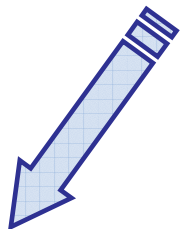
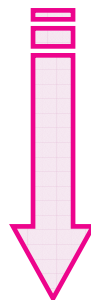
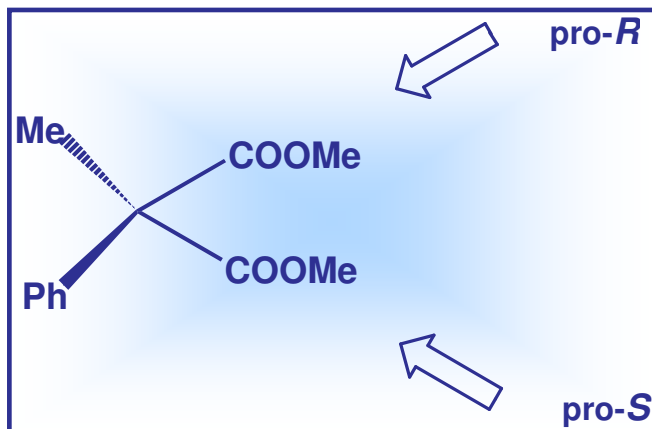


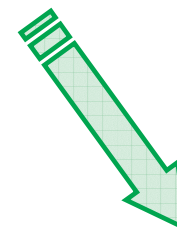
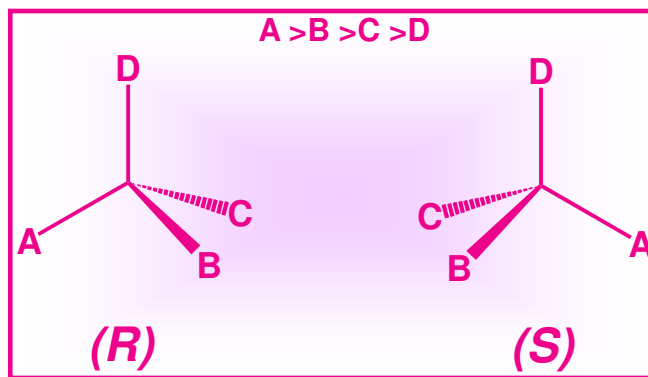
STEREOSPECIFICITA' nelle BIOTRASFORMAZIONI



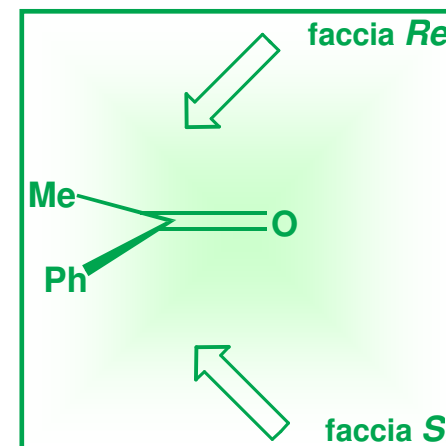
Discriminazione
enantiotopica



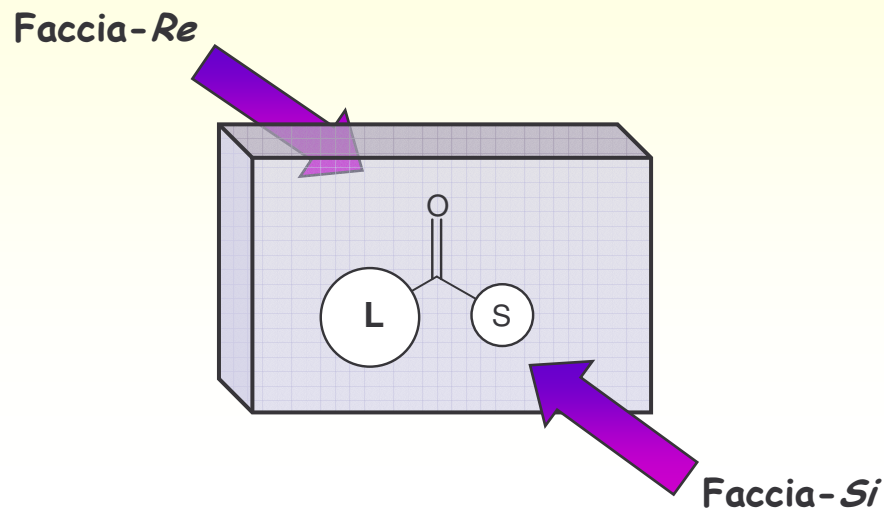
Discriminazione
enantiomerica



Discriminazione
di enantiofacce



Come variare la STEREOSELETTIVITA'



