Biotechnological applications of enzymes: lipases

Lipases

- Catalyze hydrolysis and synthesis of acyl-glycerols
- Are stable in organic solvents
- Do not require cofactors
- Have low substrate specificity
- Have high **enantioselectivity**

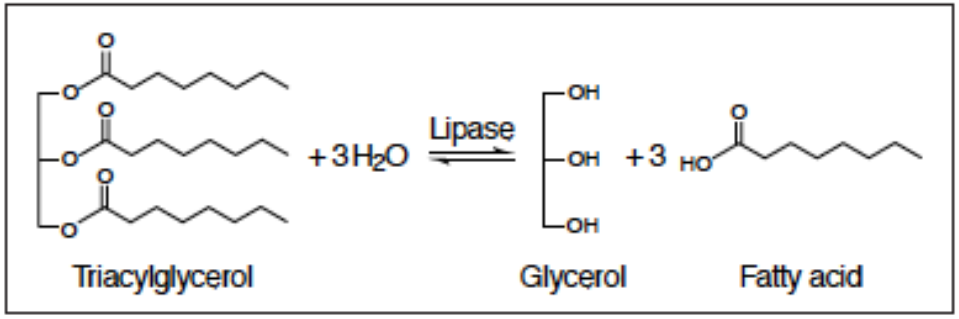


Figure 1

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.

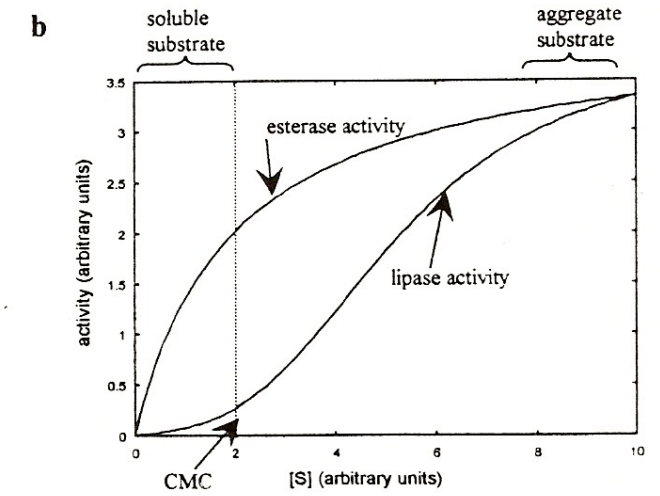


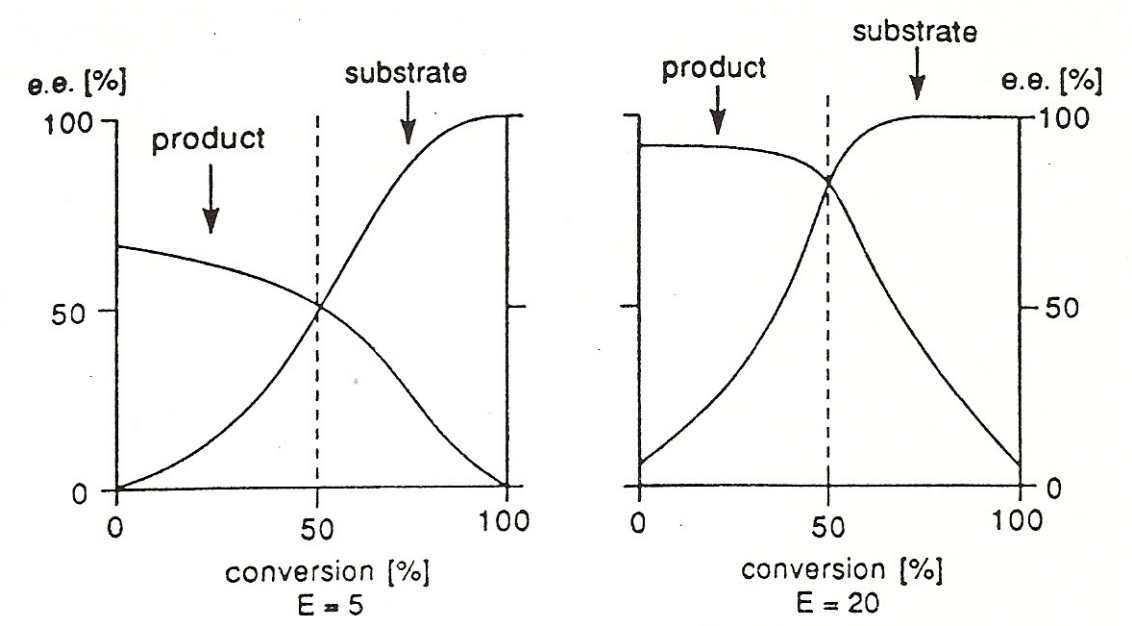
Figure 1 (a) The mechanism of action of lipases by interfacial activation at the oil–water interface. S is the substrate and P is the product. (b) The activity of esterases and lipases in aqueous solution. [S] is the substrate concentration and CMC is the critical micellar concentration of the substrate

Advantages of organic solvents

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.

Evaluation of the chiral purity of products and substrates in a reaction catalysed by an enantioselective enzyme



Enzyme with E = 5

Enzyme with E = 20

Enantiomeric ratio $E = (Vmax/Km)_{fast}/(Vmax/Km)_{slow}$

Structure of *Burkholderia cepacia* lipase

Lipases catalyze the hydrolysis of esters with a mechanism similar to serine proteases.

Lipase substrates are generally poorly soluble in water.

Catalytic activity is enhanced at the water/lipid interface.

Most industrial lipases are derived from microorganisms.

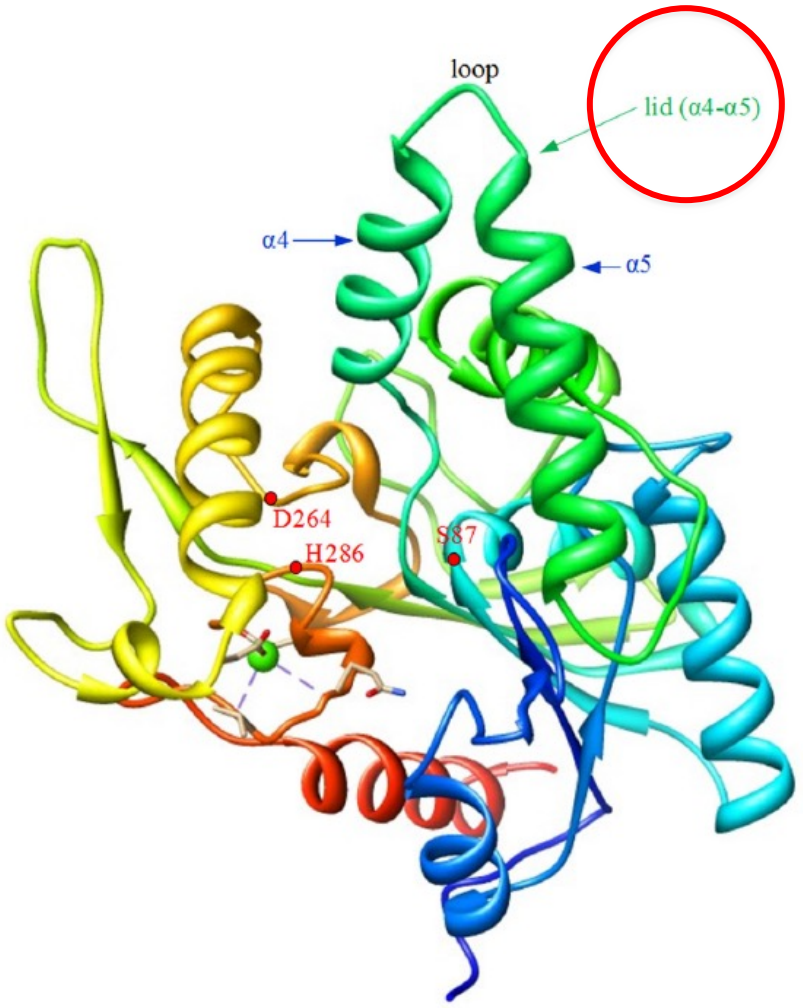


Figure 2. The structure of *Burkholderia cepacia* lipase: The lid region 118–159 (α4-loop-α5) and the catalytic triad (S87, D264, and H286) by red spheres are shown (Schrag et al., 1997).

Reaction mechanism of lipase

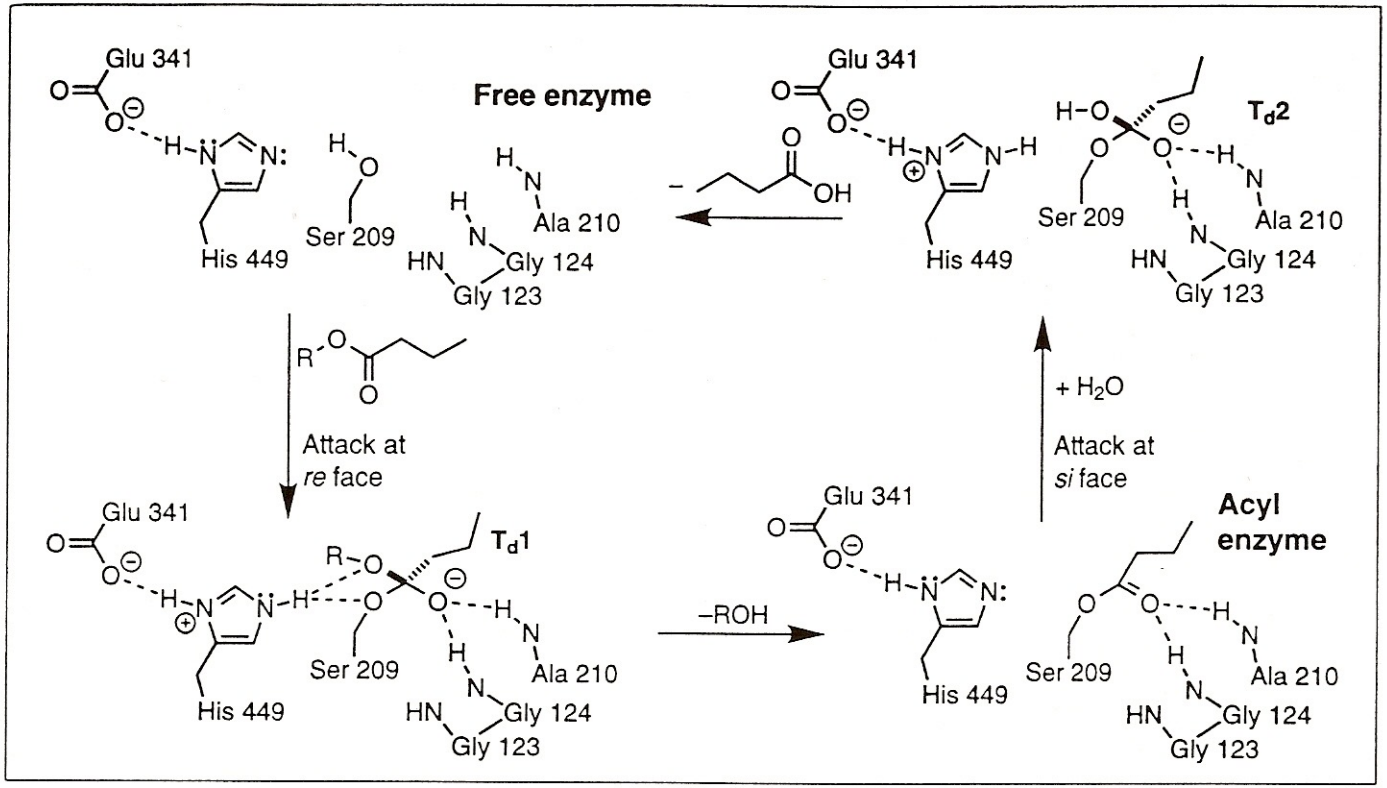


Figure 3

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).

