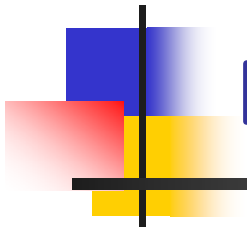


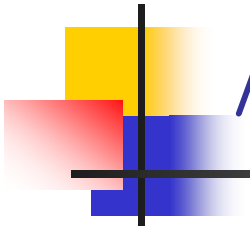
Enzymes in nonaqueous media

Enzyme Engineering

Enzymes in nonaqueous media

- Enzyme activity in organic solvent was proved in 1930's and rediscovered in 1977
- The best solvent is not water miscible acetone or methanol, but hydrophobic solvent, such as toluene or cyclohexane→ water miscible solvents strip away the remaining water, while hydrophobic solvent does not
- Advantages
  1. Increases of solubility of reactants
  2. A shift of equilibrium
  3. Easier separation of water and solvent
  4. Enhanced stability of enzyme
  5. Altered selectivity
- Lipase synthesizes ester bonds and is stable at 100 °C in organic solvent

Enzyme	Solvent	Half life at indicated temperature
Lipase (Porcine)	Aqueous (buffer)	< 1 min at 100 °C
	Organic (tributyrin)	12 h at 100 °C
Lysozyme	Aqueous (buffer)	8 mins at 100 °C
	Organic (cyclohexane)	140 h at 100 °C
Chymotrypsin	Aqueous (buffer)	< 1 min at 100 °C
	Organic (octane)	270 min at 100 °C
Ribonuclease	Aqueous (buffer)	< 10 min at 90 °C
	Organic (nonane)	> 6 h at 110 °C



Activity

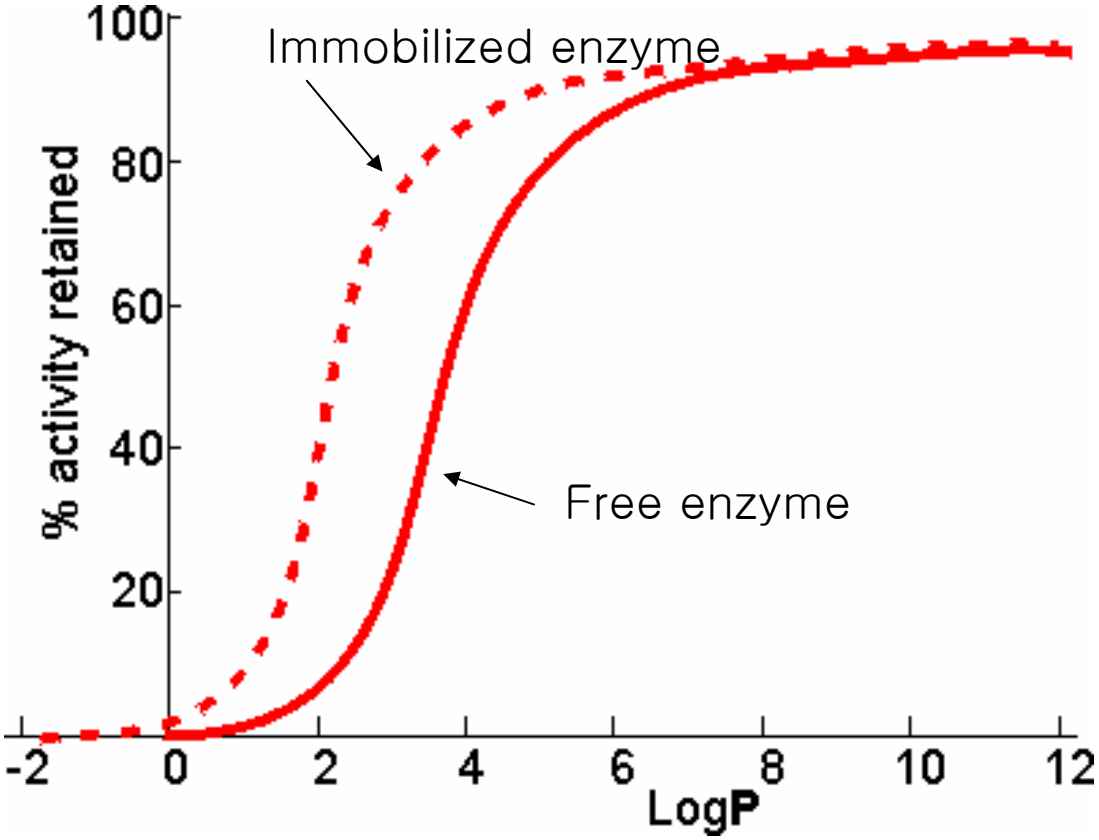
- Most of enzymes are active in organic solvent
- Activities are decreased orders of magnitude
- Some methods to improve activity
  - Sufficient hydration in organic solvent (1% v/v)
  - Lyophilization at the maximum activity in water
  - Lyophilization with protectants (polyol, phenol, ...) and strong salts such as KCl