Screening microbiologico

Ricerca di enzimi diversi

Variazione delle condizioni di biotrasformazione

Inibizione o attivazione di enzimi diversi

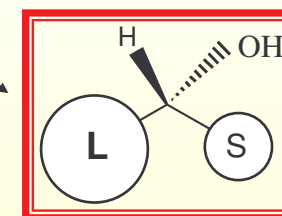
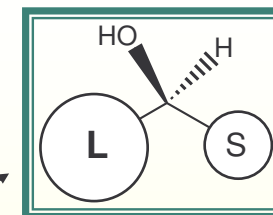
Modificazione del substrato

Variazione dell'ingombro sterico

Aggiunta di additivi

Inibizione o attivazione di enzimi

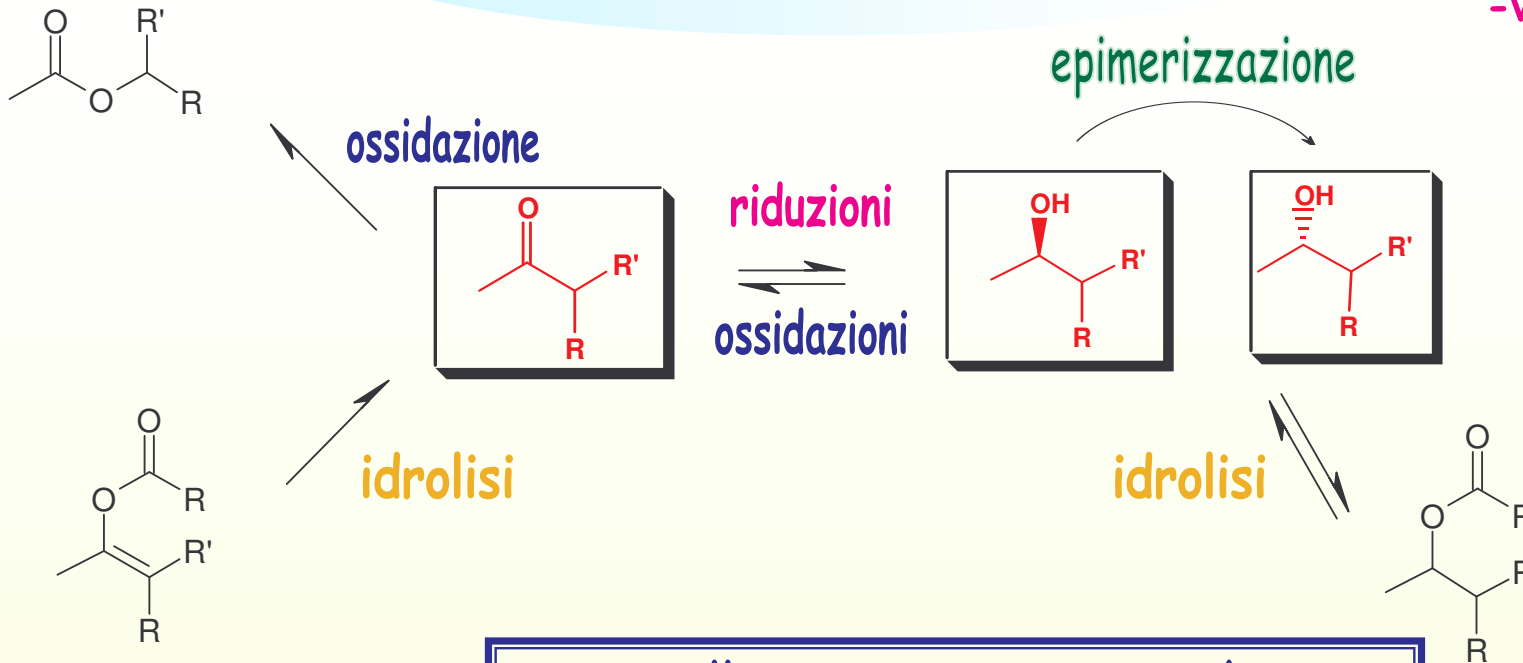
Enantiomero S



Enantiomero R

Biotrasformazioni

- microbiche
- enzimatiche
- vegetali



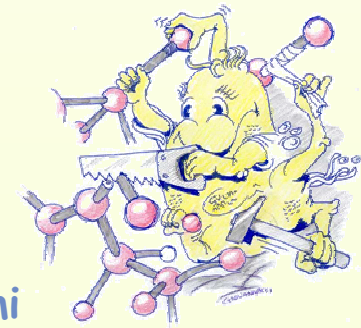
Controllo enantio e stereoselettivo

Targets

- Acidi biliari
- Terpeni
- Feromoni
- Biciclo eptenoni



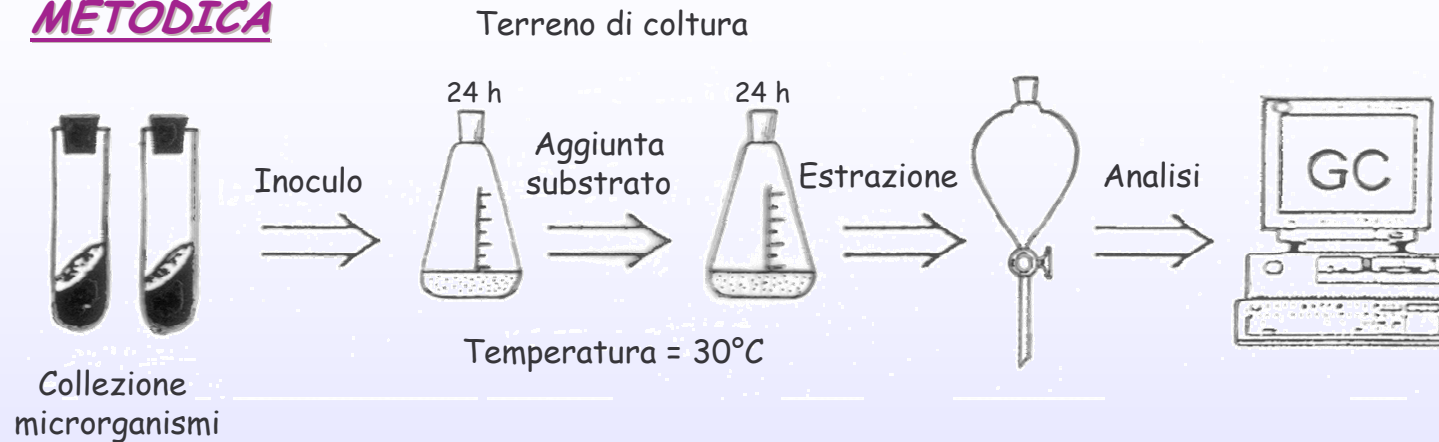