Molecular basis of enantioselectivity of *Candida rugosa* lipase

Preference of *C. rugosa* lipase for the R isomer of menthol is due to formation of a hydrogen bond between His449 and the substrate. This bond can not form with the S isomer.

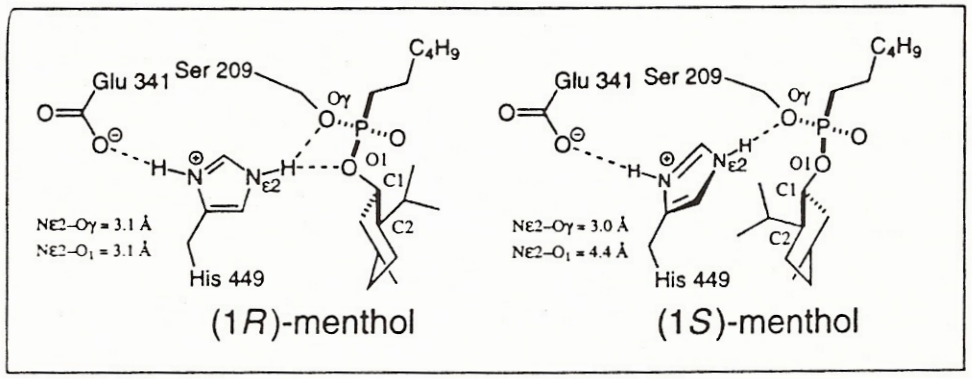


Figure 5

Different interactions between CRL and transition-state analogs containing enantiomeric menthyl groups. For the fast-reacting enantiomer, 1*R*, Nε2 of the catalytic His forms a hydrogen bond to both O_γ of Ser209, which must be deprotonated during the formation of the tetrahedral intermediate, and to O_γ of menthol, which must be protonated during the collapse of tetrahedral intermediate. The slow-reacting enantiomer, 1*S*, distorts the orientation of His so that Nε2 forms a hydrogen bond only to O_γ of Ser209.

Crystal structure obtained in the presence of a transition-state analogue

Reactions catalyzed by lipase: hydrolysis and synthesis (transesterification)

(i) *Hydrolysis*

$$\mathrm{RCOOR' + H_2O \longrightarrow RCOOH + R'OH} \qquad (1)$$

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

(a) *Esterification*

$$\mathrm{RCOOH + R'OH \longrightarrow RCOOR' + H_2O} \qquad (2)$$

(b) *Interesterification*

$$\mathrm{RCOOR' + R''COOR^* \longrightarrow RCOOR^* + R''COOR'} \qquad (3)$$

(c) *Alcoholysis*

$$\mathrm{RCOOR' + R''OH \longrightarrow RCOOR'' + R'OH} \qquad (4)$$

(d) *Acidolysis*

$$\mathrm{RCOOR' + R''COOH \longrightarrow R''COOR' + RCOOH} \qquad (5)$$

Reactions catalyzed by lipase: chiral substrates and products

Hydrolysis of esters in water

Chiral acid

$$(\pm)\text{-}R_1{}^*COOR_2 + H_2O \xrightarrow{\text{enzyme}} (R_1{}^*COOH + R_1{}^*COOR_2) + R_2OH \quad (1a)$$

racemic ester — optically active products

Chiral alcohol

$$(\pm)\text{-}R_1COOR_2{}^* + H_2O \xrightarrow{\text{enzyme}} (R_2{}^*OH + R_1COOR_2{}^*) + R_1COOH \quad (1b)$$

racemic ester — optically active products

Acylation of alcohols in organic solvents[24]

Chiral acid

$$(\pm)\text{-}R_1{}^*COOR_2 + R_3OH \xrightarrow{\text{enzyme}} (R_1{}^*COOR_3 + R_1{}^*COOR_2) + R_2OH \quad (2a)$$

racemic ester — optically active products

where R_3OH is a primary alcohol.

Chiral alcohol

$$(\pm)\text{-}R_3{}^*OH + R_1COOR_2 \xrightarrow{\text{enzyme}} (R_1COOR_3{}^* + R^*{}_3OH) + R_1COOH \quad (2b)$$

racemic alcohol — optically active products

To make the transesterification reaction irreversible, esters that give rise to products that are no longer a substrate for lipase are used as acyl donors.

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

$HOCH{=}CH_2$ is unstable and decays to CH_3CHO

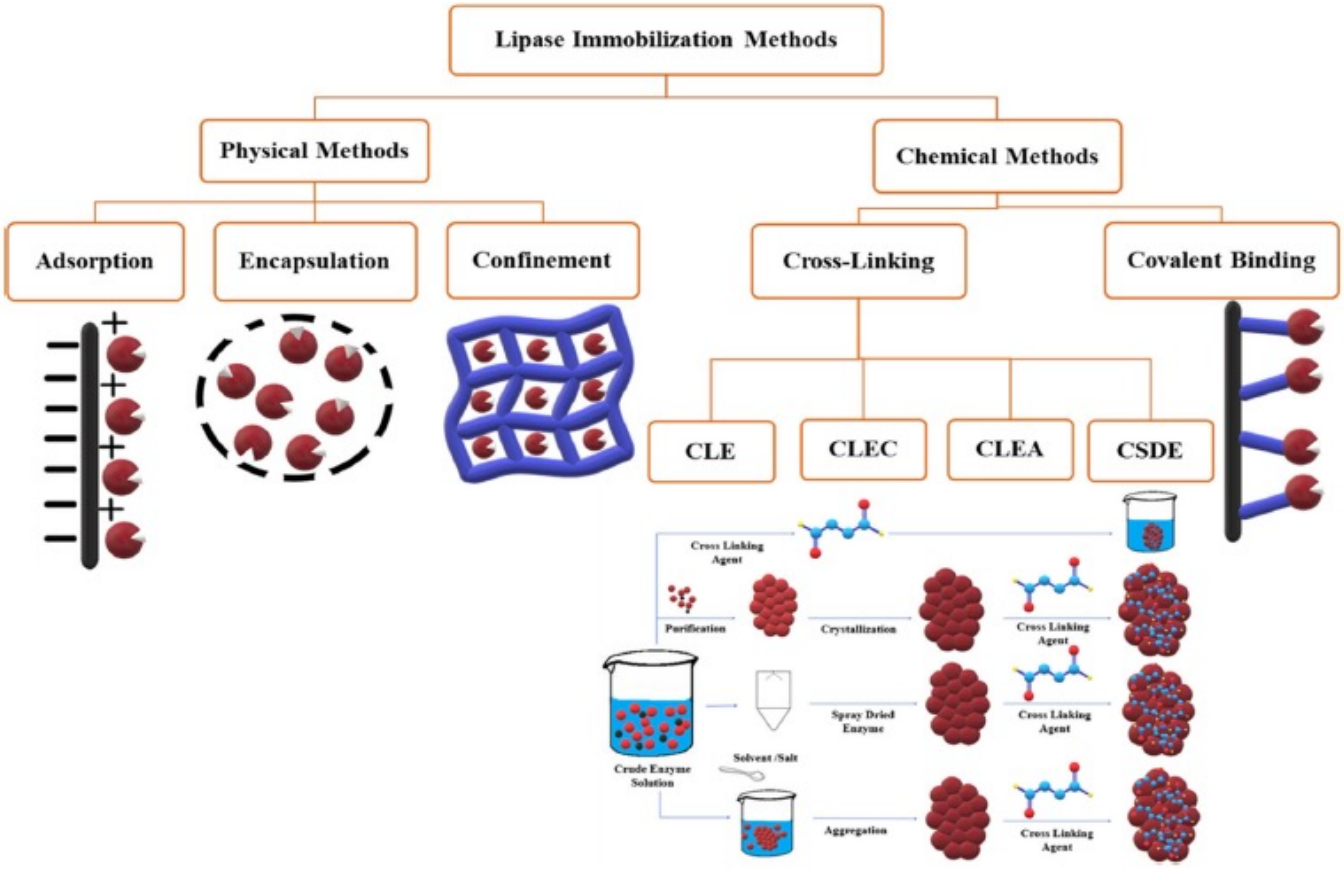
Biotechnological applications of lipases

Hydrolysis reactions
- detergent additives

Hydrolysis/transesterification reactions
- production of food ingredients
- production of cosmetics
- production of biodiesel
- production of drugs

Kinetic resolution of racemic mixtures
- dynamic kinetic resolution of racemic mixtures to obtain complete conversion of the substrate

Lipase immobilization techniques



Lipase immobilization techniques

- Cross-linked enzyme crystals (CLEC) with glutaraldehyde. CLECs are insoluble, stable in aqueous and organic solvents, highly porous, allow substrate diffusion and can be easily recovered at the end of the reaction.
- Cross-linked enzyme aggregates (CLEA) with glutaraldehyde.
- Entrapment in silica gel modified with alkyl groups $CH_3Si(OCH_3)_3$ and $Si(OCH_3)_4$ to create a hydrophobic microenvironment and retain catalytic activity
- Immobilization on activated silica nanoparticles

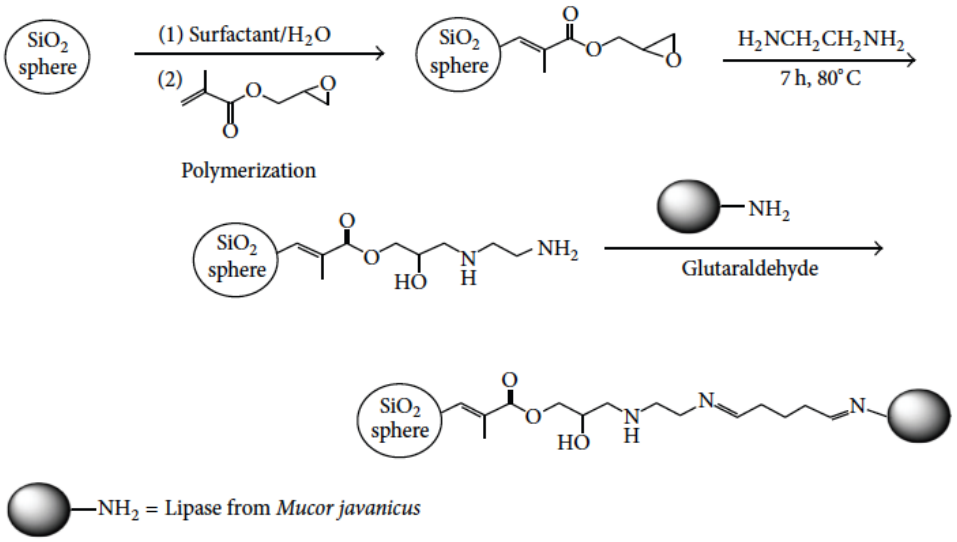


FIGURE 3: Lipase immobilization on silica nanoparticle.