Hydrophobicity of solvent

$$\mathrm{Log}\boldsymbol{P} = \mathrm{Log}_{10}\left(\frac{[Material_{octanol}]}{[Material_{water}]}\right)$$

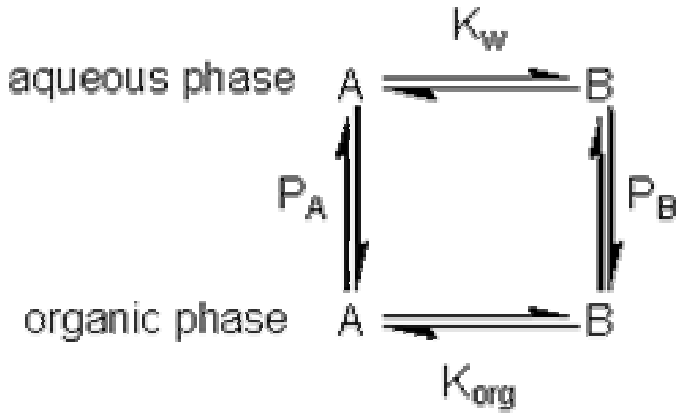
Solvent	Log***P***	Solvent	Log***P***
Butanone	0.3	1,1,1-trichloroethane	2.8
Ethyl acetate	0.7	Carbon tetrachloride	2.8
Butanol	0.8	Dibutyl ether	2.9
Diethyl ether	0.8	Cyclohexane	3.1
Methylene chloride	1.4	Hexane	3.5
Butyl acetate	1.7	Petroleum ether (60-80)	3.5
Di-isopropyl ether	2.0	Petroleum ether (80-100)	3.8
Benzene	2.0	Dipentyl ether	3.9
Chloroform	2.2	Heptane	4.0
Tetrachloroethylene	2.3	Petroleum ether (100-120)	4.3
Toluene	2.7	Hexadecane	8.7

Activities versus hydrophobicity



Sometimes loss of activity by organic solvent can be offset
by the shift of equilibrium constant : ex) glucose isomerase in ethanol

Equilibrium



$$K_W = \frac{[B_W]}{[A_W]}$$

$$K_{org} = \frac{[B_{org}]}{[A_{org}]}$$

$$P_A = \frac{[A_{org}]}{[A_w]}$$

$$P_B = \frac{[B_{org}]}{[B_w]}$$

$$K_{biphasic} = \frac{[B_t]}{[A_t]}$$

$$[A_t]V_t = [A_w]V_w + [A_{org}]V_{org}$$

$$[B_t]V_t = [B_w]V_w + [B_{org}]V_{org}$$

$$V_t = V_w + V_{org}$$

$$K_{biphasic} = \frac{[B_t]V_t}{[A_t]V_t} = \frac{[B_w]V_w + [B_{org}]V_{org}}{[A_w]V_w + [A_{org}]V_{org}}$$

