticals	45
Triglycerides	*Candida antartica*	PUFAs + glycerol	Esterification	Pharmaceuticals, nutraceuticals	46
sn-2-Phospholipids	*Pseudomonas cepacia*, *Humicola lanuginosa*	PUFAs + phospholipids	Transesterification	Anti-inflammatories, etc.	47

Abbreviations: DHA, dicosahexaenoic acid; FFA, free fatty acid; PUFAs, polyunsaturated fatty acids.

Applicazione di lipasi nella produzione di ingredienti dei cosmetici

- Lipasi come ingredienti dei cosmetici
  - Attività di idrolisi di grassi/rilascio componenti attivi
- Lipasi per la produzione di componenti dei cosmetici:
  - Esteri di acidi grassi (emulsionanti, emollienti, detergenti)
  - Esteri di zuccheri (tensioattivi)
  - Profumi e aromi
  - Ingredienti attivi (antiossidanti, filtri UV, cerammidi)

➔ Enantioselettività e regioselettività delle lipasi sono importanti per queste applicazioni

Componenti dei cosmetici ottenibili con l'uso di lipasi

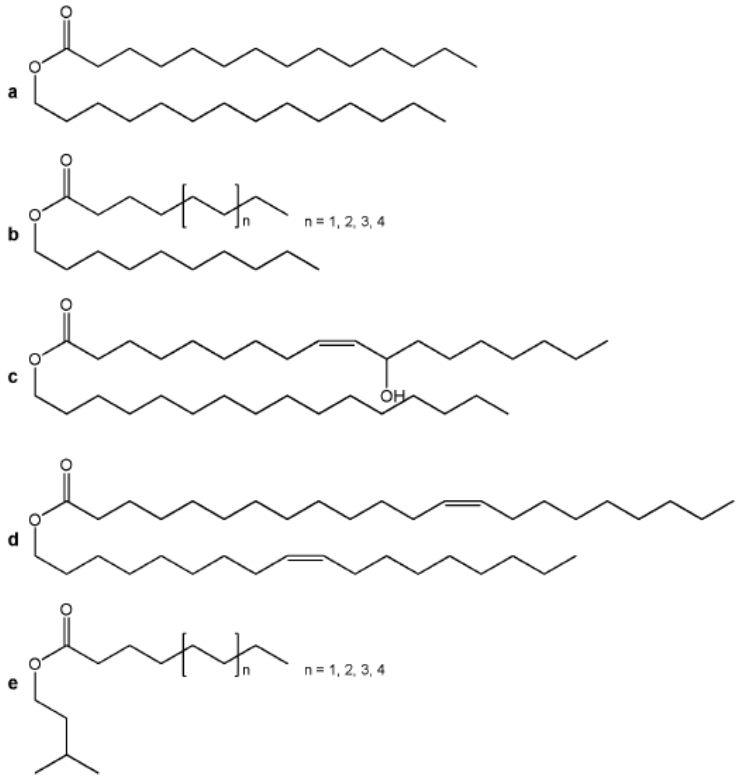


Fig. 1 Emollient esters commercialised by Evonik Industries AG. (a) myristyl myristate; (b) decyl cocoate; (c) cetyl ricinoleate; (d) oleyl erucate and (e) isoamyl cocoate.

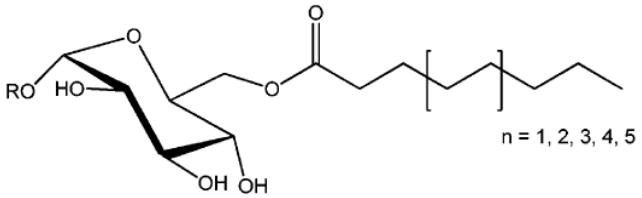


Fig. 4 Schematic of glycoside esters described by Björkling *et al.*;[63] R = short alkyl chain.

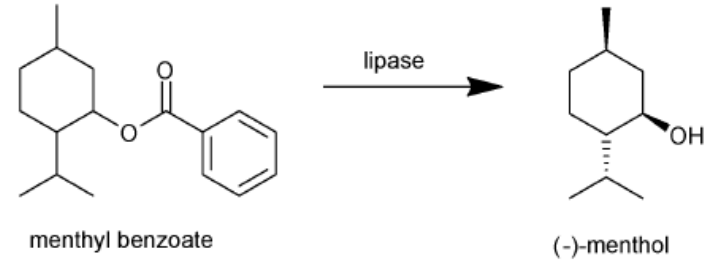


Fig. 11 Lipase-catalysed synthesis of enantiopure **(−)-menthol** according to Vorlova *et al.*[144]

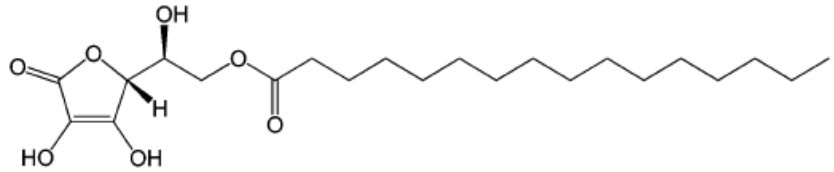


Fig. 12 Structure of 6-*O*-ascorbyl palmitate, accessible by lipase catalysis.

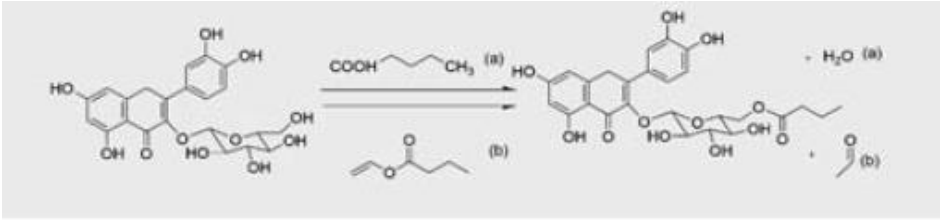


Fig. 1 Enzymatic acylation of flavonoids: a esterification, b transesterification [18].

Confronto tra procedimento convenzionale e catalizzato da lipasi per ottenere esteri degli acidi grassi utilizzati nei cosmetici

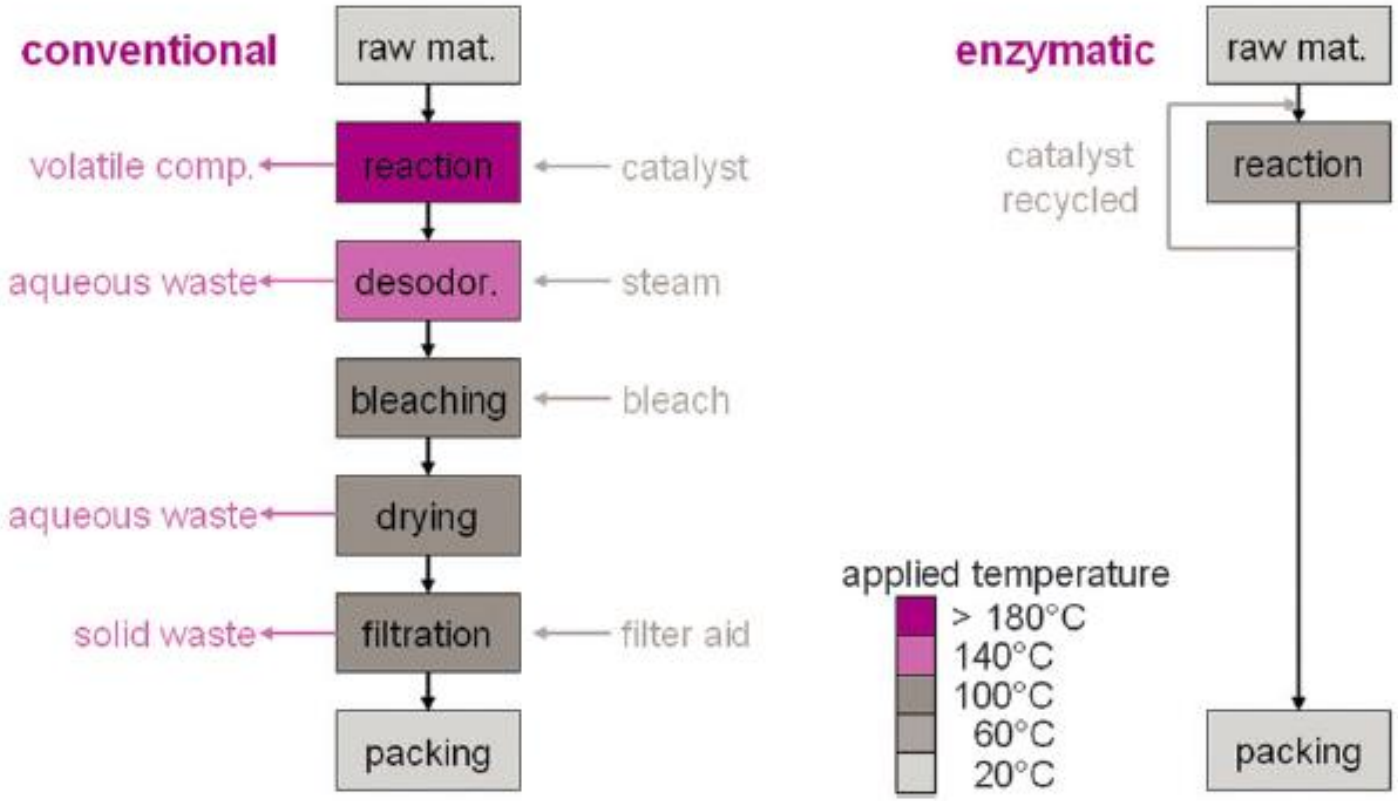


Fig. 17 Process steps of conventional (left) and enzymatic (right) esterification for production of cosmetic fatty acid esters.[29,31]

Applicazione di lipasi nella produzione di biodiesel

- Il biodiesel è costituito da esteri metilici di acidi grassi (FAME)
- La reazione di transesterificazione di trigliceridi e metanolo produce biodiesel e glicerolo