Lipase immobilization techniques

- Immobilization on functionalized magnetic nanoparticles

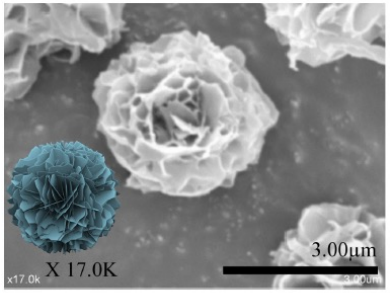


Fig. 7. Scanning electron micrographs of nanoflower.

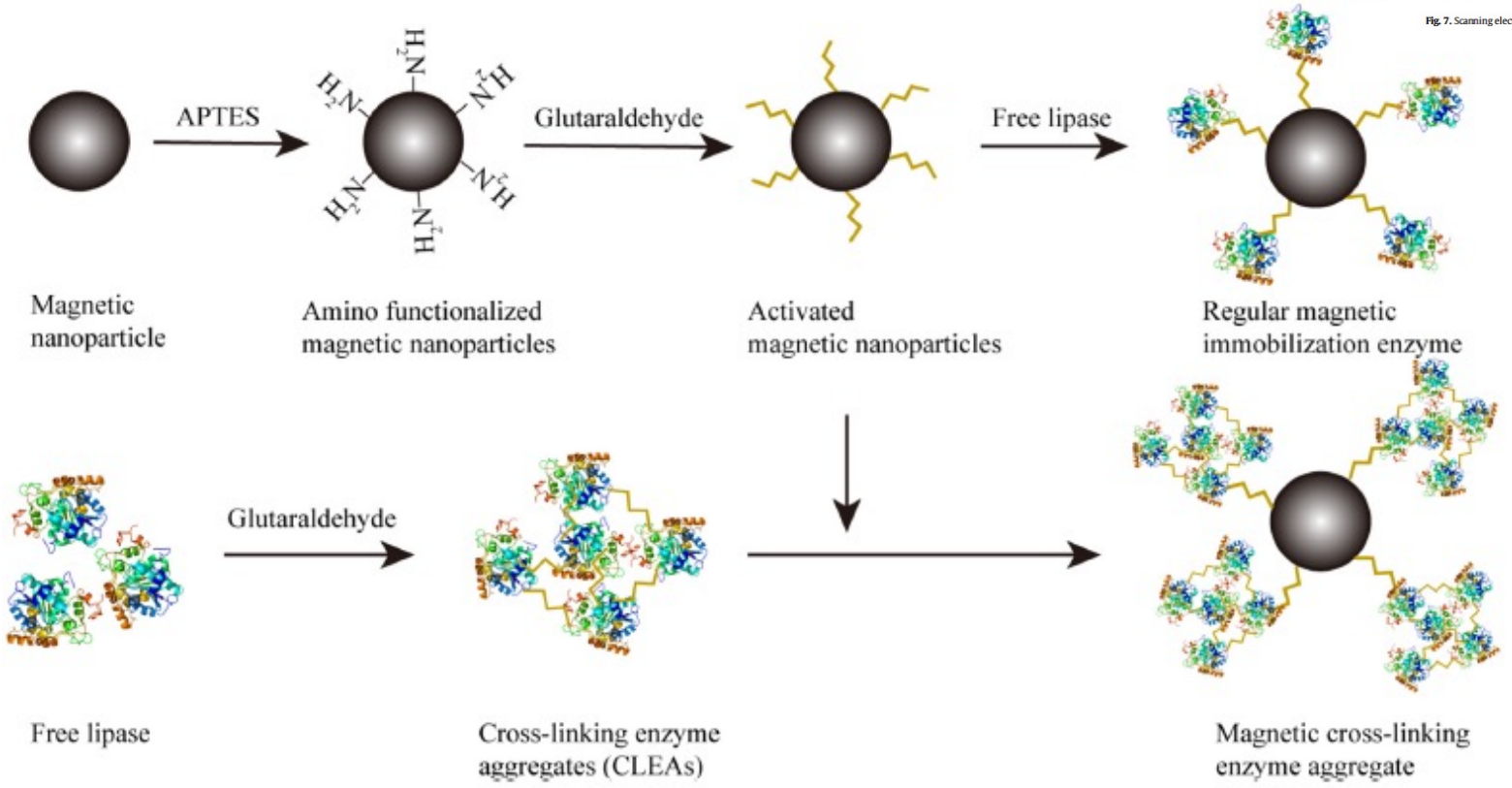


Fig. 2. Schematic representation of the synthesis of regular magnetic lipase and magnetic cross-linking enzyme aggregates.

Biotechnological applications of lipases

Hydrolysis reactions

detergent additives: low substrate specificity
stability to high T, proteases and chemical denaturation
pH optimum 10-11

Hydrolysis/transesterification reactions

production of food ingredients: poly-unsaturated fatty acids (PUFA)

production of cosmetics

production of biodiesel

production of drugs

Kinetic resolution of racemic mixtures

dynamic kinetic resolution of racemic mixtures to obtain complete conversion of the substrate ➔ drugs

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.

Enzymes encapsulated in arabic gum 3-6% and maltodextrin 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

Detergent A: free enzymes

B: encapsulated enzymes

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.

Polyunsaturated fatty acids

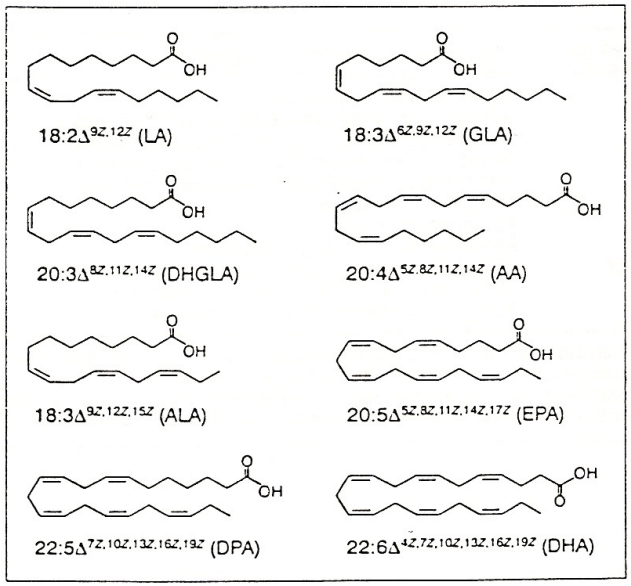
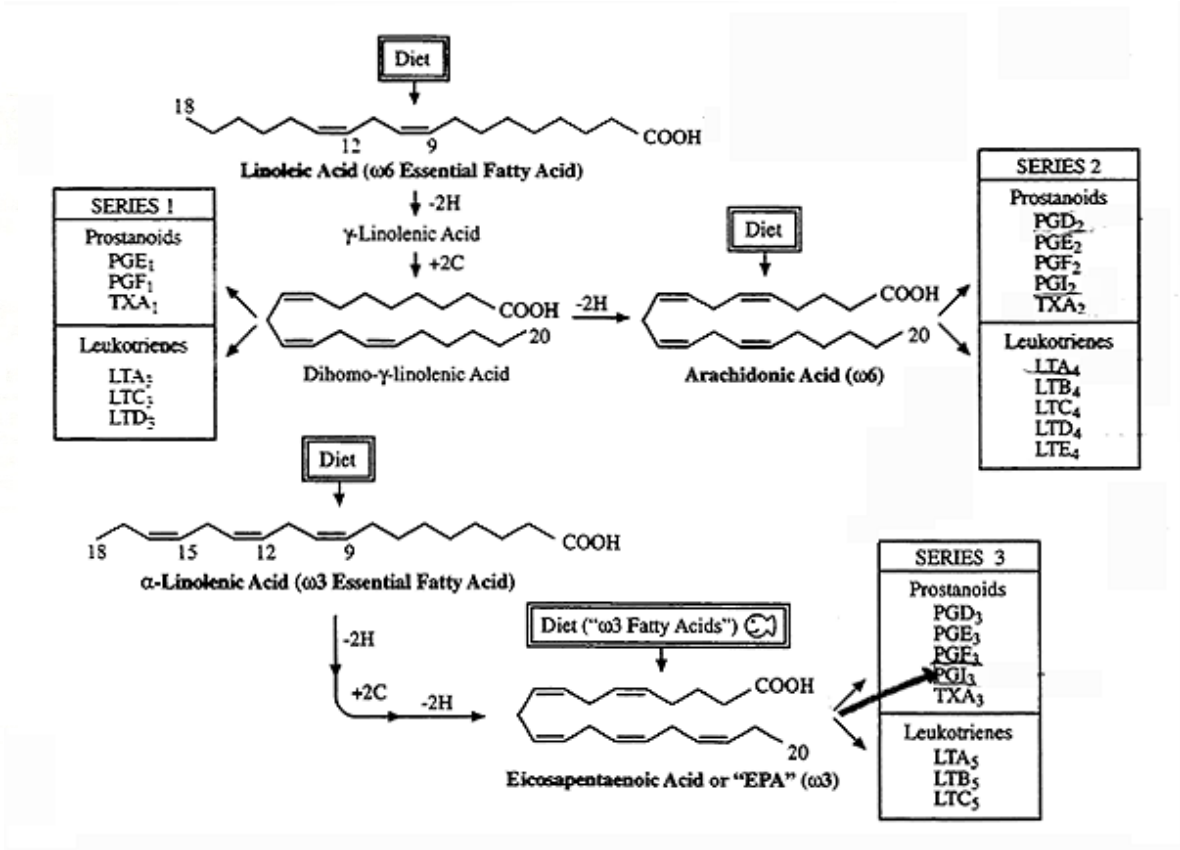
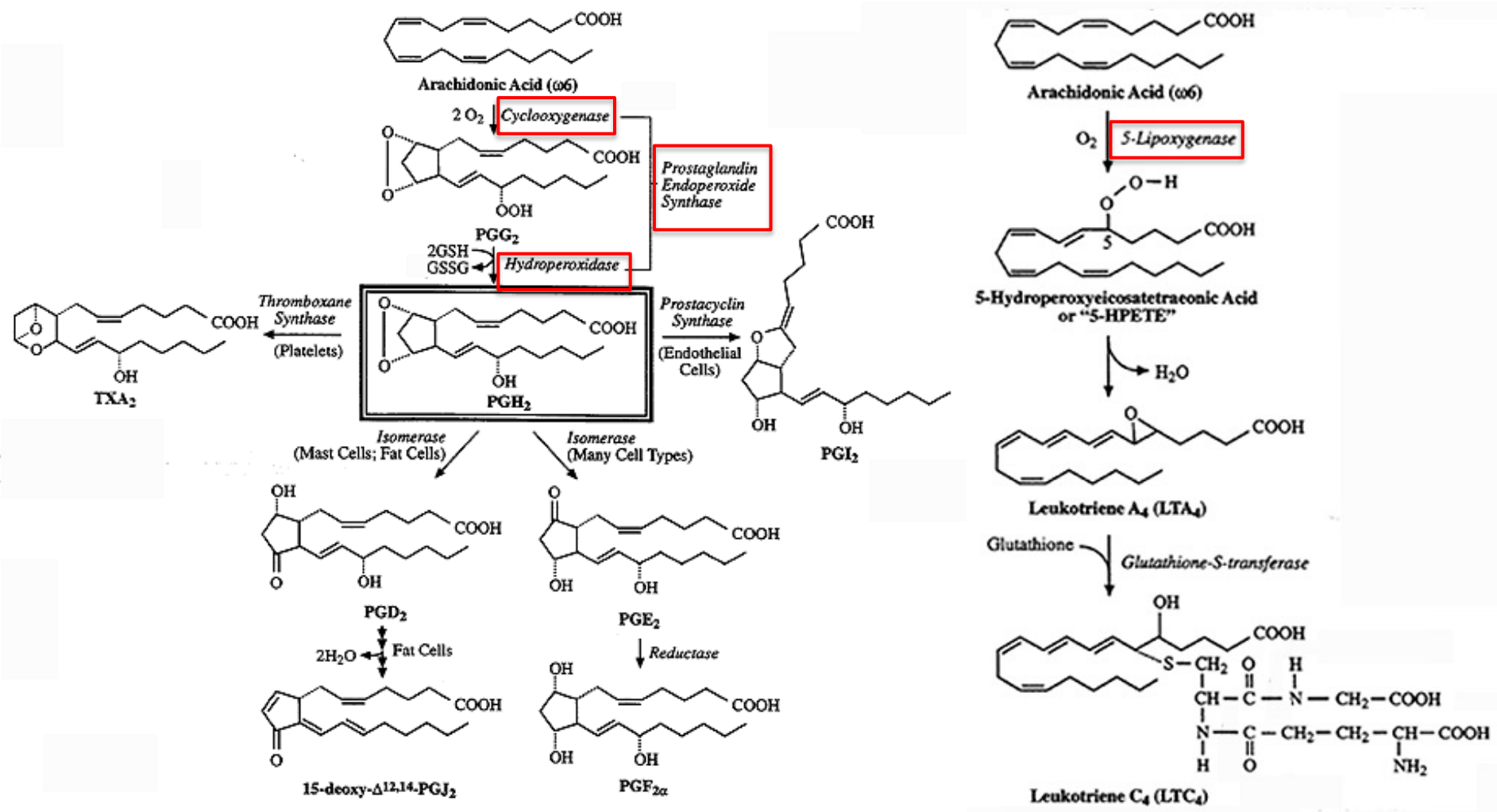