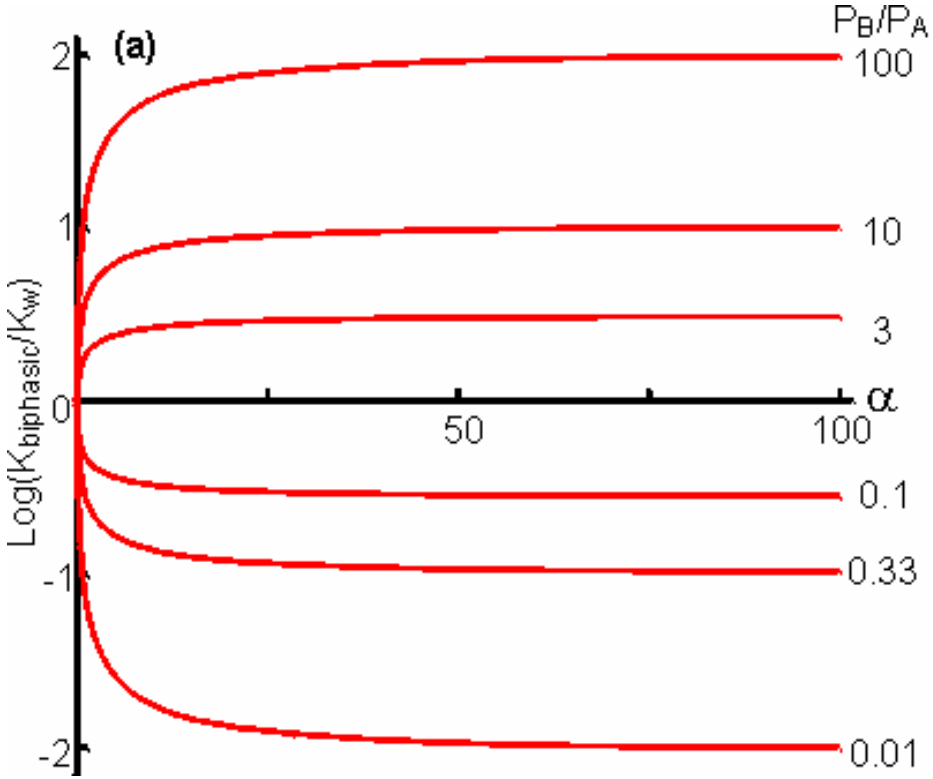
$$K_{biphasic} = \frac{\frac{[B_w]V_w}{[A_w]V_w} + \frac{[B_{org}]V_{org}}{[A_w]V_w}}{\frac{[A_w]V_w}{[A_w]V_w} + \frac{[A_{org}]V_{org}}{[A_w]V_w}} = \frac{\frac{[B_w]}{[A_w]} + \frac{[B_{org}]}{[B_w]}\frac{[B_w]}{[A_w]}\frac{V_{org}}{V_w}}{1 + \frac{[A_{org}]}{[A_w]}\frac{V_{org}}{V_w}}$$