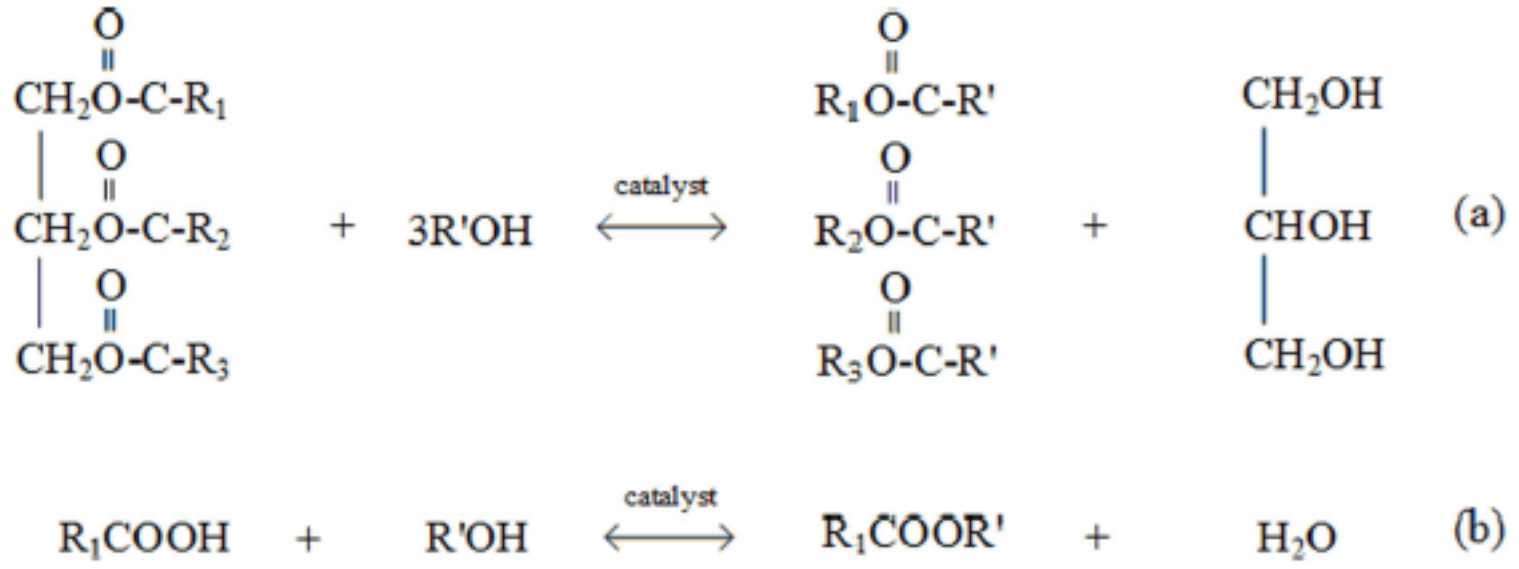


Figure 1. Typical reactions in biodiesel production, (**a**) transesterification of triglycerides (TG), and (**b**) esterification of free fatty acids (FFA).

Applicazione di lipasi nella produzione di biodiesel

- Catalisi alcalina (NaOH) vs catalisi enzimatica

Table 4
Comparison of enzymatic technology versus conventional alkaline technology for biodiesel production.

Key issue	Enzymatic process	Alkaline process
Presence of free fatty acid in the starting oil	Free fatty acids are transformed to biodiesel.	Free fatty acids are transformed to soaps.
Water content of starting oil	It is not deleterious for lipase.	Impact on the catalyst by forming soaps. It may hydrolyze the oil and ultimately more soaps are formed.
Biodiesel yield[a]	High, usually around 90%.	High, usually >96%.
Glycerol recovery	Easy, high grade glycerol.	Complex, low grade glycerol.
Catalyst recovery and reusage	Easy or not necessary when operating in a PBR. Reusability not sufficiently studied.	Difficult or not profitable, usually it is neutralized by adding an acid after transesterification. It is partially lost as soaps or in the successive washing steps.
Energy costs	Low, temperature range 20–50 °C.	Medium, temperature range 60–80 °C.
Catalyst cost	High	Low
Environmental impact	Low, waste water treatment not needed.	Medium, alkaline and saline effluents are generated. Wastewater treatment needed.
Process productivity[b]	Low	High

[a] Percentage of starting oil transformed to biodiesel.
[b] Mass of biodiesel produced per volume of reactor and per unit of time.

Applicazione di lipasi nella produzione di biodiesel

- Lipasi purificate o microrganismi che producono elevati livelli dell'enzima (ricombinante o naturale)
- Biocatalizzatore immobilizzato
- Fonti di trigliceridi: oli vegetali commestibili (olio di girasole, soia, palma) e non commestibili, microrganismi ricchi di lipidi (microalghe, batteri o lieviti), oli di scarto (alimentari, lavorazione carta o tabacco)

- Variabili da controllare: temperatura, alcool (inattivazione dell'enzima in eccesso di metanolo), presenza di acqua e solventi, rigenerazione della lipasi, concentrazione di acidi grassi liberi

Applicazione di lipasi nella produzione di biodiesel

Table 5
Examples of research works on enzymatic production of biodiesel by transesterification.

Oil	Enzyme	Acyl-acceptor	Solvent	Yield (%)
Sunflower	Novozym-435	Methanol	No	3
		Methanol	Petroleum ether	79
		Ethanol	No	82
Tallow	Lipozyme IM-60	Primary alcohols	Hexane	94.8–98.5
Soybean	Novozym	Secondary alcohols	Hexane	61.2–83.8
Rapeseed	Lipozyme IM	Methanol	No	19.4
	Lipozyme IM	Ethanol	No	65.5
Soybean	*Rhizopus* oryzae lipase	Methanol	No, water 4–30% by wt.	80–90
Palm	Lipase PS-30	Methanol	No	15
		Ethanol	No	72
Soybean	Novozym-435 preincubated 0.5 h in ethyl oleate	Methanol	No	97
Soybean (crude)	*Candida antarctica* lipase	Methanol	No	93.8
Soybean	Novozym-435	Methyl acetate	No	92
Triolein	Various commercial lipases	Linear and branched alcohols Fusel oil-like alcohol mixture	No	near 100
Soybean	Lipase PS (immobilized)	Methanol	No	67
		Ethanol	No	65
Vegetable oils	*Candida* sp. lipase (immobilized)	Methanol	No	96–93
Frying oils				92
Rapeseed	Lipozyme TL IM	Methanol	*t*-butanol	95
	Novozym-435	Methanol	*t*-butanol	95
Jatropha Sunflower	Novozym-435	2-propanol	Hexane	92.8–93.4
Jatropha Sunflower	Novozym-435	Ethyl acetate	No	91.3
		Ethyl acetate	No	92.7
Microalgae	*Candida* sp. lipase (immobilized)	Methanol	Hexane	98
Cotton	Novozym-435	Methanol	*t*-butanol	97
Vegetable oils	Novozym 435 Lipozyme TL IM	Methanol Ethanol	No	near 100
Microalgae	Various commercial lipases	Long-chain alcohols	Hexane	–
Waste edible oil (2.5% free fatty acids)	Novozym 435	Methanol	No	>90
Acid oil (77.9% free fatty acids)	Novozym 435	Methanol	No	>90
Soybean oil deodorizer distillate (28% free fatty acids)	Novozym 435 Lipozyme TL IM	Methanol	*t*-butanol	around 95%

Applicazione di lipasi immobilizzate nella produzione di biodiesel

Table 1
Biodiesel production with various immobilized lipase (Jegannathan et al., 2008).

Immobilized method	Carrier used	Lipase origin	Oil	Acyl acceptors	Yield (%)
Adsorption	Acrylic resin	*Candida antartica*	Vegetable oil, waste cooking oil	Methanol, 1-propanol, methyl acetate	>90
Adsorption	Textile membrane	*Candida* sp. 99–125	Lard, waste oil, salad oil	Methanol	>87
Adsorption	Toyonite 200-M, polypropylene	*Pseudomonas fluorescens*	Vegetable oil	Methanol	>87
Adsorption	Celite, Diatomaceous earth	*Pseudomonas cepacia,*	Jatropha oil, vegetable oil	Ethanol, 2-butanol	>98
Adsorption	Anion resin, celite-545	*Porcine pancreatic, Rhizomucor Miehei, Chromobacterium viscosum*	Sunflower oil, soybean oil, Jatropha oil	Ethanol, methanol	>80
Covalent bond	Silica-PVA styrene-divinylbenzene	*Burkholderia cepacia, Thermomyces lanuginosus*	Babassu oil, canola oil	Ethanol, methanol	>97%
Entrapment	Hydrophobic sol–gel support	*Pseudomonas cepacia,* NS44035	Soybean oil, triolein,	Methanol, ethanol	60
Cross-linking	Glutaraldehyde	*Pseudomonas cepacia*	Mahua oil	Ethanol	92

Table 1
Lipase immobilization on/in nanomaterials for biodiesel production.

Enzyme	Nano-support	Methodology	Feedstocks	Solvents
Thermomyces lanuginosa lipases	APTES modified Fe_3O_4	Covalently attach	Soybean oil	Solvent-free
Candida antarctica lipase B	APTES modified Fe_3O_4	Covalently attach	Rapeseed oil	Solvent-free
Lipase	APTES modified Fe_3O_4	Covalently attach	*Aspergillus* lipid	Hexane
Aspergillus niger lipase	Fe_3O_4 coated with APTES/MPTMS modified mesoporous silicon	Covalently attach	Soybean oil	Solvent-free
Candida rugosa lipase	Fe_3O_4 coated with poly(styrene-methacrylic acid)	Covalently attach	Soybean oil	Solvent-free
Candida rugosa lipase	Hollow Fe_3O_4 coated with mesoporous dopamine	Adsorption	Oleic acid	Solvent-free
Candida rugosa lipase	Fe_3O_4 coated with chitosan	Covalently attach	Soybean oil	Hexane
Candida rugosa lipase	Fe_3O_4 coated with graphene oxide	Covalently attach	Soybean oil	Solvent-free
Thermomyces lanuginosa lipases	Snowman-like Fe_3O_4/Au nanoparticles	Adsorption	Tomato seed oil	Solvent-free

Stabilizzazione di lipasi vs metanolo

Table 3. Protein engineering toward stabilization to methanol.