Figure 1
Representative examples of naturally occurring polyunsaturated fatty acids.

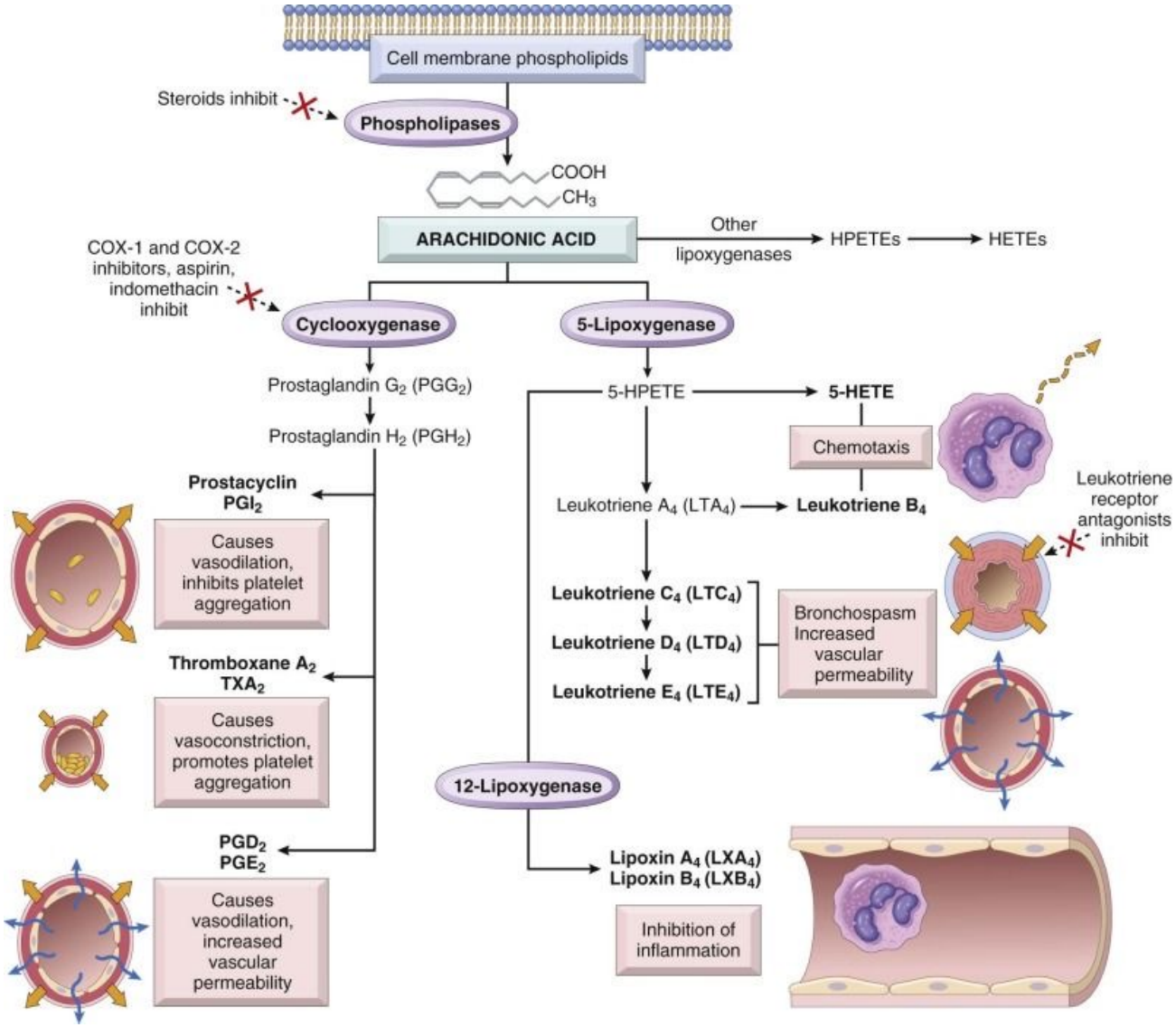


Linoleic acid (18:2) and linolenic acid (18:3) are essential.
They are precursors of eicosanoids arachidonic acid (20:4) and eicosapentaenoic acid (20:5).

Arachidonic acid is the precursor of prostaglandins, thromboxanes and leukotrienes



Biological role of arachidonic acid metabolites



Applications of lipase for production of polyunsaturated fatty acids (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.

Properties and applications fatty acid esters

Ester type	Application(s)
Carbohydrates fatty acid esters	Antitumorals [121], cosmetics [122], anticaries properties [123], and insecticidals [124]
Fatty acid esters of hydroxyl acids (lactic acid, citric acid, and alkyl lactates)	Surfactants in food industry [125] and cosmetics [126]
Flavonoids, a group of polyphenolic compounds, found ubiquitously in fruits and vegetables	Broader application like dietetic, nutritional, pharmacological/cosmetic [127, 128], and antioxidants [129, 130]
Fatty acid esters of sugars/sugar alcohol	Surfactant/emulsifier used in food, detergent, cosmetics, and pharmaceutical industries [117, 124]
Esters of long-chain acids with long-chain alcohols (12–20 carbon atoms)	Plasticizers and lubricants [39]
Aminoacyl esters of carbohydrates	Sweetening agents, surfactants, microcapsules in pharmaceutical preparations, active nucleoside amino acid esters, antibiotics, and in the delivery of biological active agents [131, 132]
Canola phytosterols oleate	Cholesterol lowering agents [133]
L-Ascorbyl linoleate	Preservative, crumb softening agent, and inhibition of cancer [134]
FAME	Crude palm oil transesterification [135]
Cinnamic acid	Antioxidant activity [136]
Esters of gallic acid	Free radical scavenger showing astringent activity [137]
Esters of ferulic acid	Flavor/fragrance compounds, precursors of pharmaceuticals, and as additives in foods, cosmetics, and sunscreens [114]
Starch esters	Used in the food, pharmaceutical, and biomedical applications industries [138]
Hydroxycinnamic acids and their analogues such as 4-hydroxycinnamic (*p*-coumaric), 3,4-dihydroxycinnamic (caffeic), and 4-hydroxy-3,5-dimethoxycinnamic (sinapic) acids including their medium- or long-chain alkyl esters	Antioxidant capacity, particularly against oxidative attacks by their radical-scavenging activity [139]

Applications of lipase for production of cosmetic ingrendients

- Lipases as cosmetic ingredients
  - Hydrolysis of fats/release of active components
- Lipases for production of cosmetic components:
  - Fatty acid esters (emulsifiers, emollients, detergents)
  - Sugar esters (tensioactives)
  - Fragrances and aromas
  - Active ingredients (antioxidants, UV filters, ceramides)

➔ Enantioselectivity and regioselectivity of lipases are key for these applications

Components of cosmetics that can be obtained with lipases

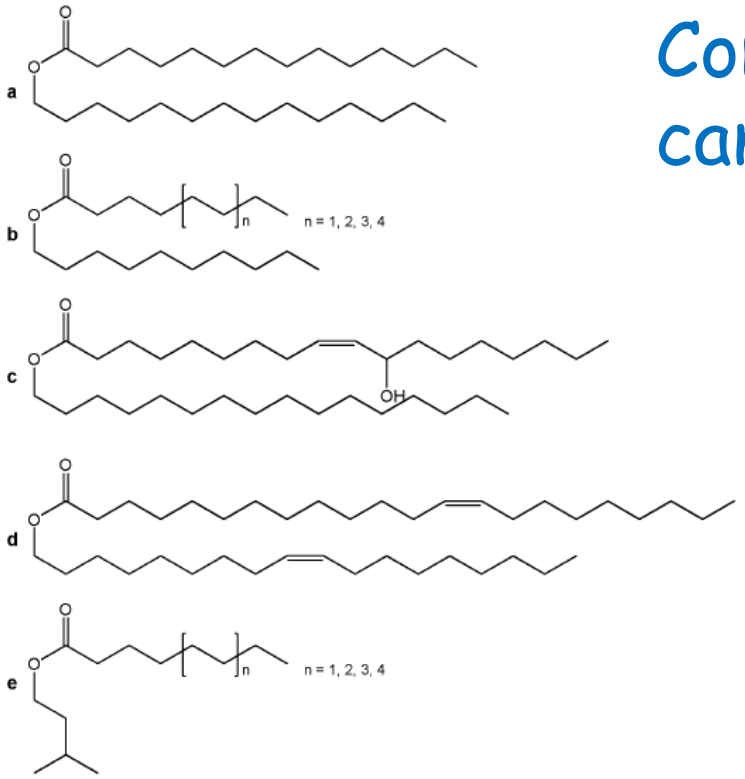


Fig. 1 Emollient esters commercialised by Evonik Industries AG. (a) myristyl myristate; (b) decyl cocoate; (c) cetyl ricinoleate; (d) oleyl erucate and (e) isoamyl cocoate.