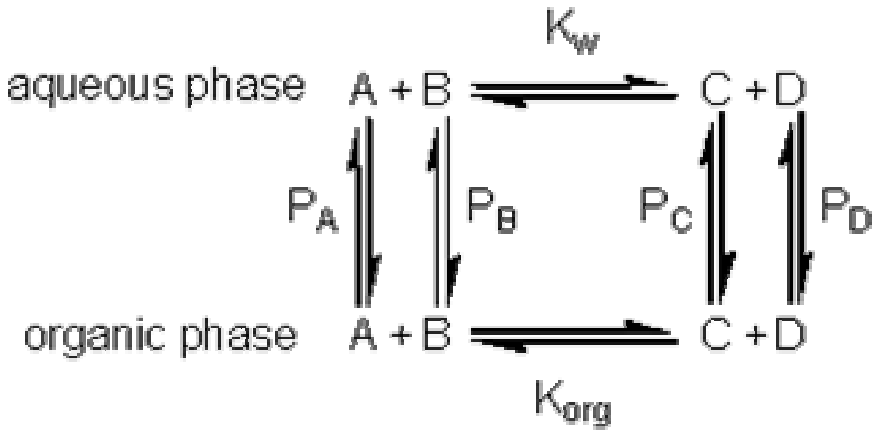
$$\alpha = \frac{V_{org}}{V_w}$$

$$K_{biphasic} = K_w \frac{(1+\alpha P_B)}{(1+\alpha P_A)}$$

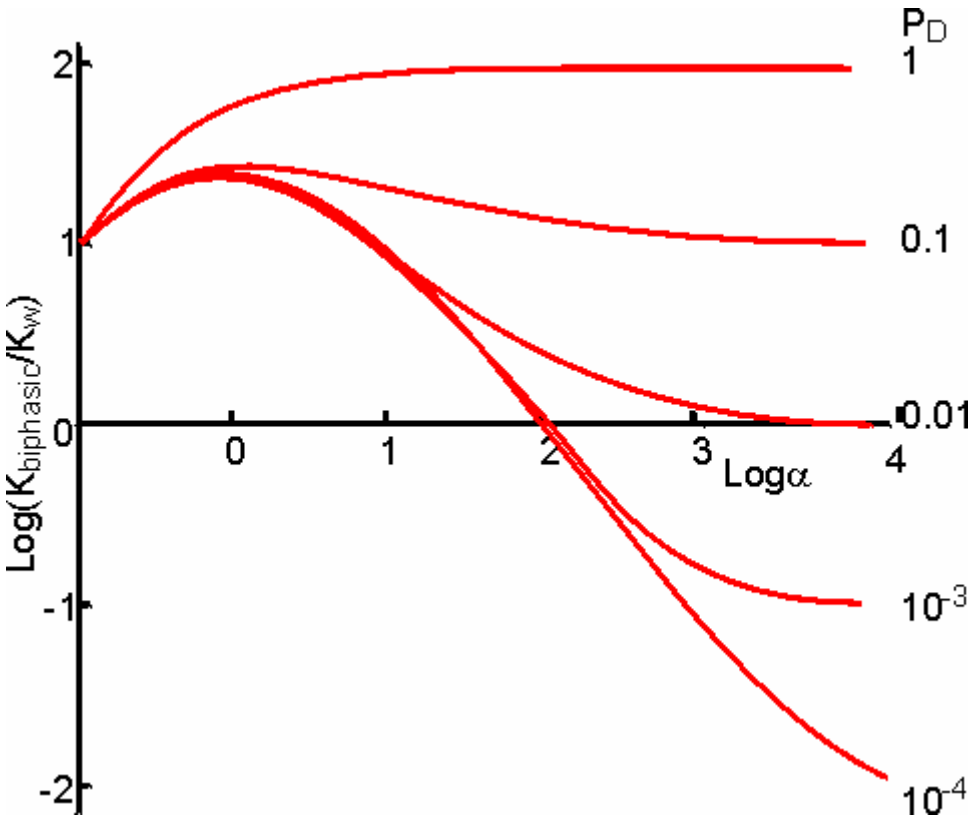
If α is large,

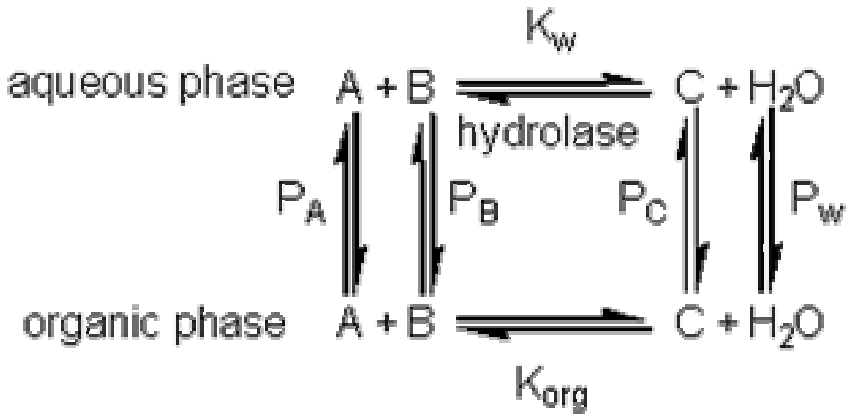
$$K_{biphasic} = K_w \frac{P_B}{P_A} \qquad K_{biphasic} = \frac{[B_{org}]}{[A_{org}]} = K_{org}$$





$$K_{biphasic} = K_w \frac{(1+\alpha P_C)(1+\alpha P_D)}{(1+\alpha P_A)(1+\alpha P_B)}$$