Enzyme	Wild type[a)]	Mutagenesis	Improvement in stability[a)]	Structural changes	Substrate for transesterification	Conversion	Ref.
Proteus mirabilis lipase (stabilized with S-S bond)	Inactive after 2 h incubation in 70% methanol	Ep-PCR + SDM	80% residual activity after 16 h incubation in 70% methanol	11 substitutions	Canola oil 5:1 molar ratio	76% in 20 h (wt 47.7%)	[91]
				Additional polar interactions and salt bridges		Can be recycled (the wt not)	
Geobacillus stearothermophilus lipase	4 min half-life in 70% methanol	-Consensus-guided	324 min half-life in 70% methanol (87×)	H86Y/A269T/R374W	soybean oil 1.5:1 methanol to oil molar ratio + other substrates	46% in 24 h[b)]	[88,89]
		-Ep-PCR		Hydrogen bonds network and structural water molecules		Wt: 8.6% in 24 h	
		-Substitution of surface charged residues					
Thermomyces lanuginosum lipase	28% residual activity after 1 h incubation in 75% methanol	Mutagenesis of residues with high B-factor	71% residual activity after 1 h incubation in 75% methanol	S105C/D27R	Waste grease	With whole cells	[87]
				New hydrogen bond that stabilizes a flexible loop structure		S105C/D27R	
						90% in 24 h	
						Wt: 82% in 24 h	

[a)] Measured as activity of methanol-incubated biocatalysts in hydrolysis reactions. [b)] Highest conversion possible 50%.

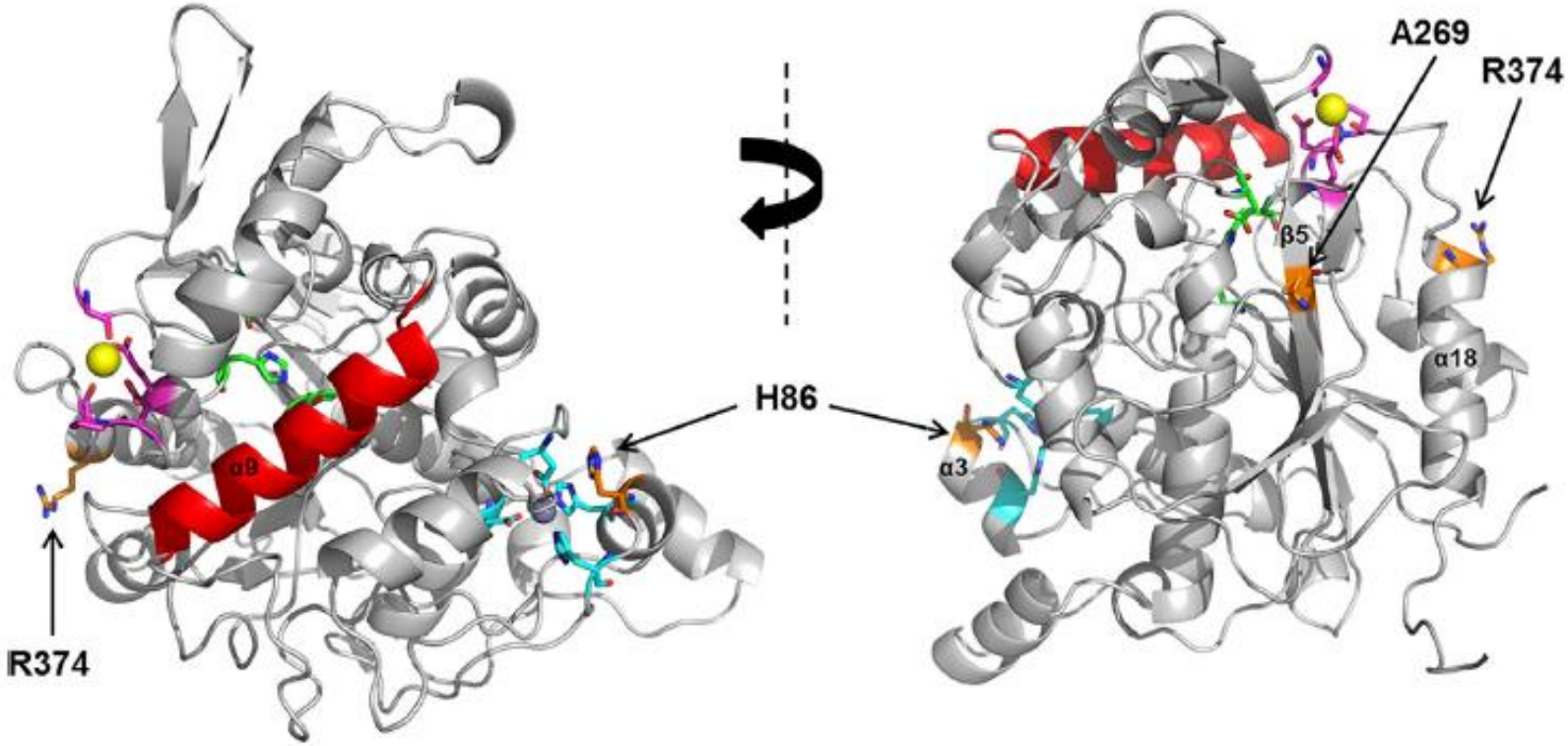


Figure 2. Crystal structure of wild-type lipase T6 with the mutated residues (H86, A269, R374) shown in orange sticks, catalytic triad residues (Ser114, Asp318, and His359) in green, calcium-binding residues (Glu361, Gly287, Pro367, and Asp366) in magenta, zinc-binding residues (Asp62, His88, Asp239, and His82) in cyan, α-helix lid and α9 in red and gray spheres, respectively. Reproduced with permission.[89] Copyright 2015, Springer Science + Business Media.

Risoluzione cinetica di miscele racemiche di precursori di farmaci

- I farmaci anti-infiammatori non steroidei (FANS) sono inibitori competitivi delle cicloossigenasi.
- I FANS derivati di acidi aril-propionici contengono un centro chirale. L'isomero S si lega al sito attivo di COX-2.

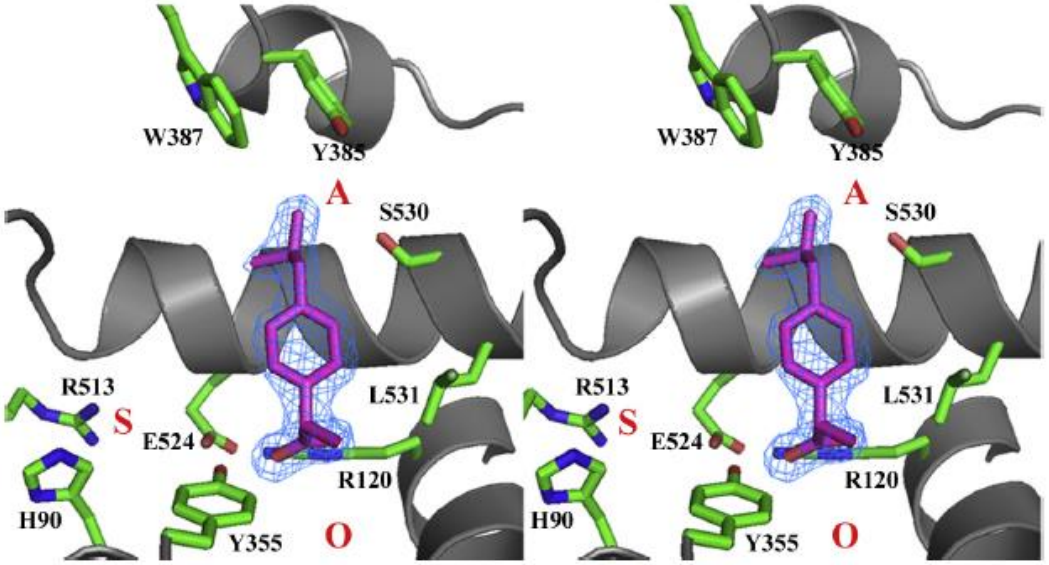
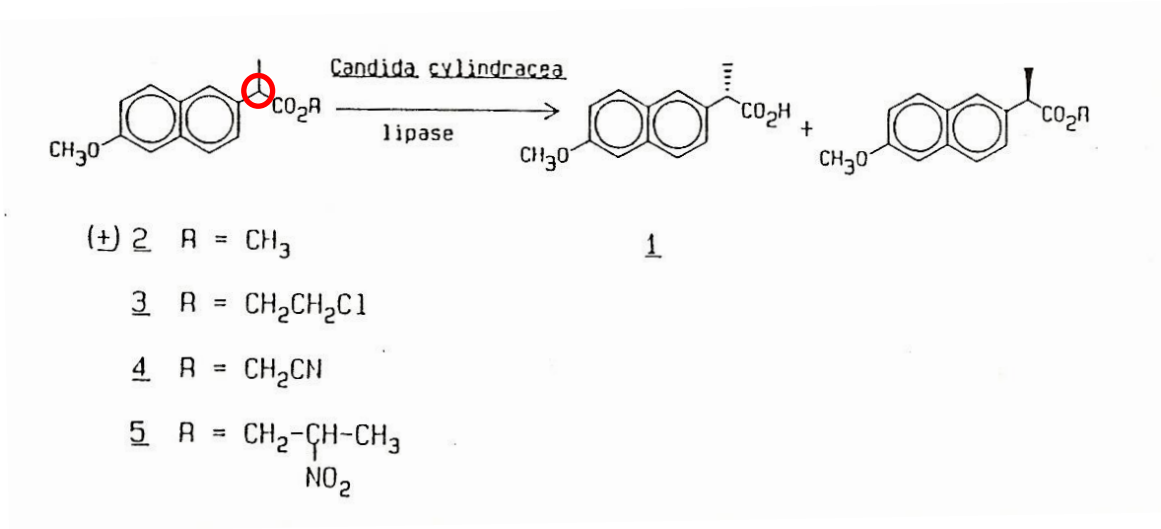


Fig.1. IBP bound in the cyclooxygenase channel of COX-2. Stereo view of IBP bound within the cyclooxygenase channel of monomer A of the muCOX-2:IBP crystal structure. F_o–F_c simulated annealing omit map electron density (light blue), contoured at 3.5σ, is shown with the final refined model of IBP (pink). Residues lining the cyclooxygenase channel, along with the spatial locations of the channel opening (O), channel apex (A), and COX-2 specific side pocket (S) are labeled accordingly. Carbon atoms of residues lining the channel are colored green, while nitrogen, and oxygen atoms are colored blue and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Risoluzione cinetica di miscele racemiche di precursori di farmaci

Il Naproxen (acido metil-2-(6-metossi-2-naftil) propionico) è un farmaco anti-infiammatorio non steroideo che contiene un centro chirale. L'isomero S è 28 volte più attivo dell'isomero R. Può essere ottenuto per idrolisi enantiospecifica della miscela racemica dell'estere precursore della molecola attiva.