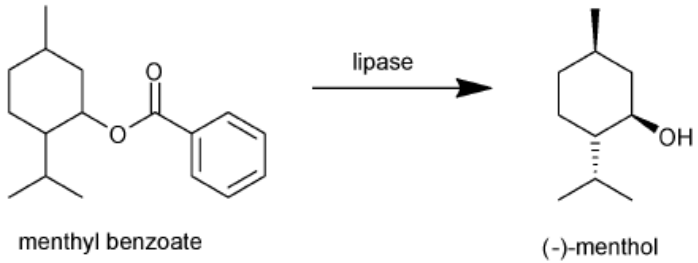


Fig. 11 Lipase-catalysed synthesis of enantiopure **(−)-menthol** according to Vorlova *et al.*[144]

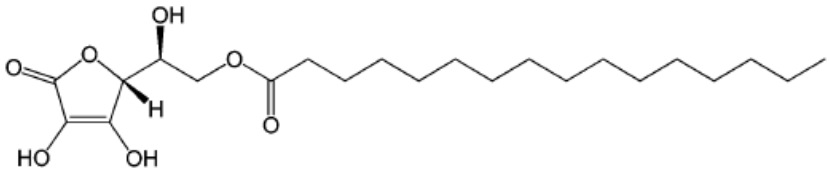


Fig. 12 Structure of 6-*O*-ascorbyl palmitate, accessible by lipase catalysis.

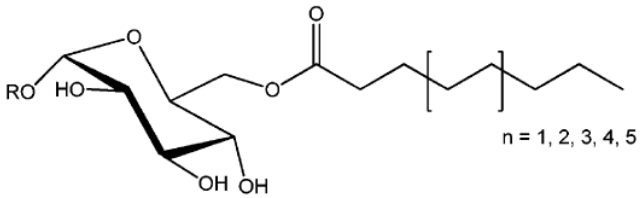
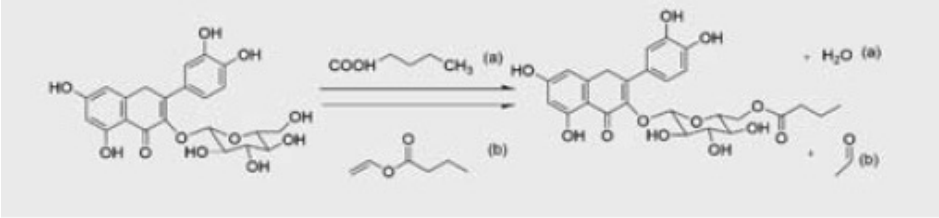


Fig. 4 Schematic of glycoside esters described by Björkling *et al.*;[63] R = short alkyl chain.



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

Comparison between conventional and lipase-catalyzed procedure for production of cosmetic fatty acid esters

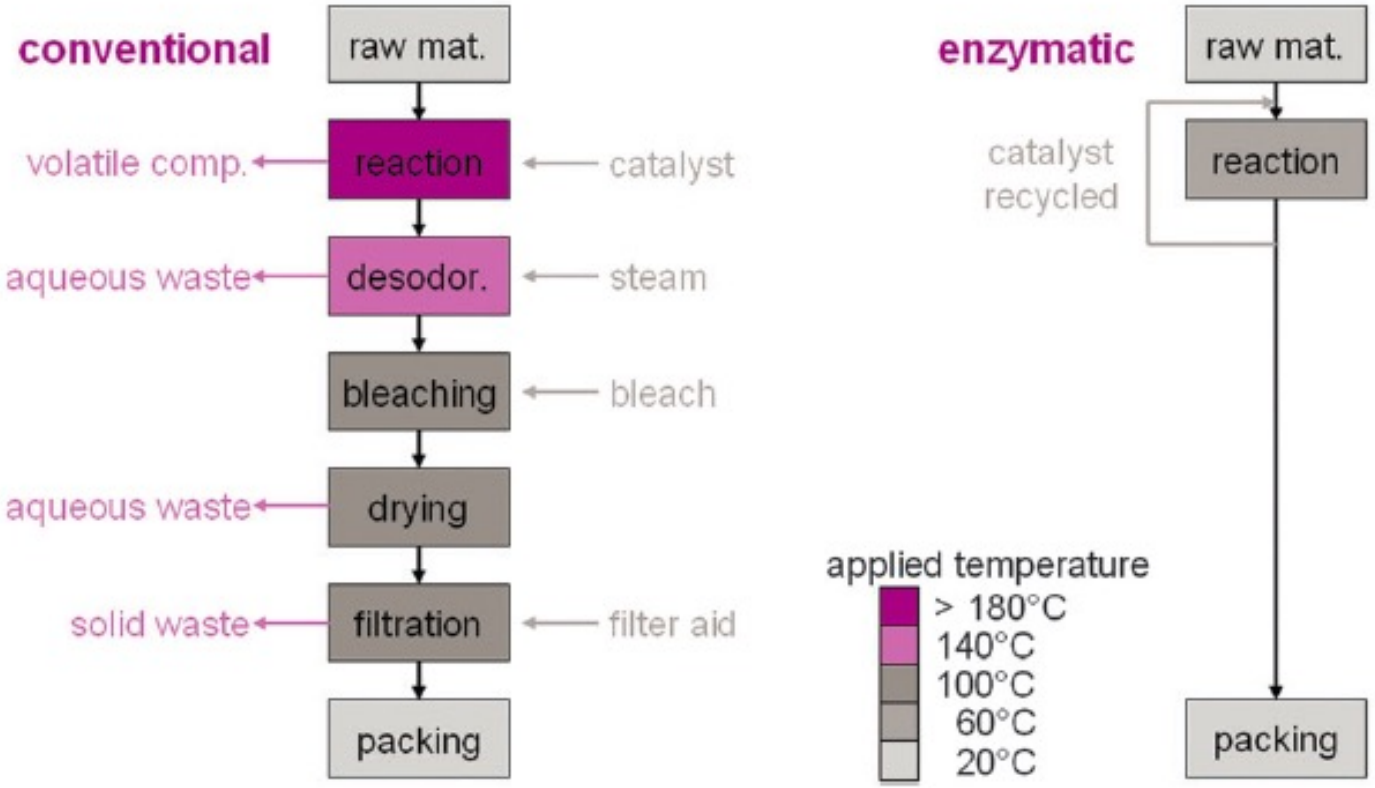


Fig. 17 Process steps of conventional (left) and enzymatic (right) esterification for production of cosmetic fatty acid esters.[29,31]

Application of lipase for biodiesel production

- Biodiesel is constituted by fatty acid methyl esters (FAME)
- The transesterification reaction of triglycerides with methanol produces biodiesel and glycerol

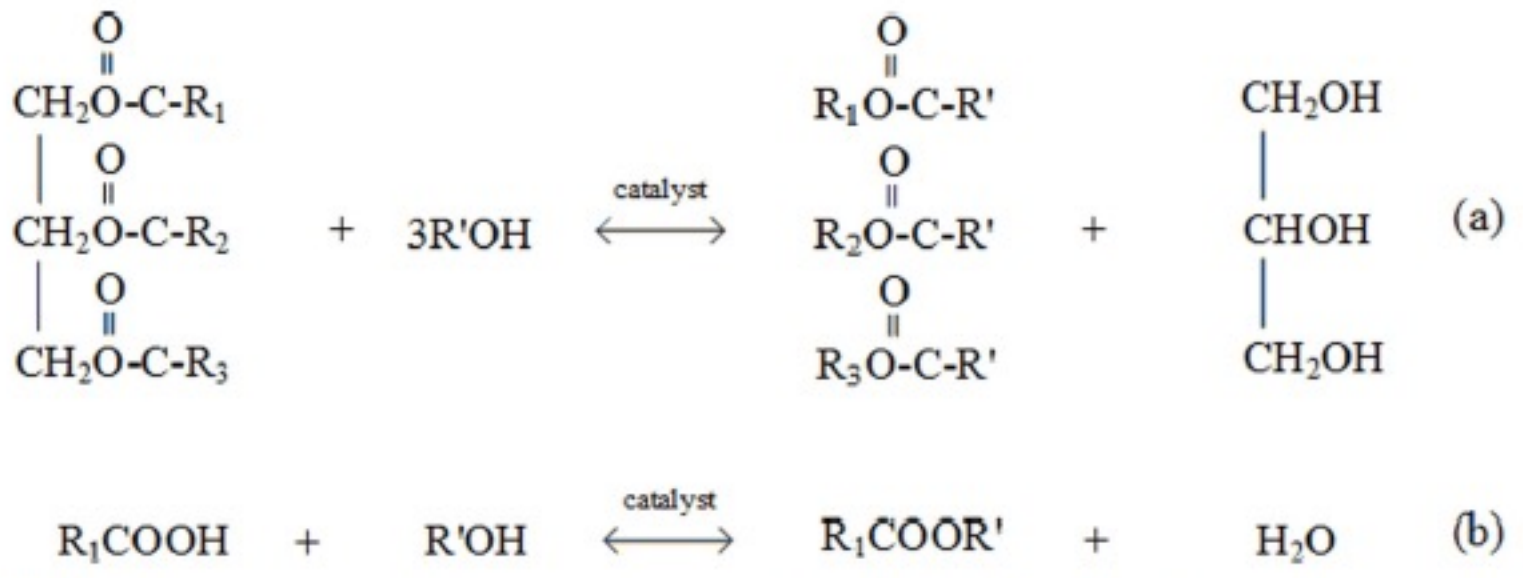


Figure 1. Typical reactions in biodiesel production, (**a**) transesterification of triglycerides (TG), and (**b**) esterification of free fatty acids (FFA).

Application of lipase for biodiesel production

- Alkaline catalysis (NaOH) vs enzymatic catalysis

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.