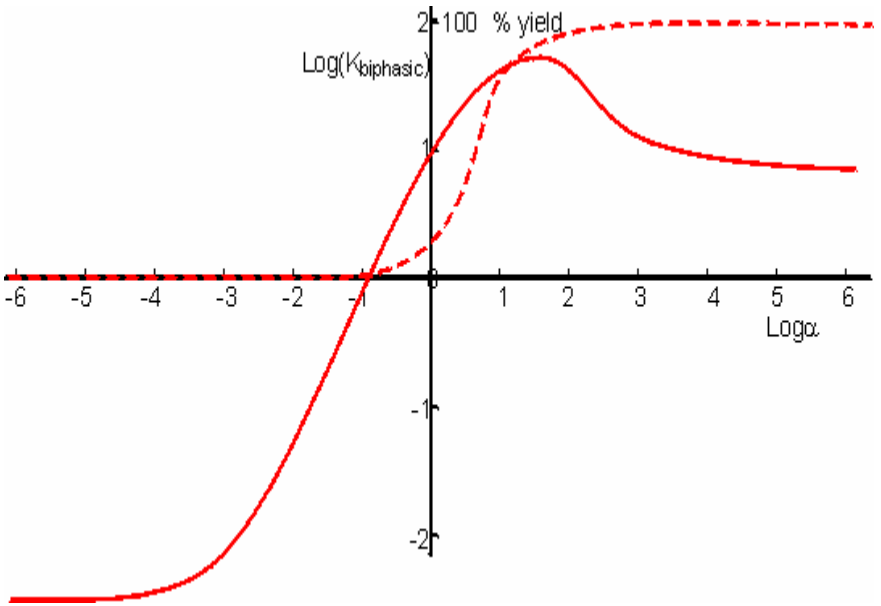


$$[(H_2O)_t](V_w + V_{org}) = [(H_2O)_w]V_w + [(H_2O)_{org}]V_{org}$$

$$[(H_2O)_t] = \frac{55.5(1+\alpha P_w)}{1+\alpha}$$

$$K_{biphasic} = \frac{[A_t][B_t]}{[C_t][(H_2O)_t]}$$

$$[C_t] = K_w \frac{(1+\alpha P_C)(1+\alpha)[A_t][B_t]}{55.5(1+\alpha P_A)(1+\alpha P_B)}$$



Separation

- Evaporating organic solvent

Table 12.1 Boiling points and enthalpies of evaporation for common organic solvents.