(±) 2 R = CH_3

3 R = CH_2CH_2Cl

4 R = CH_2CN

5 R = CH_2-CH(NO_2)-CH_3

1

La lipasi di *C. cylindracea* (*C. rugosa*) mostra preferenza stereochimica per l'isomero S

TABLE 1. Enantiospecific hydrolysis of (±)-methyl-2-(6-methoxy-2-naphthyl)propionate (2) by microbial lipases.

Lipase Source[1]	Stereochemical Preference	Extent of Conversion (%)	Enantiomeric Excess (%) Ester	Acid	E
Candida cylindracea[1a]	S	39	63	>98	>100
Mucor meihei[1b]	R	18	21	95	51
Rhizopus arrhizus[1c]	R	11	13	97	78
Rhizopus sp.[1d]	R	19	21	92	27
Rhizopus oryzae[1e]	R	11	10	76	8

[1] To one ml of 0.2 M potassium phosphate buffer, pH 8.0, was added 244 mg (1 mmol) of (±)2 and varying amounts of different enzyme preparations. The contents were incubated at 22°C for 120-216 h under gentle stirring. [a] 1 mg of pure enzyme[9] isolated from the Sigma type VII preparation, 216 h; [b] 200 mg of Amano MAP10 powder, 120 h; [c] 10 mg of enzyme of Boehringer-Mannheim, 120 h; [d] 150 mg of powder from Serva, 120 h; [e] 200 mg of Amano FAP powder, 120 h.

[2] E is the ratio of the specificity constants (k_{cat}/K_m) of the two enantiomers.[10]

La velocità di reazione dipende dalla natura del gruppo uscente

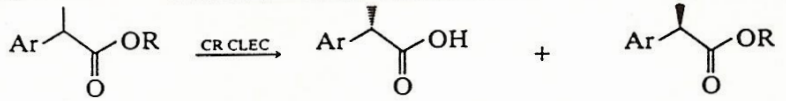
TABLE 2. Relative rates of enzymatic hydrolysis.

Compound	Relative rate	Enantiomeric ratio (E)
2	1	>100
3	15	>100
4	6	>100
5	3	81

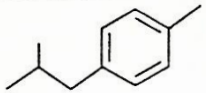
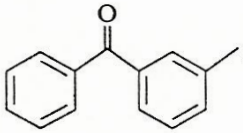
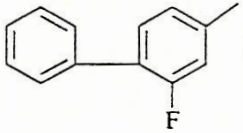
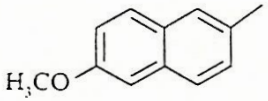
Immobilizzazione della lipasi di *C. rugosa* per la risoluzione di miscele racemiche di esteri di acidi arilpropionici

Metodo di immobilizzazione: cristalli di enzima reticolati con glutaraldeide (CLEC). La cristallizzazione avviene in 2-metil-2,4-pentandiolo, che permette di mantenere l'attività enzimatica e l'accessibilità del sito attivo.

TABLE I
CR CLEC LIPASE-CATALYZED RESOLUTION OF ARYLPROPIONIC ACID ESTERS



(*R,S*)-α-Arylpropionate ester (*S*)-Arylpropionic acid (*R*)-Arylpropionate ester

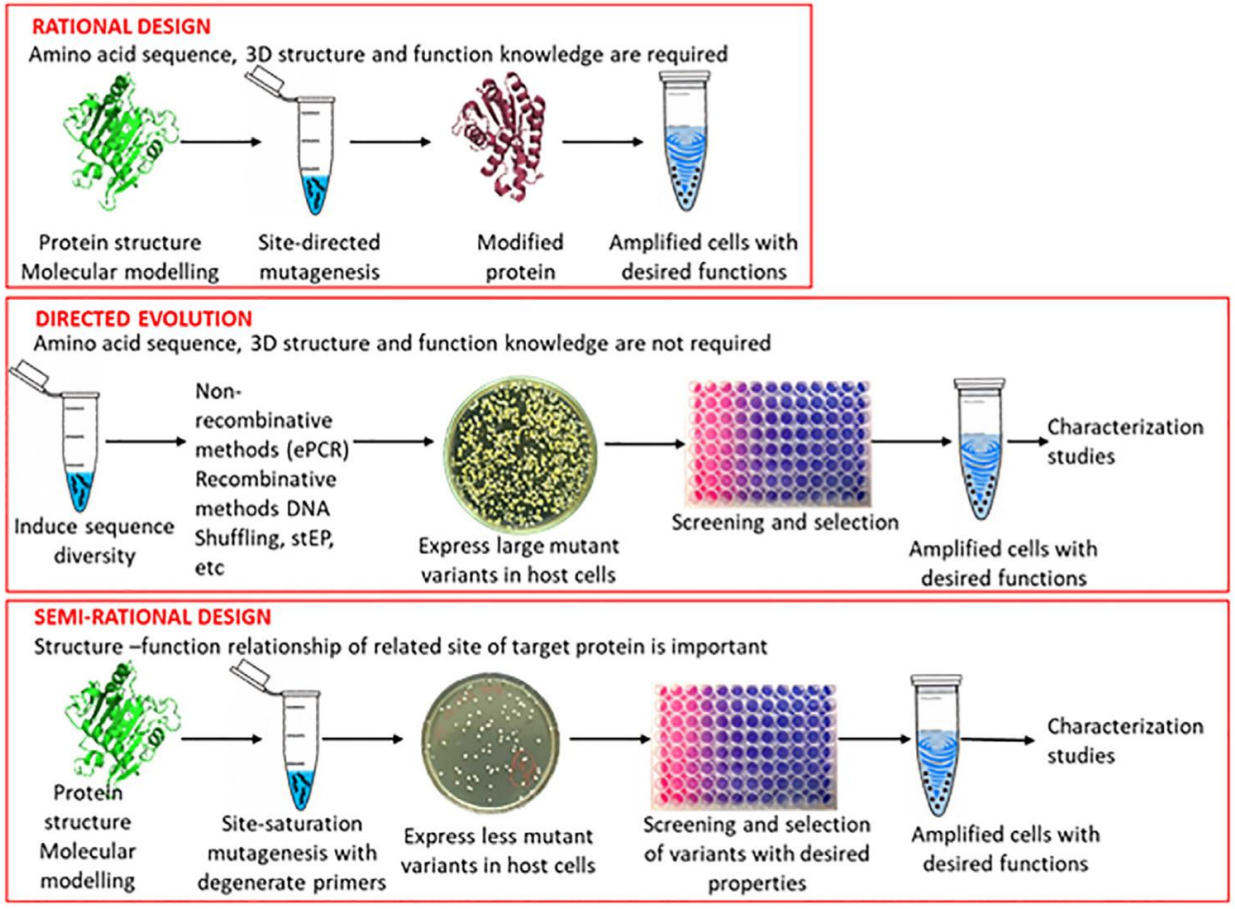
Ar	% Enantiomeric excess (% conv.) CR CLEC	% Enantiomeric excess (% conv.) Crude	*E* CR CLEC	*E* Crude
 **1a:** R = H **ibuprofen** **b:** R = Me	94.6 *S*-**1a** (22)[a]	81.9 *S*-**1a** (39.3)[a]	47	17
 **2a:** R = H **ketoprofen** **b:** R = CH_2CH_2Cl	91.1 *S*-**2a** (49.3)[b]	64.5 *R*-**2b** (66)[b]	66	5
 **3a:** R = H **flurbiprofen** **b:** R = CH_2CH_2Cl	94.3 *S*-**3a** (34.4)[c]	61.1 *S*-**3a** (34)[c]	55	6
 **4a:** R = H **naproxen** **b:** R = Me	97.3 *S*-**4a** (39)[d]	76.2 *S*-**4a** (46.8)[d]	>100	12

[a] Reaction buffer 0.1 *M* pH 6 sodium acetate.
[b] Reaction buffer 0.1 *M* pH 5 sodium acetate.
[c] Reaction buffer 0.1 *M* pH 7 sodium phosphate.
[d] Reaction buffer 50% PEG 1000/50% pH 5 ammonium acetate.

Strategie di ingegnerizzazione delle lipasi

Quali proprietà migliorare?

- Enantioselettività
- Stabilità in ambienti 'esotici' (solventi organici)