Application of lipase for biodiesel production

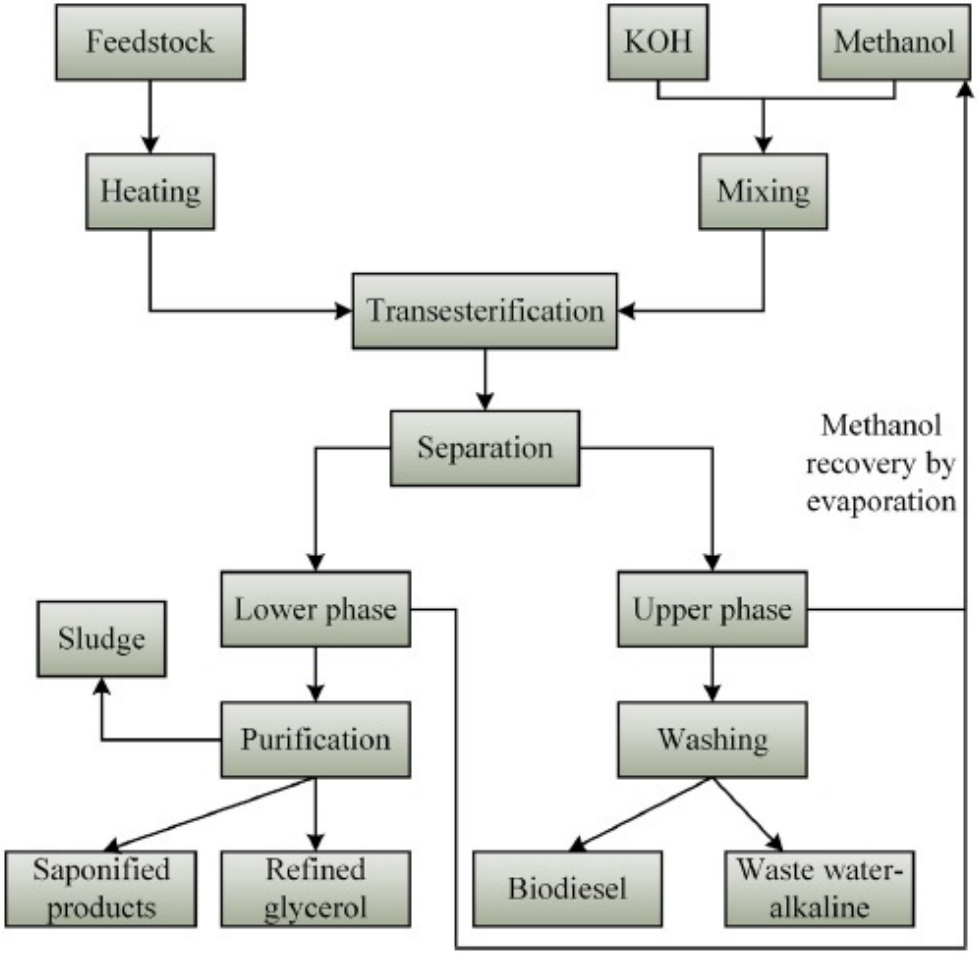


Fig. 9. Biodiesel production using alkali-catalyzed transesterification process [154].

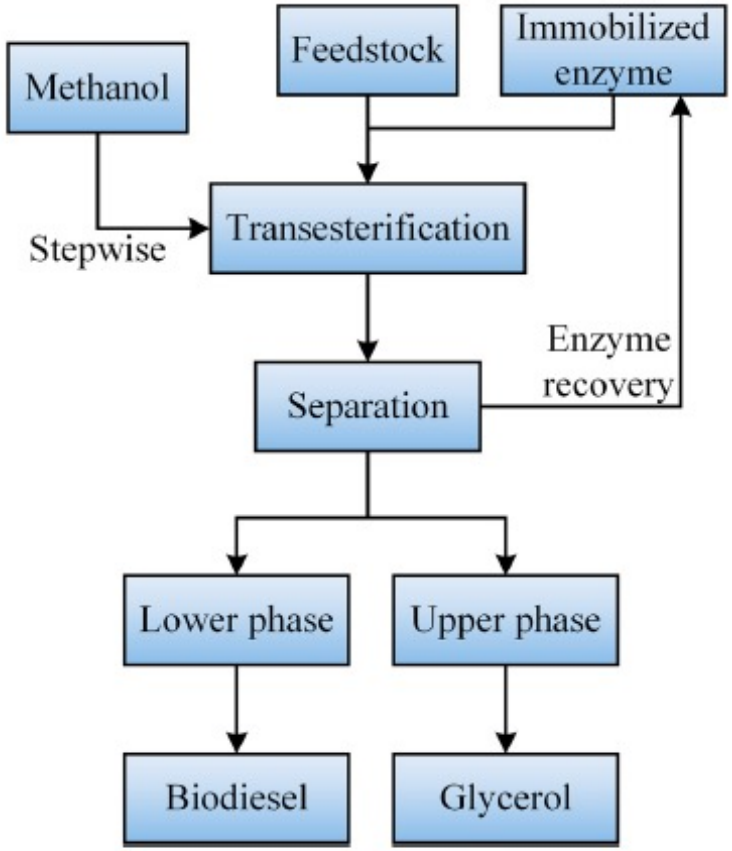


Fig. 10. Biodiesel production using enzymatic-catalyzed transesterification process.

Application of lipase for biodiesel production

- Purified lipase or microrganisms that produce high levels of the enzyme (recombinant or natural)
- Immobilized biocatalyst
- Triglyceride sources: edible vegetable oils (sunflower, soy and palm oil) non edible oils, lipid-rich microrganisms (microalgae, bacteria or yeast), waste oils (food, paper and tobacco manufacture)

- Variables to be controlled: temperature, alcohol (inactivation of the enzyme in excess methanol), presence of water and solvents, regeneration of lipase, concentration of free fatty acids

Application of lipase for biodiesel production

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%

Application of immobilized lipase for biodiesel production

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

Stabilization of lipase to methanol

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%.

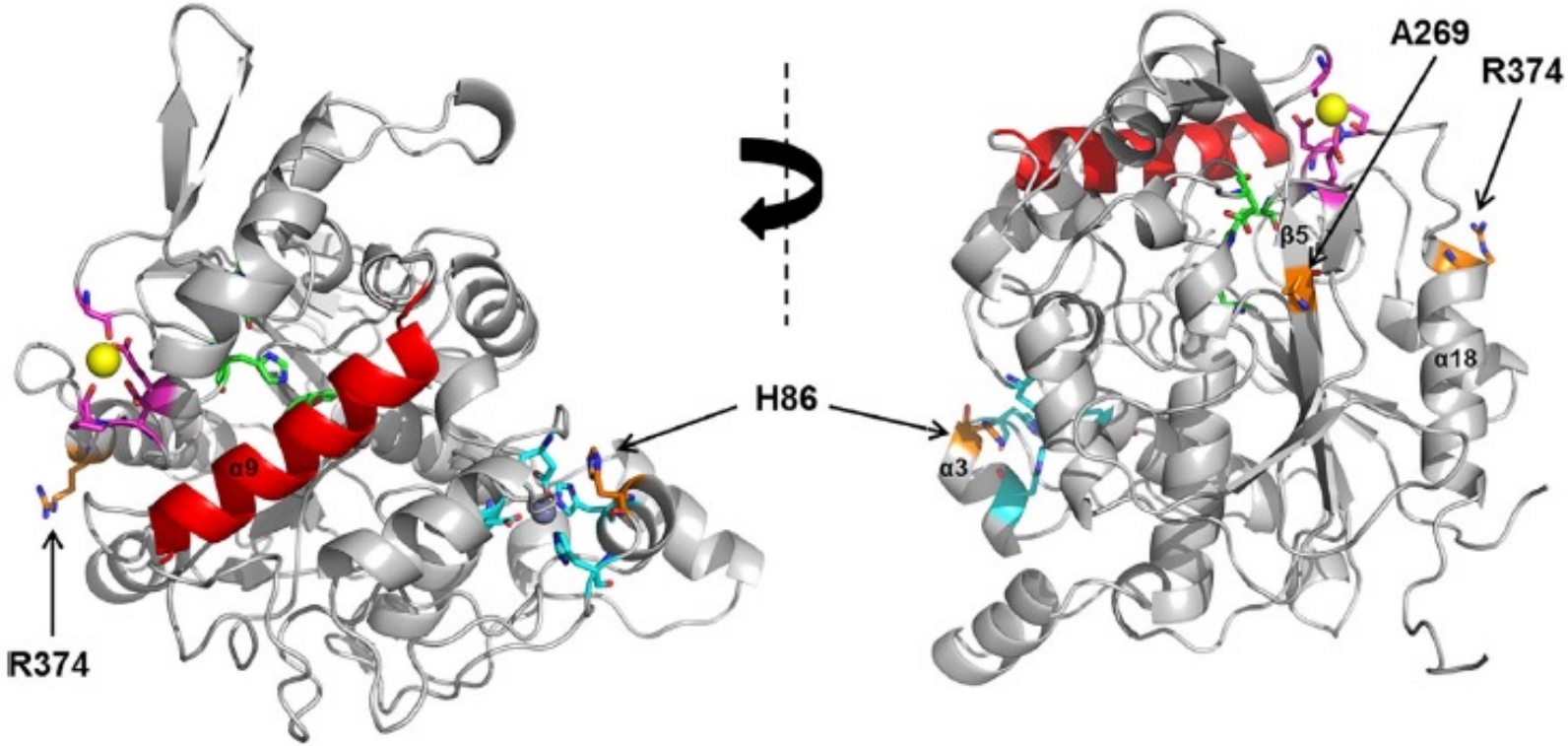


Figure 2. Crystal structure of wild-type lipase T6 with the mutated residues (H86, A269, R374) shown in orange sticks, catalytic triad residues (Ser114, Asp318, and His359) in green, calcium-binding residues (Glu361, Gly287, Pro367, and Asp366) in magenta, zinc-binding residues (Asp62, His88, Asp239, 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.

Kinetic resolution of racemic mixtures of drug precursors

- Non steroid anti-inflammatory drugs (NSAID) are competitive inhibitors of cycloxygenase.
- NSAID derived from aryl-propionic acids contain a chiral center. The S isomer binds the active site of the target COX-2.

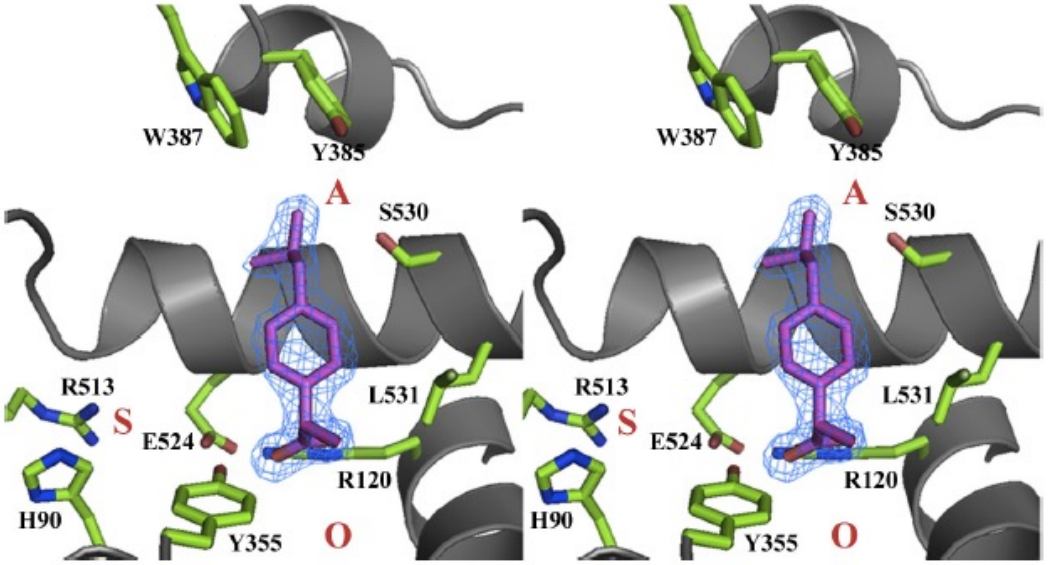
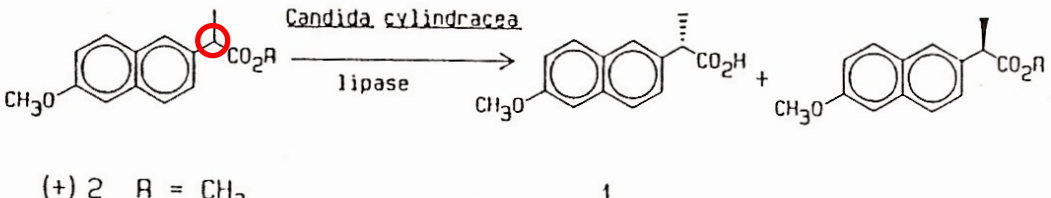


Fig.1. IBP bound in the cyclooxygenase channel of COX-2. Stereo view of IBP bound within the cyclooxygenase channel of monomer A of the muCOX-2:IBP crystal structure. F_o–F_c simulated annealing omit map electron density (light blue), contoured at 3.5σ, is shown with the final refined model of IBP (pink). 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.)