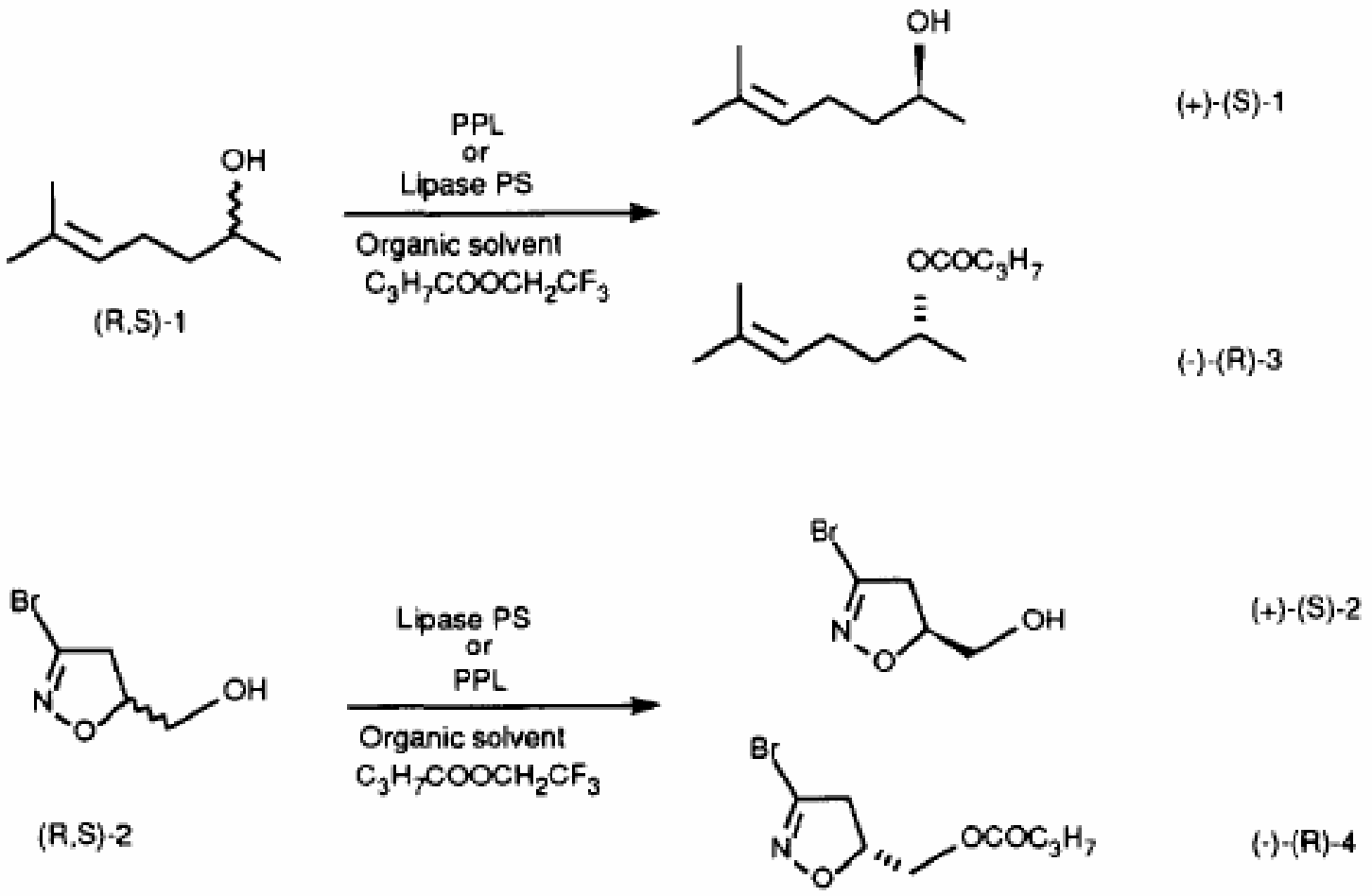
	Solvent										
	MTBE	**Acetone**	**CH_3OH**	**THF**	**Hexane**	**DIPE**	**EA**	**C_2H_5OH**	**C_6H_{12}**	**Toluene**	**DMF**
B.p. [°C]	55.2	56.2	65.0	67.0	69.0	69.0	77.1	78.5	80.7	110.6	149
ΔH_{vap} [kJ mol^{-1}]	29.70	32.00	39.26	28.76	31.93	32.56	34.75	40.50	32.79	39.23	60.45

MTBE: methyl *t*-butyl ether; THF: tetrahydrofuran; EA: ethyl acetate; C_6H_{12}: cyclohexane; DIPE: diisopropyl ether; DMF: dimethylformamide.

Importance of water

- The required water molecules per protein is several hundreds per protein, which is less than monolayer outside of protein
- Water activity (thermodynamic term)
  - *Pseudomonas cepacia* lipase in n-octanol has maximum activity at aw between 0.11 – 0.38
  - *Mucor miehei* lipase has maximum activity at 0.55

Strategies using lipase in organic solvents



Extremophile enzymes

Table 10.11 Various categories of extremophiles and the habitats in which they live