Evoluzione in vitro di lipasi per aumentare l'enantioselettività dell'enzima

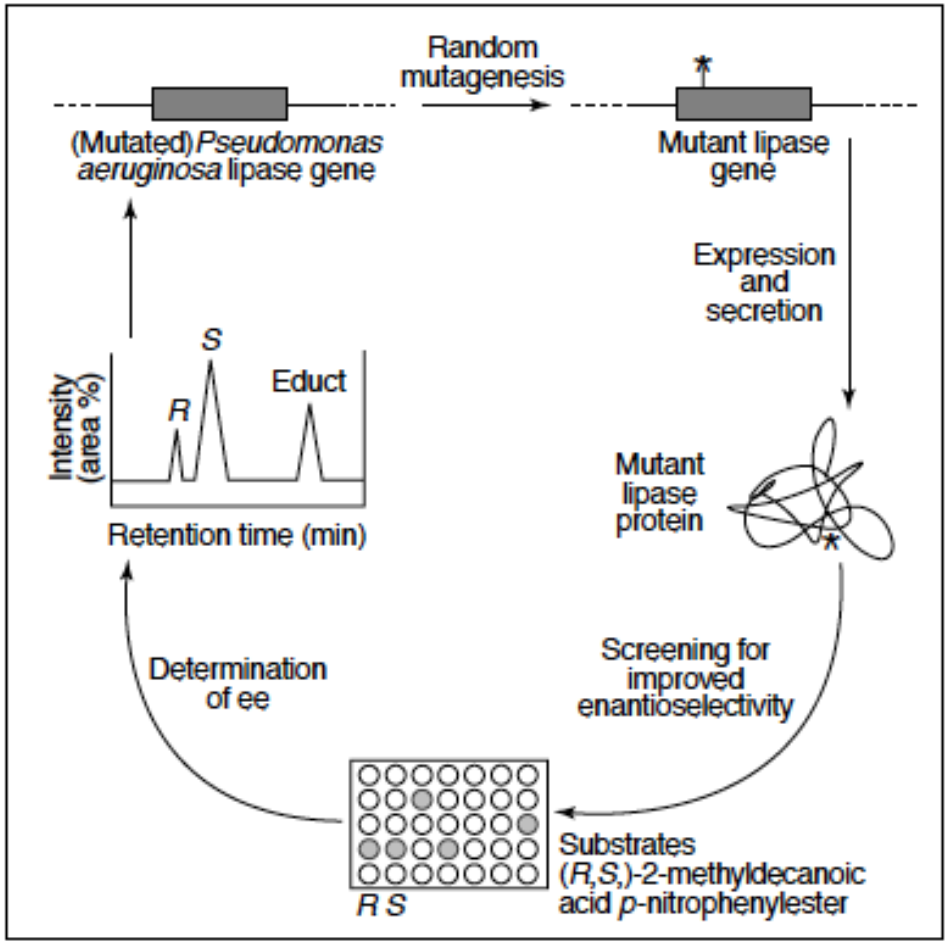


Figure 5

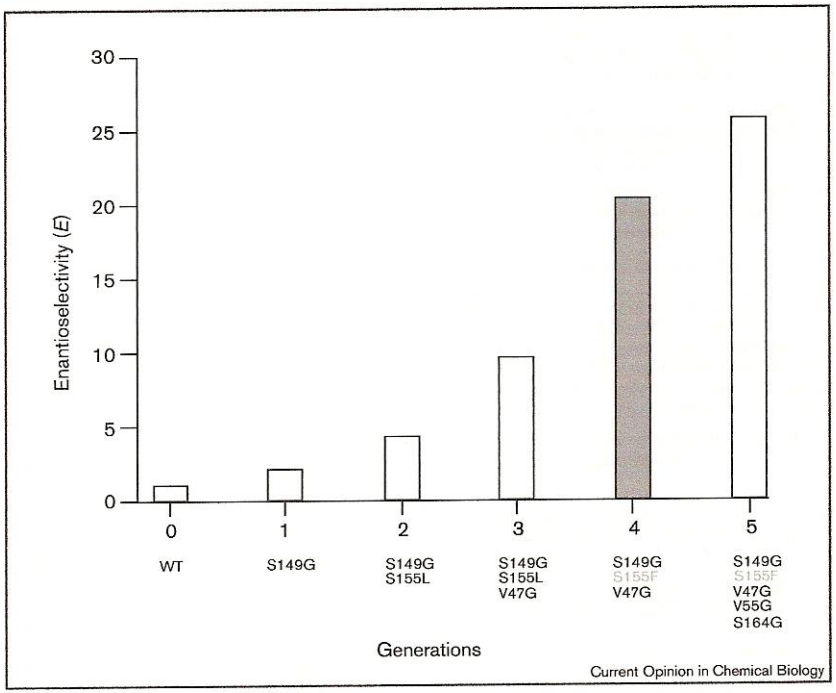
Strategy to create an enantioselective lipase by directed evolution. Intensity (area %) refers to the amount of *R*- and *S*-enantiomer as measured by chiral chromatography.

Evoluzione in vitro di lipasi per aumentare l'enantioselettività dell'enzima

Il mutante ottenuto alla 5 generazione presenta la mutazione S155F e 4 sostituzioni in glicina che rendono l'enzima più flessibile, modificando alcune interazioni nel sito catalitico e nel sito dell'ossianione

Figure 4

Creation of an enantioselective lipase by directed evolution. The lipase gene from *P. aeruginosa* was subjected to random mutagenesis by ep-PCR. Mutant proteins were identified by UV/Vis spectrophotometry using 2-methyldecanoate *p*-nitrophenylester as the substrate, and mutations leading to improved enantioselectivity (white bars) were identified by DNA-sequencing. The mutations present in each generation are shown along the x-axis in single letter code for amino acids. Subsequent saturation mutagenesis at previously identified amino acid positions lead to a further increase in enantioselectivity for mutant S155F (shaded bar), which proved to be superior over S155L previously identified in the second generation which was generated by ep-PCR. This improvement is highlighted on the x-axis using grey text.



Mutagenesi sito-specifica del sito attivo della lipasi di *Yarrowia lipolitica* per modificare l'enantioselettività dell'enzima

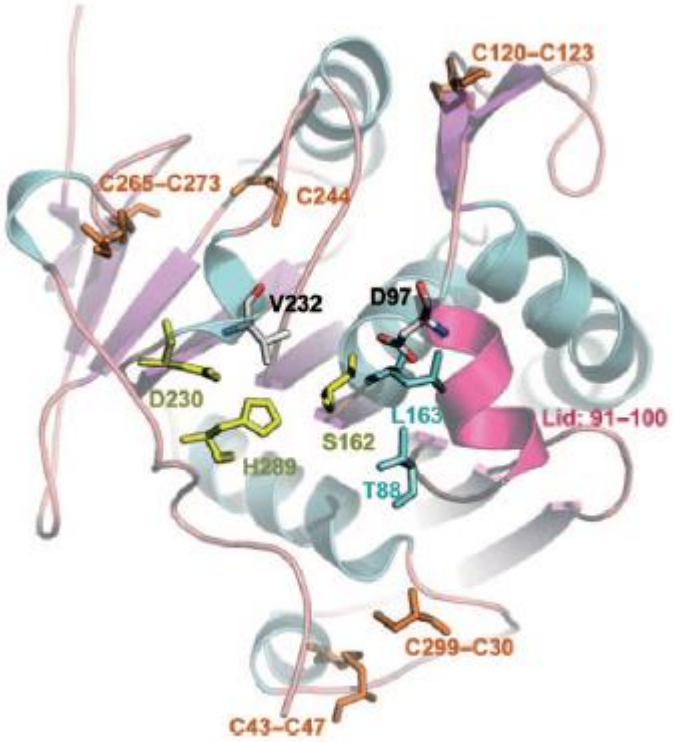


Figure 3. Overall representation of the Lip2p homology model. Hydrogen atoms on amino acid residues have been omitted for clarity purpose.

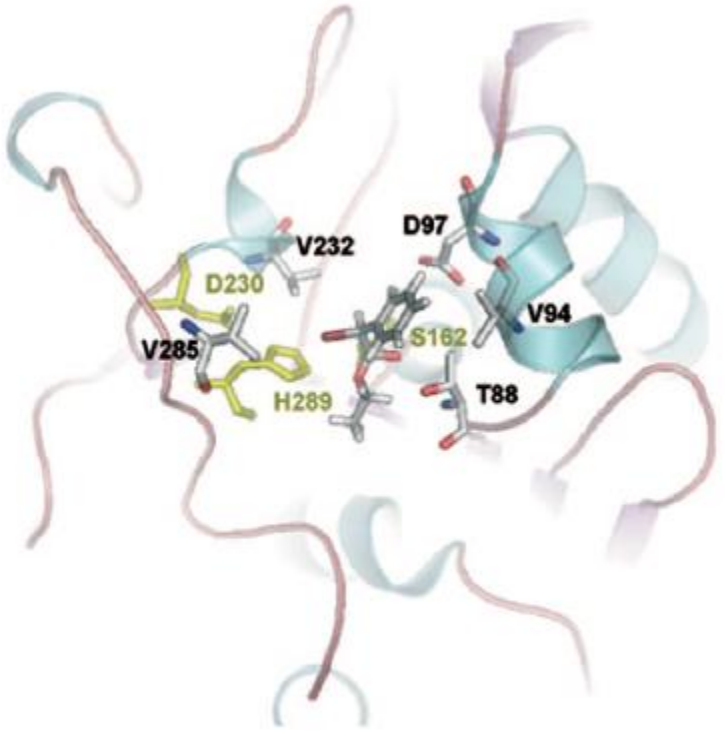


Figure 5. Representation of Lip2p amino acid residues selected for site-directed mutagenesis. The S-2-bromo-phenylacetic acid ethyl ester covalently bound to Ser162 is shown in the active site.

Mutagenesi sito-specifica di lipasi per modificare l'enantioselettività dell'enzima

Table 1. *p*-Nitrophenol butyrate hydrolysis activity of wild-type Lip2p and its variants.

Enzyme	WT	T88S	T88X[c]	V94A	V94L	V285A	V285L	V232A	V232L	D97A
Initial rate[a,b]	64.0	21.3	0	52.8	42.5	46.0	45.6	47.4	40.3	9.8

[a] µmol of *p*NP liberated per minute and mL of enzyme. [b] Each experiment was carried out in triplicate. [c] X = A, V, L.

Table 2. 2-bromo-phenylacetic acid ethyl ester hydrolysis activity of wild-type Lip2p and its variants.

Enzyme	WT	T88S	V94A	V94L	V285A	V285L	V232A	V232L	D97A
viS[a]	1.71	2.13	1.41	1	0.97	1.3	8.8	0.017	0.010
viR[a]	0.58	1.04	0.39	0.44	0.4	0.4	0.101	0.31	0.34
E value[b]	3(*S*)	2(*S*)	4(*S*)	2(*S*)	2(*S*)	3(*S*)	87(*S*)	18(*R*)	34(*R*)
conversion [%]	54.7 (8 h)						52.9 (8.5 h)		
ee_s[c] [%]	53.5						99.6		
ee_p[d] [%]	43.7						88.7		

[a] µmol of 2-bromo-phenylacetic acid liberated per hour and mL of enzyme. [b] *E* value = viS/viR or viR/viS according to enantiomer preference; viR, viS: initial rates. [c] Substrate enantiomeric excess. [d] Product enantiomeric excess.