Kinetic resolution of racemic mixtures of drug precursors

Naproxen (methyl-2-(6-methoxy-2-naftyl) propionic acid) is a non-steroid anti-inflammatory drug that contains a chiral center. The S isomer is 28 times more active than the R isomer. It can be obtained by enantioselective hydrolysis of the racemic mixture of the ester precursor of the active molecule.



(±) 2 R = CH_3 1

3 R = CH_2CH_2Cl

4 R = CH_2CN

5 R = CH_2-$\underset{NO_2}{CH}$-CH_3

C. cylindracea (*C. rugosa*) lipase shows stereochemical preference for the S isomer

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]

Reaction rate depends on the leaving group

TABLE 2. Relative rates of enzymatic hydrolysis.

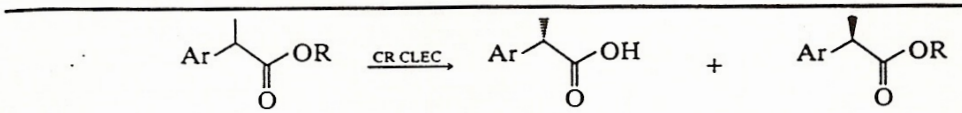
Compound	Relative rate	Enantiomeric ratio (E)
2	1	>100
3	15	>100
4	6	>100
5	3	81

C. rugosa lipase immobilization for resolution of racemic mixtures of arylpropionic acid esters

Immobilization method: cross-linked enzyme crystals with glutaraldehyde (CLEC). Crystallization in 2-methyl-2,4-pentandiol allows to retain catalytic activity and active site accessibility.

TABLE I

CR CLEC LIPASE-CATALYZED RESOLUTION OF ARYLPROPIONIC ACID ESTERS



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

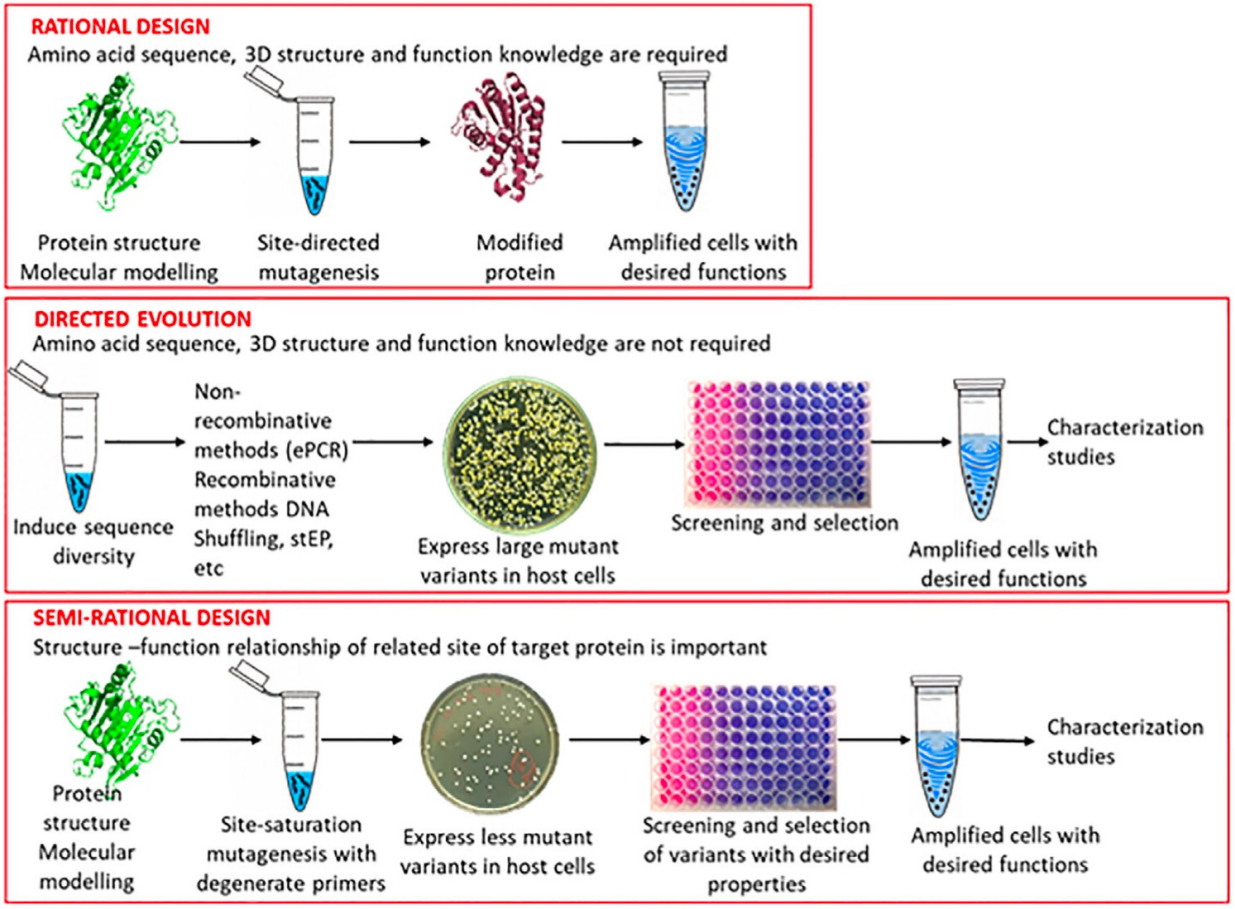
Ar	% Enantiomeric excess (% conv.) CR CLEC	% Enantiomeric excess (% conv.) Crude	*E* CR CLEC	*E* Crude
1a: R = H **ibuprofen** **b:** R = Me	94.6 *S*-**1a** (22)[a]	81.9 *S*-**1a** (39.3)[a]	47	17
2a: R = H **ketoprofen** **b:** R = CH_2CH_2Cl	91.1 *S*-**2a** (49.3)[b]	64.5 *R*-**2b** (66)[b]	66	5
3a: 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.

Strategies for lipase engineering

Which properties do you want to improve?

- Enantioselectivity
- Stability in 'exotic' environment (organic solvent)



In vitro directed evolution to improve enantioselectivity of lipase

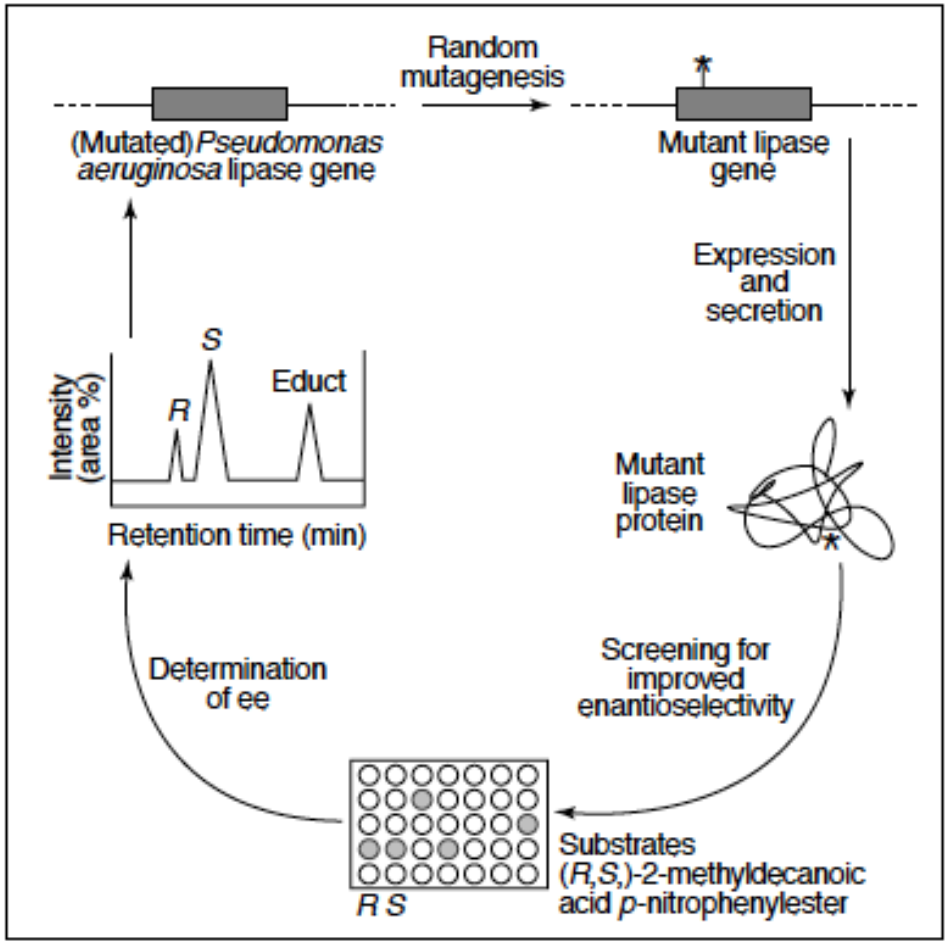


Figure 5

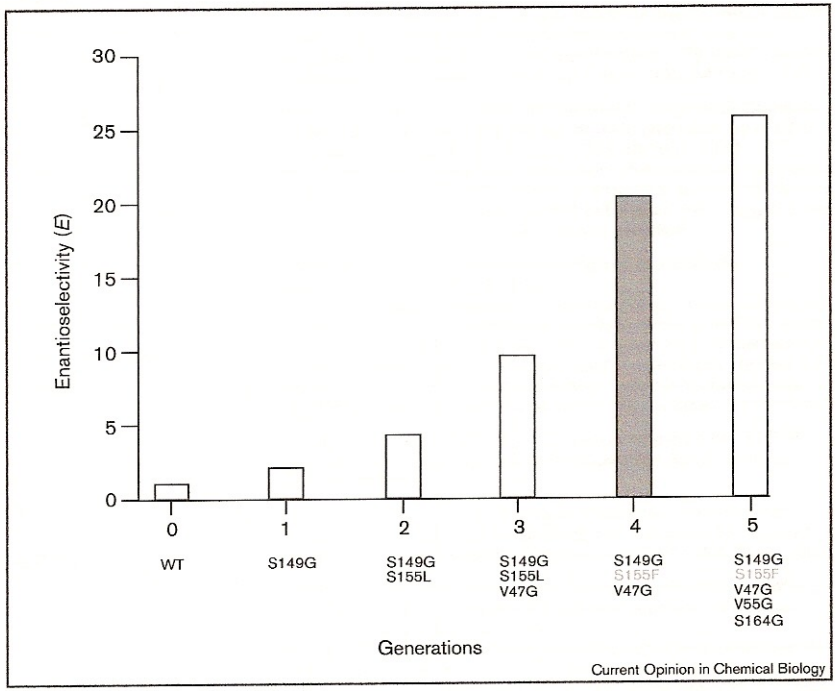
Strategy to create an enantioselective lipase by directed evolution. Intensity (area %) refers to the amount of *R*- and *S*-enantiomer as measured by chiral chromatography.