Extremophile type	Natural habitat	Relevant growth parameter	Example microbe
Hyperthermophiles	Geothermal habitats (e.g. hot springs)	Opt. temp. for growth at or above 80 °C	*Pyrococcus furiosus*
Halophiles	Hypersaline waters	Grow in upto 5 M NaCl	*Halobacterium halobium*
Psycrophiles	Extremely cold environments (e.g. Antarctic sea water)	Grow at temperatures as low as −2 °C	*Alteromonas* sp.
Alkaliphiles	Alkaline environments	Grow at pH values above 9	*Natronobacterium* sp.
Acidophiles	Acidic environments	Grow at pH values lower than 4	*Thermoplasma acidophilum*

More than 20 different genera are living at the temperature above 100°C

Most of them are anaerobic and use peptides as a source of carbon and nitrogen

Cell architecture and metabolism are novel (for example, no fatty acid ester group)

Table 10.12 (Hyper)thermophilic archaea and their respective maximum growth temperatures

Order	Genus (max. growth temp.)	
Thermococcales	*Pyrococcus*	(105 °C)
	Thermococcus	(97 °C)
Sulfolobales	*Sulfolobus*	(87 °C)
	Acidianus	(96 °C)
	Desulfurolobus	(87 °C)
	Metallosphaera	(80 °C)
	Styoiolobus	(88 °C)
Thermoproteales	*Pyrodictium*	(110 °C)
	Thermodiscus	(98 °C)
	Desulfurococcus	(90 °C)
	Staphylothermus	(98 °C)
	Thermoproteus	(92 °C)
	Pyrobaculum	(102 °C)
	Thermofilum	(100 °C)
Thermoplasmatales	*Thermoplasma*	(67 °C)
Methanogenic Archaea	*Methanothermus*	(97 °C)
	Methanococcus	(91 °C)
	Methanopyrus	(110 °C)
Sulfate-reducing archaea	*Archaeoglobus*	(95 °C)
Unclassified	*Hyperthermus*	(110 °C)
	ES-1	(91 °C)
	ES-4	(108 °C)
	GE-5	(102 °C)
	GB-D	(103 °C)

Thermophile enzyme

- Advantage
  - Enzyme reactor in high temp can reduce viscosity, inhibit bacterial growth, increase solubility of substrates
  - More resistant to detergents, organic solvents, and proteases
- Disadvantage
  - High temp operation can increase energy costs
  - Certain food industry use heat for inactivation
  - Many are oxygen sensitive
  - Difficult to cultivate to high cell density → DNA recombinant method

Examples

Table 10.13 Temperature optimum and stability of selected enzymes isolated from various hyperthermophiles

Enzyme	Source	T_{opt} (°C)	Thermostability (T_{50})
Protease	*Thermobacteroides vulgaris*	85 °C	NR
Protease	*Pyrococcus furiosus*	>115 °C	33 h at 98 °C
DNA Polymerase	*Pyrococcus furiosus*	>75 °C	20 h at 95 °C
Amylase	*Pyrococcus woesei*	100 °C	6 h at 100 °C
Amylase	*Pyroccus furiosus*	100 °C	2 h at 120 °C
Amylopullulanase	*Thermoproteus tenax*	118 °C	20 h at 98 °C
Xylanase	*Thermotoga* sp.	105 °C	1.5 h at 95 °C
Cellobiohydrolase	*Thermotoga* sp.	105 °C	1 h at 108 °C
Glucose Isomerase	*Thermotoga neapolitana*	95 °C	NR
α-Glucosidase	*Pyrococcus furiosus*	100 °C	48 h at 98 °C
β-Glucosidase	*Pyrococcus furiosus*	105 °C	85 h at 100 °C or 13 h at 110 °C
Glutamate dehydrogenase	*Pyrococcus furiosus*	95 °C	10 h at 100 °C
Lactate dehydrogenese	*Thermotoga maritima*	>90 °C	1.5 h at 90 °C

Most such enzymes would have potential industrial applications. NR, not recorded. T_{50}, time required to lose 50% of catalytic

Other extremophiles

- Psychrophiles are living at −1.8 °C
  - Antifreeze glycoproteins
  - Maximum activity at 0 °C
  - Can be used for heat labile substrate
  - Low temp food processing can reduce contamination
- Halophiles
  - Active at low water activity
  - Can be used for reactions in organic solvent