Substrato: esteri dell'acido 2-bromo-fenilacetico, intermedi nella sintesi di farmaci

Mutagenesi sito-specifica di lipasi per modificare l'enantioselettività dell'enzima

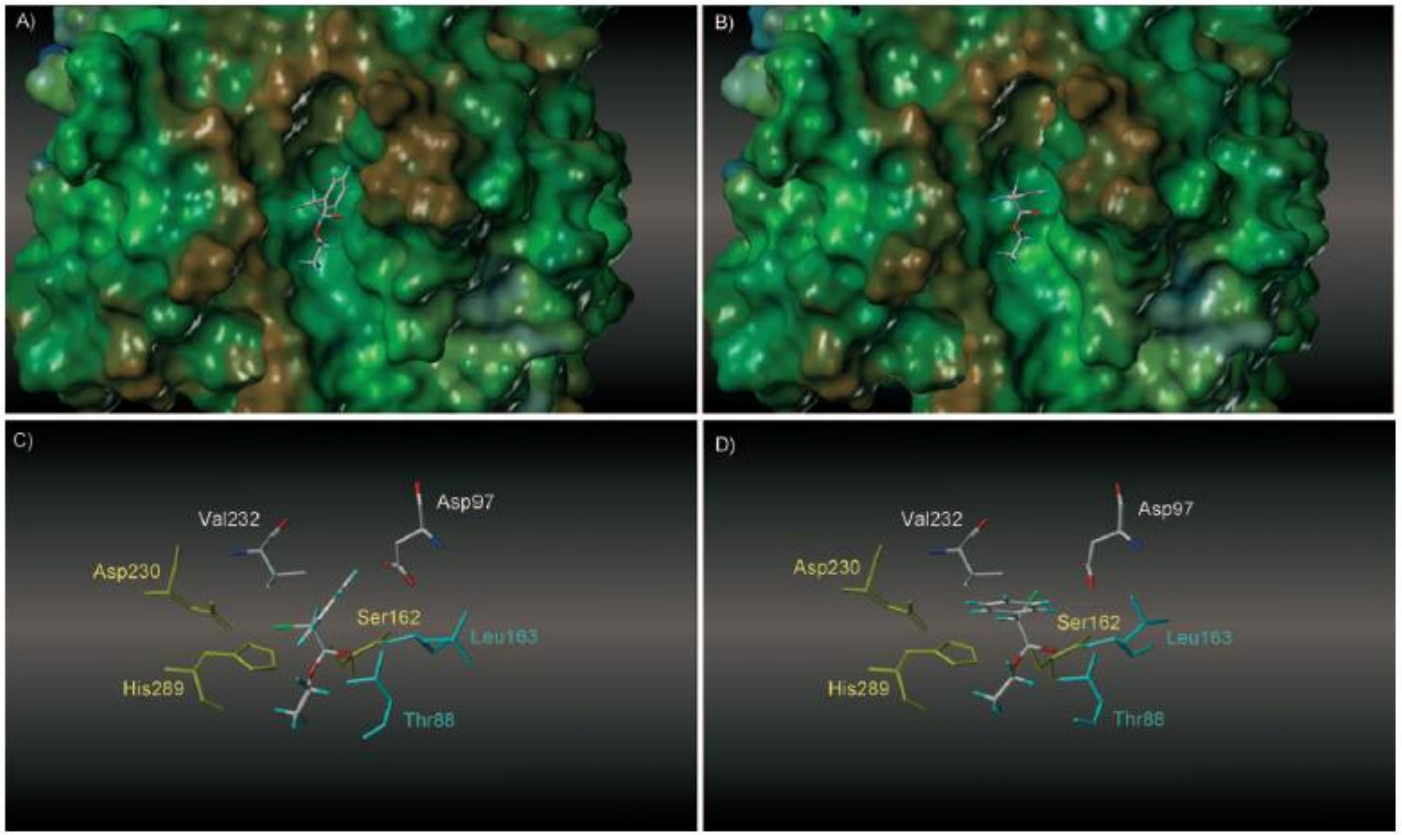


Figure 6. Representation of (*R,S*)-2-bromo-phenylacetic acid ethyl ester enantiomers covalently bound to catalytic Ser162 of Lip2p. A), B) *S* and *R* enantiomers are respectively shown. Lip2p is shown as a Connolly surface mapped with the lipophilic potential, as calculated by the MOLCAD module implemented in SybyL7.3 (Tripos, Saint Louis, USA). The protein surface is colour-coded (brown colour indicates more lipophilic regions whereas blue codes for more polar ones). C), D) Arrangement of the *S* (left) and *R* (right) enantiomers with respect to the catalytic triad (coloured in yellow) as well as the residues forming the oxyanion hole (cyan coloured) and the two key positions (V232 and D97) playing a role on enantio-discrimination.

Mutagenesi sito-specifica di lipasi per modificare l'enantioselettività dell'enzima

Le dimensioni del residuo in posizione 232 determinano la preferenza della lipasi per un enantiomero rispetto all'altro.
Aminoacidi piccoli: S
Aminoacidi grandi: R

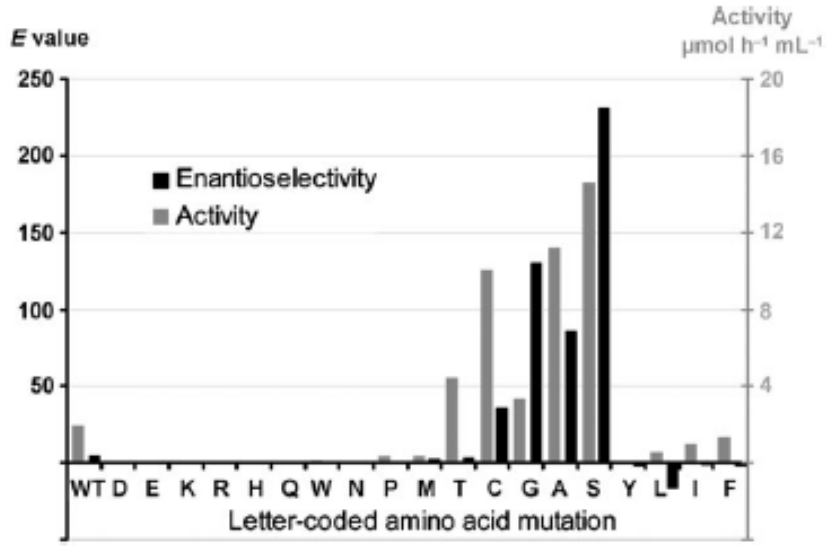
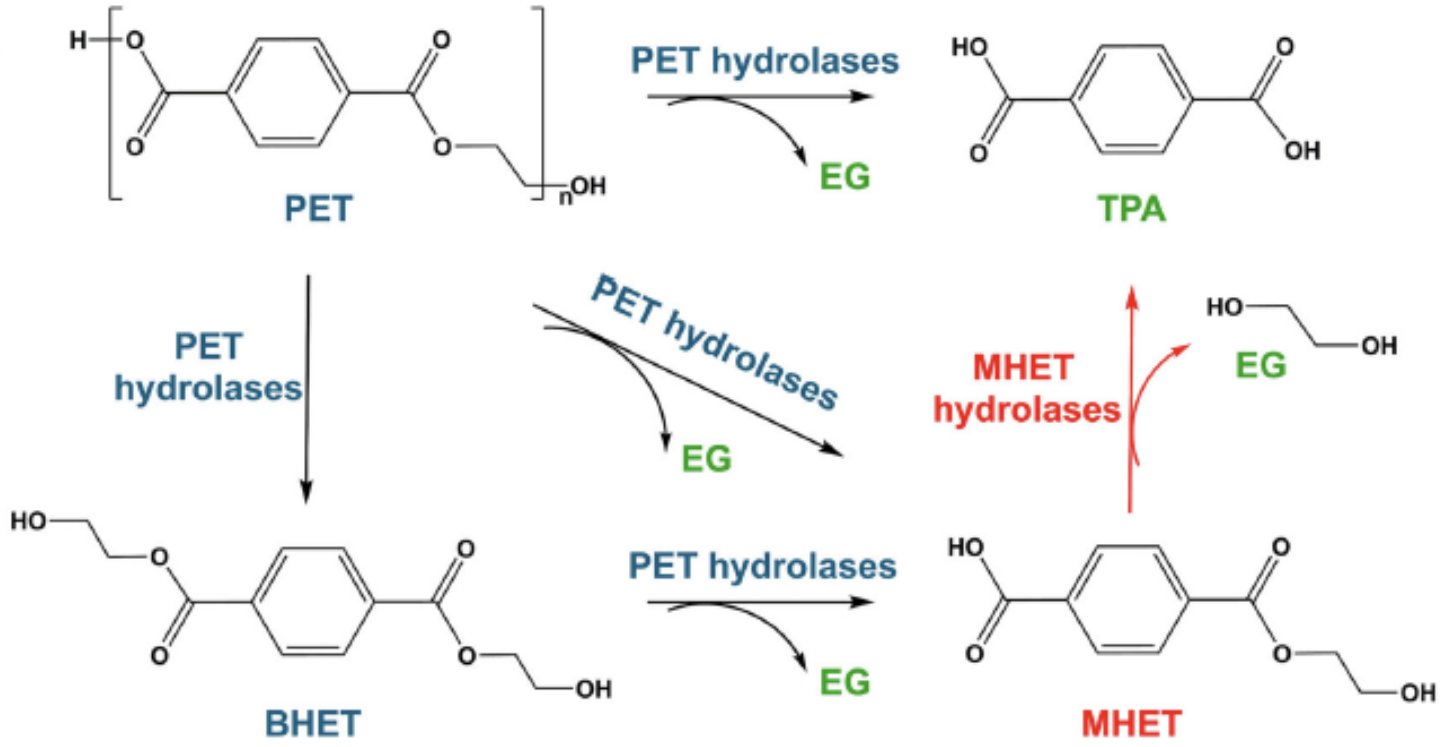
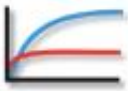


Figure 7. Activity and enantioselectivity of V232 variants in 2-bromo-phenyl-acetic octyl ester racemate hydrolysis reaction. WT: wild-type Lip2p. A positive *E* value corresponds to *S* selectivity, a negative *E* value to *R* selectivity.

Biodegradazione del PET



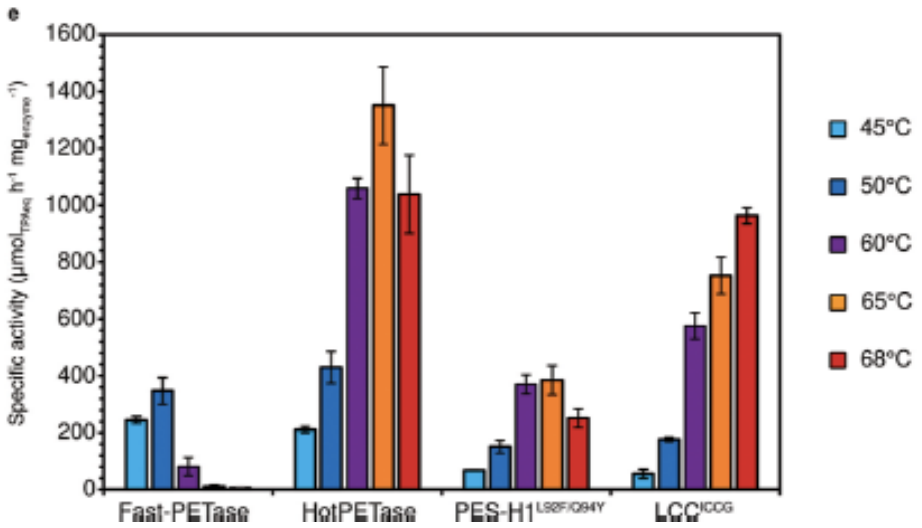
PET: polyethylene terephthalate
TPA: terephthalic acid
EG: ethylene glycol