In vitro directed evolution to improve enantioselectivity of lipase

The variant obtained at the 5th generation shows mutation S155F and 4 glycine substitutions that make the enzyme more flexible, modifying some interactions in the active site and in the oxyanion site.

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.



Site-specific mutagenesis of the active site of *Yarrowia lipolitica* lipase to modify enantioselectivity

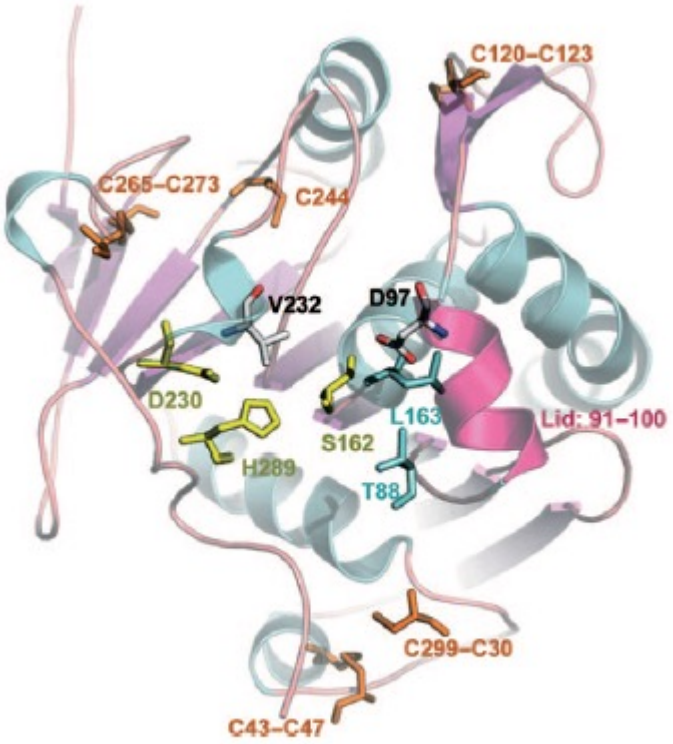


Figure 3. Overall representation of the Lip2p homology model. Hydrogen atoms on amino acid residues have been omitted for clarity purpose.

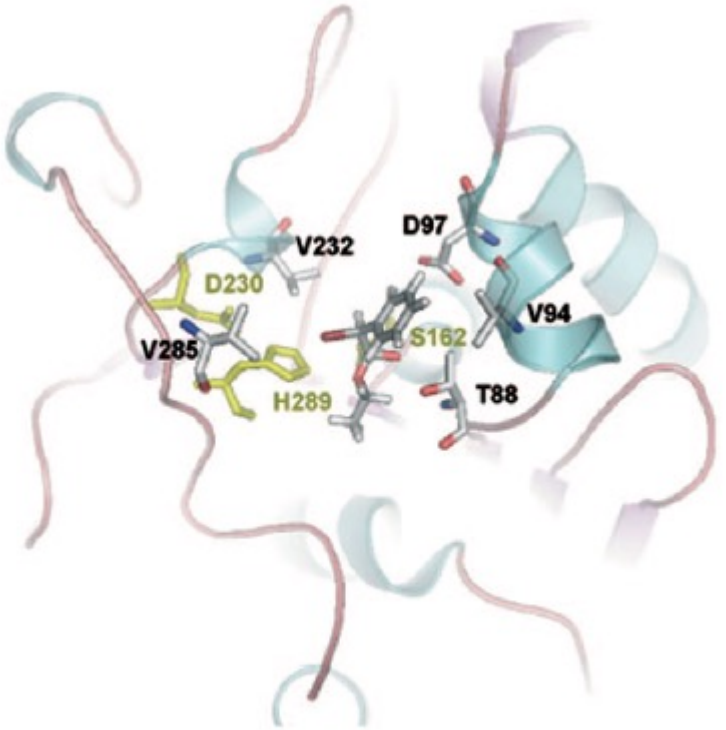


Figure 5. Representation of Lip2p amino acid residues selected for site-directed mutagenesis. The *S*-2-bromo-phenylacetic acid ethyl ester covalently bound to Ser162 is shown in the active site.

Site-specific mutagenesis of lipase to modify enantioselectivity

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
vi*S*[a]	1.71	2.13	1.41	1	0.97	1.3	8.8	0.017	0.010
vi*R*[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 = vi*S*/vi*R* or vi*R*/vi*S* according to enantiomer preference; vi*R*, vi*S*: initial rates. [c] Substrate enantiomeric excess. [d] Product enantiomeric excess.

Substrate: 2-bromo-phenylacetic acid esters, intermediates for drug synthesis

Site-specific mutagenesis of lipase to modify enantioselectivity

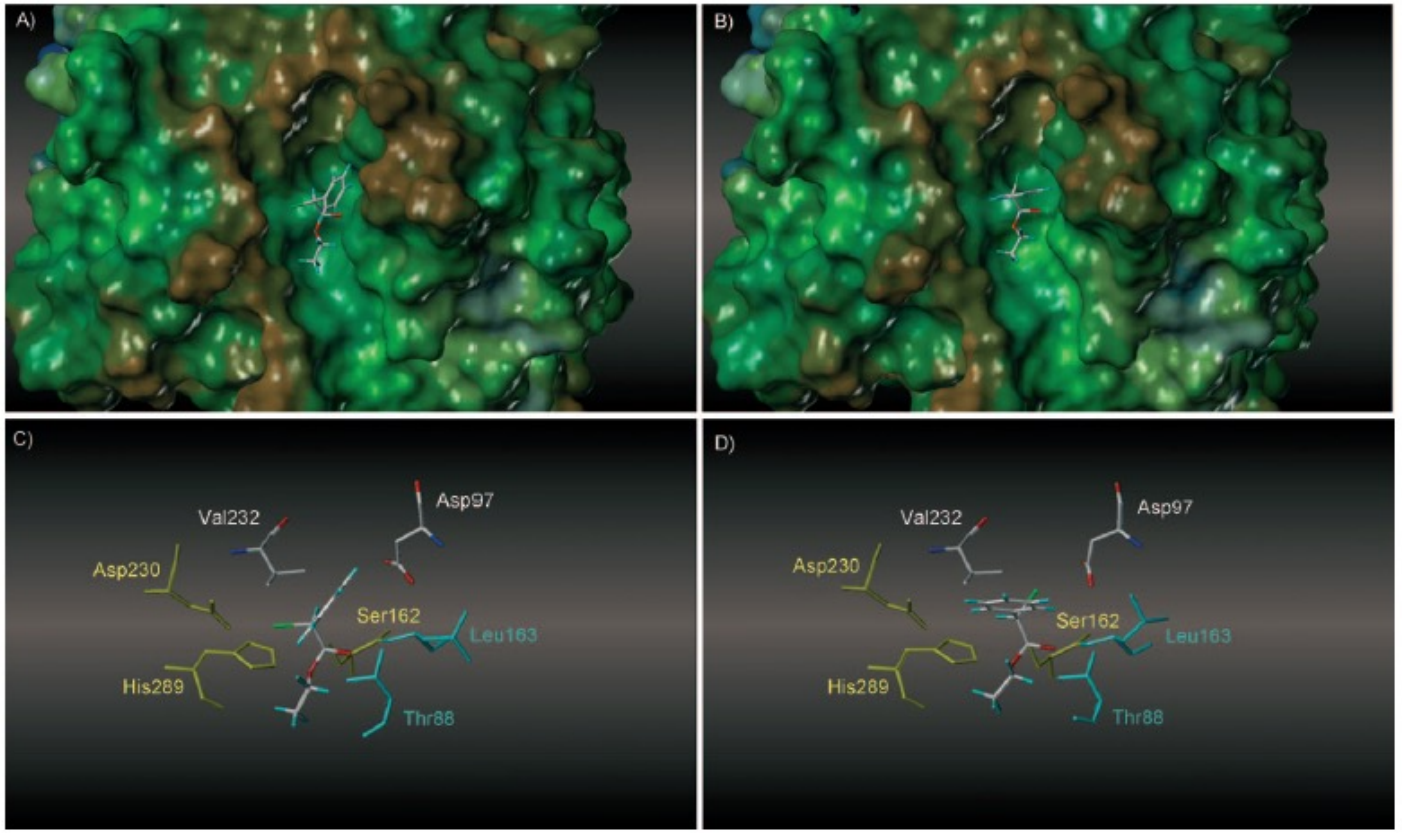


Figure 6. Representation of (*R,S*)-2-bromo-phenylacetic acid ethyl ester enantiomers covalently bound to catalytic Ser162 of Lip2p. A), B) *S* and *R* enantiomers are respectively shown. Lip2p is shown as a Connolly surface mapped with the lipophilic potential, as calculated by the MOLCAD module implemented 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.

Site-specific mutagenesis of lipase to modify enantioselectivity

The size of the residue in position 232 determine lipase preference of one enantiomer.
Small amino acids: S
Large amino acids: R

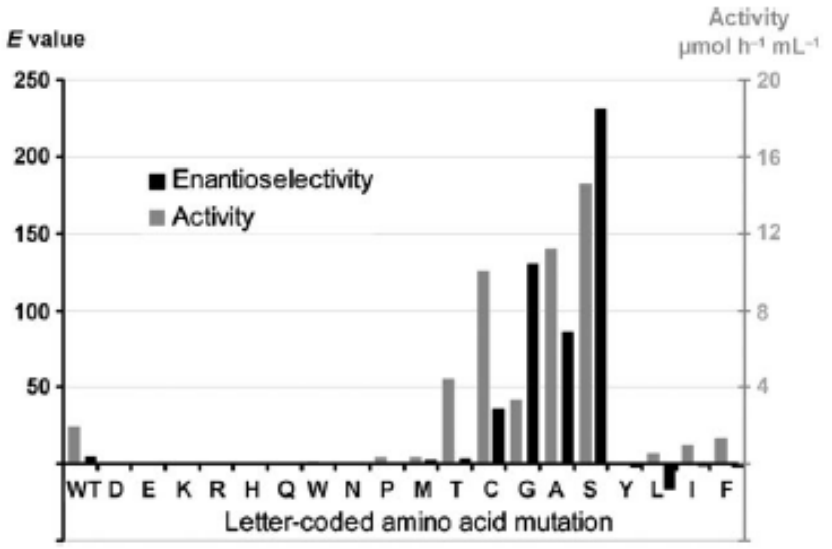


Figure 7. Activity and enantioselectivity of V232 variants in 2-bromo-phenyl-acetic octyl ester racemate hydrolysis reaction. WT: wild-type Lip2p. A positive *E* value corresponds to *S* selectivity, a negative *E* value to *R* selectivity.