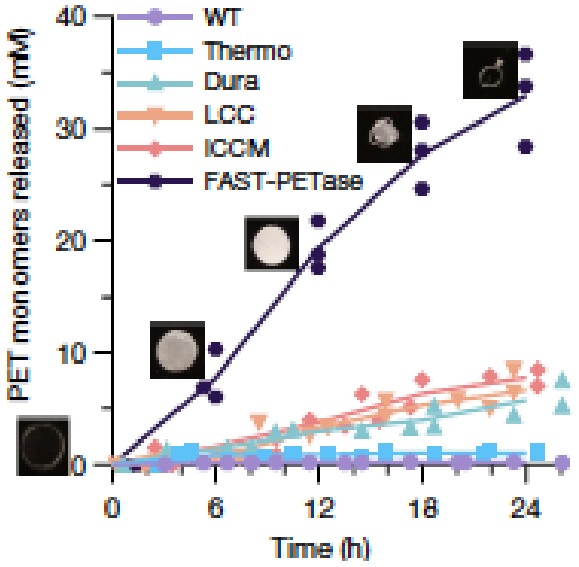
	Parameter	Critical constraint	Proposed solutions
	**PET crystallinity**	Recalcitrance of highly crystalline PET regions to enzymatic hydrolysis.	Use of amorphized PET samples (Goodfellow PET film and pre-treated post-consumer waste amorphized by extrusion).
	**Surface of exchange**	Low enzyme performance with large PET substrate pieces due to limited reaction surface.	Micronization (< 500 µm) of substrate to increase the reaction surface and to enable high efficiency of the depolymerization.
	**Temperature**	At temperatures largely below T_g of PET, the availability of productive substrate conformations (dihedral angles around the ester bond) is limited.	According to Arrhenius' law and substrate peculiarities, maximal enzyme performance is reached when PET depolymerization is performed at temperatures close to the T_g of PET. Enzyme thermostability must be high enough.
	**Enzyme efficiency *vs* PET crystallization**	Re-crystallization phenomena of PET when incubated at temperatures close or higher than the T_g.	A highly efficient enzyme is required to overcome the kinetic of PET re-crystallization.
	**PET concentration**	Commonly used concentrations of PET are far below the requirement for a high-productivity process.	Initial PET waste load to maximize the productivity per batch without loss of TPA needs to be assessed according to running operation conditions.
	**Yield of depolymerization**	Initial rate of the reaction is not the only parameter to consider. Enzyme activity deteriorates during the reaction either due to instability or product inhibition.	Enzyme efficiency should allow near 100% PET conversion to maximize productivity per batch. Enzyme stability in highly concentrated PET solution upon completion of depolymerization is mandatory.
	**Composition of the products**	Yield and productivity of an enzyme-based PET depolymerization process is negatively affected by the presence of MHET or soluble oligomers that might be lost during the purification scheme.	Final product composition should be thoroughly determined (e.g., by HPLC) to assess the purity of the products (e.g. TPA and MEG) of the PET depolymerization. Enzyme performance must ensure a complete hydrolysis of PET into TPA and MEG.
	**Availability of the enzyme**	Instability, aggregation and low expression rates of soluble protein may limit an upscaling of the reaction to reach industrial scales.	The availability of the enzyme in large quantities has to be explored early on. Industrial expression hosts are required to obtain sufficient enzyme quantities for large scale reactions

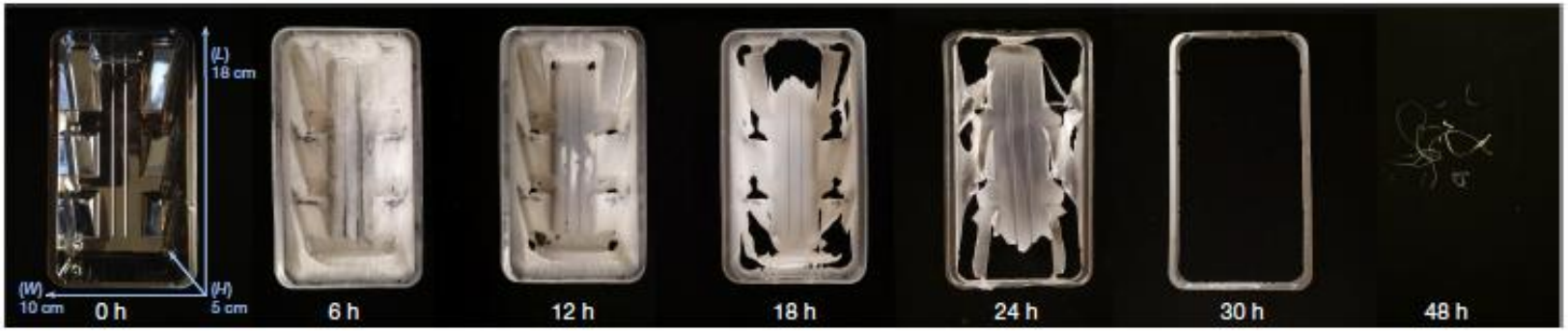
Ottimizzazione di PETasi

- LCC^{ICCG} da leaf-branch compost cutinase
  - mutagenesi sito-specifica di residui che legano il substrato e introduzione di un ponte disolfuro (F243I/D238C/S283C/Y127G -> ICCG)
- FAST (functional, active, stable, tolerant)-PETase da *Ideonella sakaiensis*
  - machine-learning (mutazioni N233K/R224Q/S121E from prediction e D186H/R280A from scaffold)
- HotPETase da *Ideonella sakaiensis*
  - evoluzione in vitro (a partire da S121E/D186H/R280A/ N233C, S282C introdotte altre 16 mutazioni)
- PES-H1 da libreria metagenomica da compost
  - L92F/F94Y

Confronto PETasi per la capacità di degradare PET a TPA ed EG







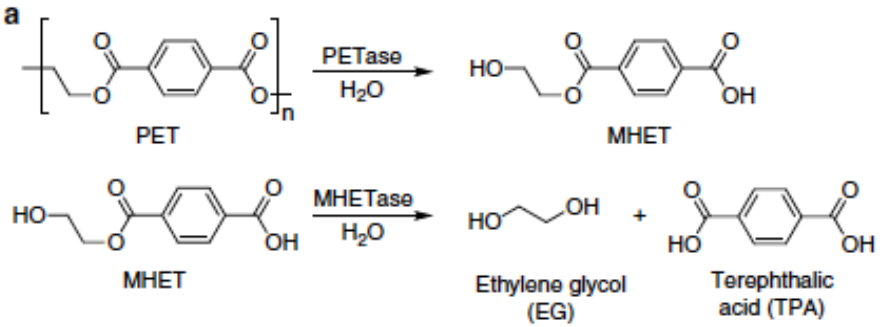
nature COMMUNICATIONS

ARTICLE

https://doi.org/10.1038/s41467-019-09326-3 OPEN

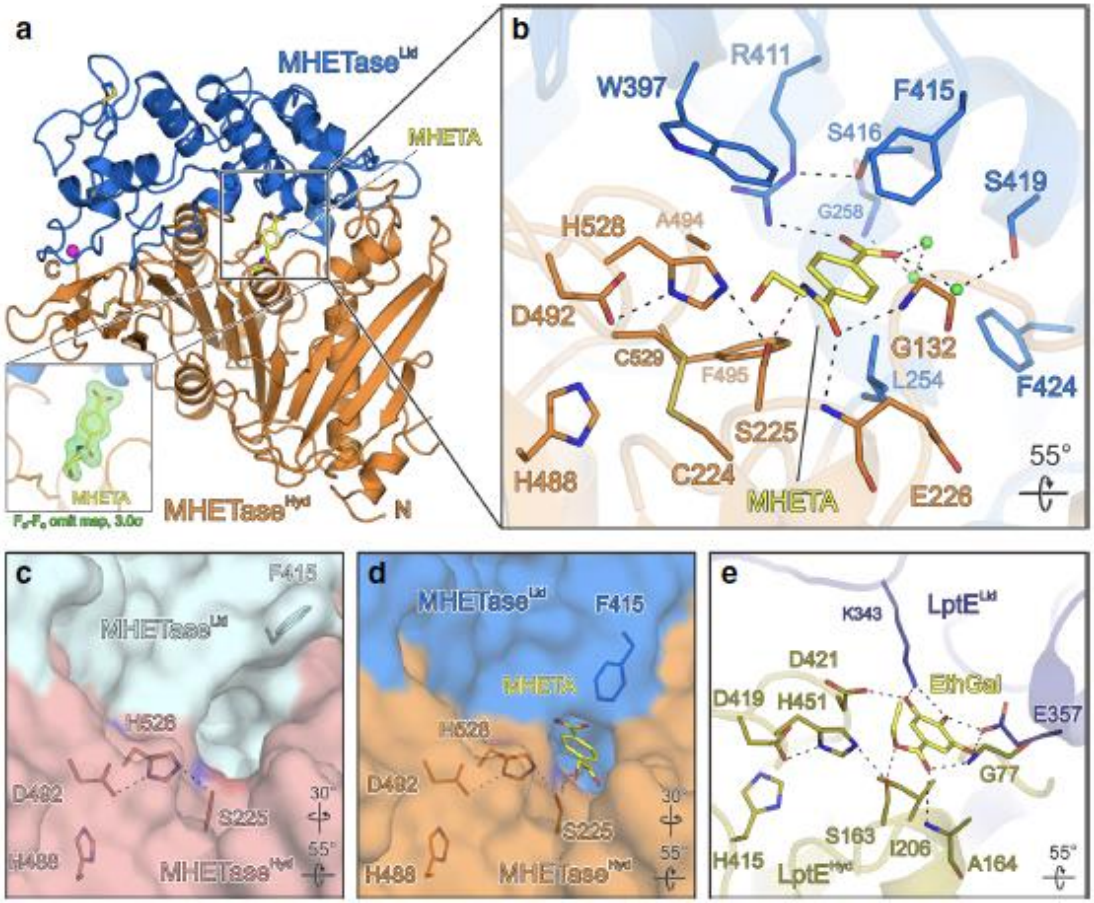
Structure of the plastic-degrading *Ideonella sakaiensis* MHETase bound to a substrate

Gottfried J. Palm[1], Lukas Reisky[2], Dominique Böttcher[2], Henrik Müller[2], Emil A.P. Michels[1], Miriam C. Walczak[2], Leona Berndt[1], Manfred S. Weiss[3], Uwe T. Bornscheuer[2] & Gert Weber[1,4]



La MHETasi appartiene alla famiglia delle α/β idrolasi

Dominio Hyd catalitico
Dominio Lid cambia accessibilità del sito attivo quando si lega il substrato

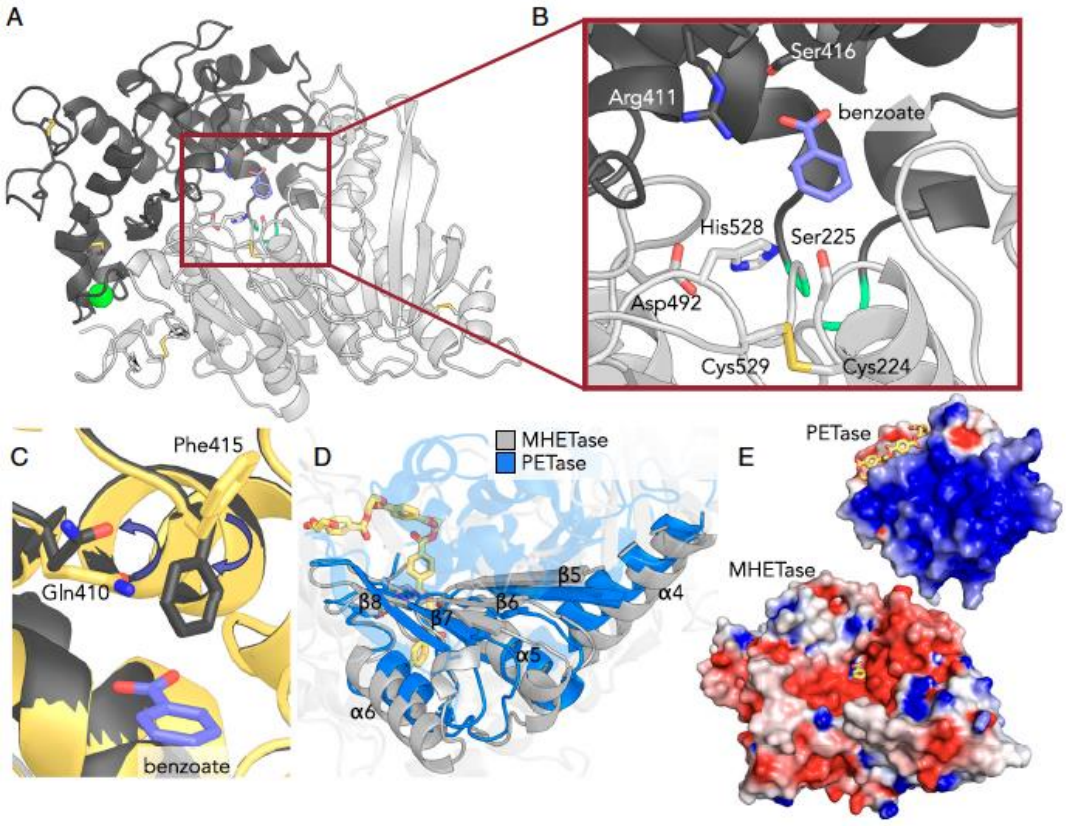


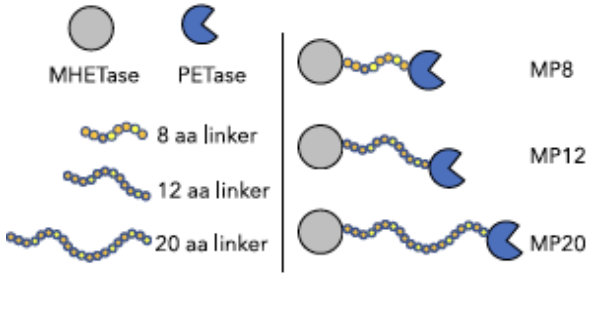
Characterization and engineering of a two-enzyme system for plastics depolymerization

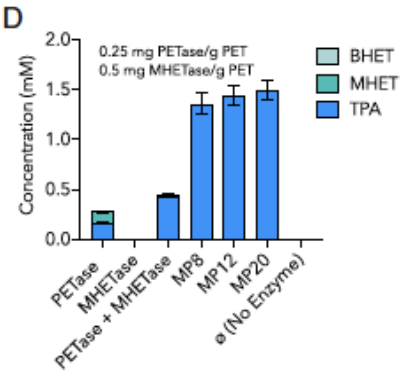
2020

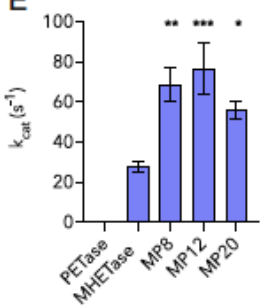
Brandon C. Knott[a,1], Erika Erickson[a,1], Mark D. Allen[b,1], Japheth E. Gado[a,c,1], Rosie Graham[b], Fiona L. Kearns[d], Isabel Pardo[a], Ece Topuzlu[a,e], Jared J. Anderson[a], Harry P. Austin[b], Graham Dominick[a], Christopher W. Johnson[a], Nicholas A. Rorrer[a], Caralyn J. Szostkiewicz[a], Valérie Copié[e], Christina M. Payne[c], H. Lee Woodcock[d], Bryon S. Donohoe[f], Gregg T. Beckham[a,2], and John E. McGeehan[b,2]

Proteina di fusione MHETase-PETase









communications biology

2023

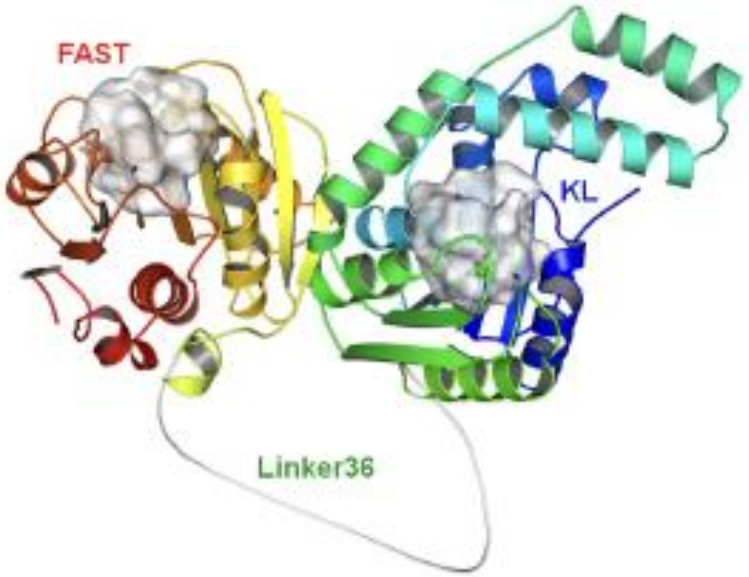
ARTICLE

https://doi.org/10.1038/s42003-023-05523-5 OPEN

Computational design of highly efficient thermostable MHET hydrolases and dual enzyme system for PET recycling

Jun Zhang[1,2,5], Hongzhao Wang[1,5], Zhaorong Luo[3,5], Zhenwu Yang[1], Zixuan Zhang[1], Pengyu Wang[2], Mengyu Li[1], Yi Zhang[3], Yue Feng[3], Diannan Lu[2✉] & Yushan Zhu[1,4✉]

Recently developed enzymes for the depolymerization of polyethylene terephthalate (PET) such as FAST-PETase and LCC-ICCG are inhibited by the intermediate PET product mono(2-hydroxyethyl) terephthalate (MHET). Consequently, the conversion of PET enzymatically into its constituent monomers terephthalic acid (TPA) and ethylene glycol (EG) is inefficient. In this study, a protein scaffold (1TQH) corresponding to a thermophilic carboxylesterase (Est30) was selected from the structural database and redesigned in silico. Among designs, a double variant KL-MHETase (I171K/G130L) with a similar protein melting temperature (67.58 °C) to that of the PET hydrolase FAST-PETase (67.80 °C) exhibited a 67-fold higher activity for MHET hydrolysis than FAST-PETase. A fused dual enzyme system comprising KL-MHETase and FAST-PETase exhibited a 2.6-fold faster PET depolymerization rate than FAST-PETase alone. Synergy increased the yield of TPA by 1.64 fold, and its purity in the released aromatic products reached 99.5%. In large reaction systems with 100 g/L substrate concentrations, the dual enzyme system KL36F achieved over 90% PET depolymerization into monomers, demonstrating its potential applicability in the industrial recycling of PET plastics. Therefore, a dual enzyme system can greatly reduce the reaction and separation cost for sustainable enzymatic PET recycling.



ACS Synthetic Biology 2025

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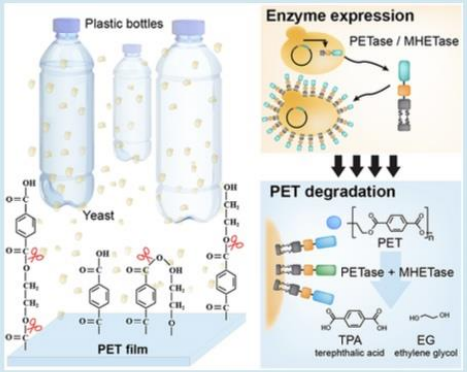
Research Article

Engineered Yeasts Displaying PETase and MHETase as Whole-Cell Biocatalysts for the Degradation of Polyethylene Terephthalate (PET)

Caiping Jiang, Kairui Zhai, R. Clay Wright, and Juhong Chen*

Cite This: ACS Synth. Biol. 2025, 14, 2810−2820
Read Online

ABSTRACT: Due to its low cost of manufacturing, poly(ethylene terephthalate) (PET, a polyester plastic) has been the most widely used plastic material for food packaging. However, PET is nonbiodegradable. It can take years to degrade when it is discarded into the environment. In recent years, plastic pollution has received much attention and has become a major environmental issue. In this study, we engineered yeast surfaces to display two PET-degrading enzymes (PETase and MHETase) to degrade PET plastics. The enzymes displayed on the yeast surface were characterized by using confocal microscopy and flow cytometry. The reaction conditions to degrade PET plastics using the engineered yeasts were optimal at pH 9 and 30 °C. In addition, the engineered yeasts showed great stability and reusability to degrade PET films. Furthermore, we demonstrated that the engineered yeasts as whole-cell catalysts can be used to degrade drinking water bottles into value-added products. This study provides a novel whole-cell biocatalyst using engineered yeasts to degrade plastic waste, offering a new strategy to solve plastic pollution and recycling challenges.



KEYWORDS: *polyethylene terephthalate (PET), plastic waste, PETase, MHETase, whole-cell biocatalyst, yeast*