- * Microrganismo (screening)
- * Fonte di carbonio
- * Solvente
- * Pressione
- * Tipo/cambio di reazione
- * Combinazione di microrganismi (reazioni)





Screening: scelta del microrganismo

METODICA



OBIETTIVO

- identificazione del microrganismo più adatto
- "ceppoteca"

PARAMETRI OSSERVATI:

- resa della trasformazione
- eccesso stereoisomerico (enantio e diastereo)

Metodologie di isolamento da una matrice:

- aspecifica
- selettiva per tipo di microrganismo
- aspecifica, ma da "luoghi di lavoro"
- selezione per somministrazione del substrato come unica fonte di carbonio

screening

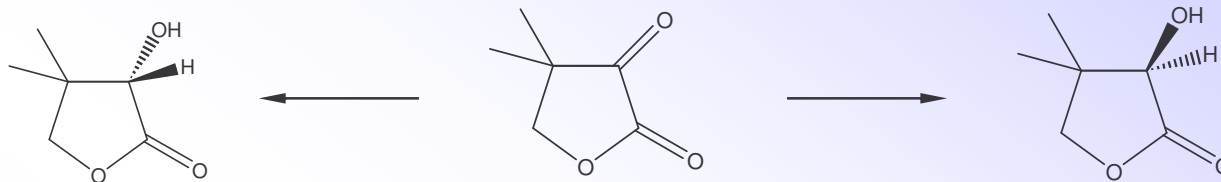
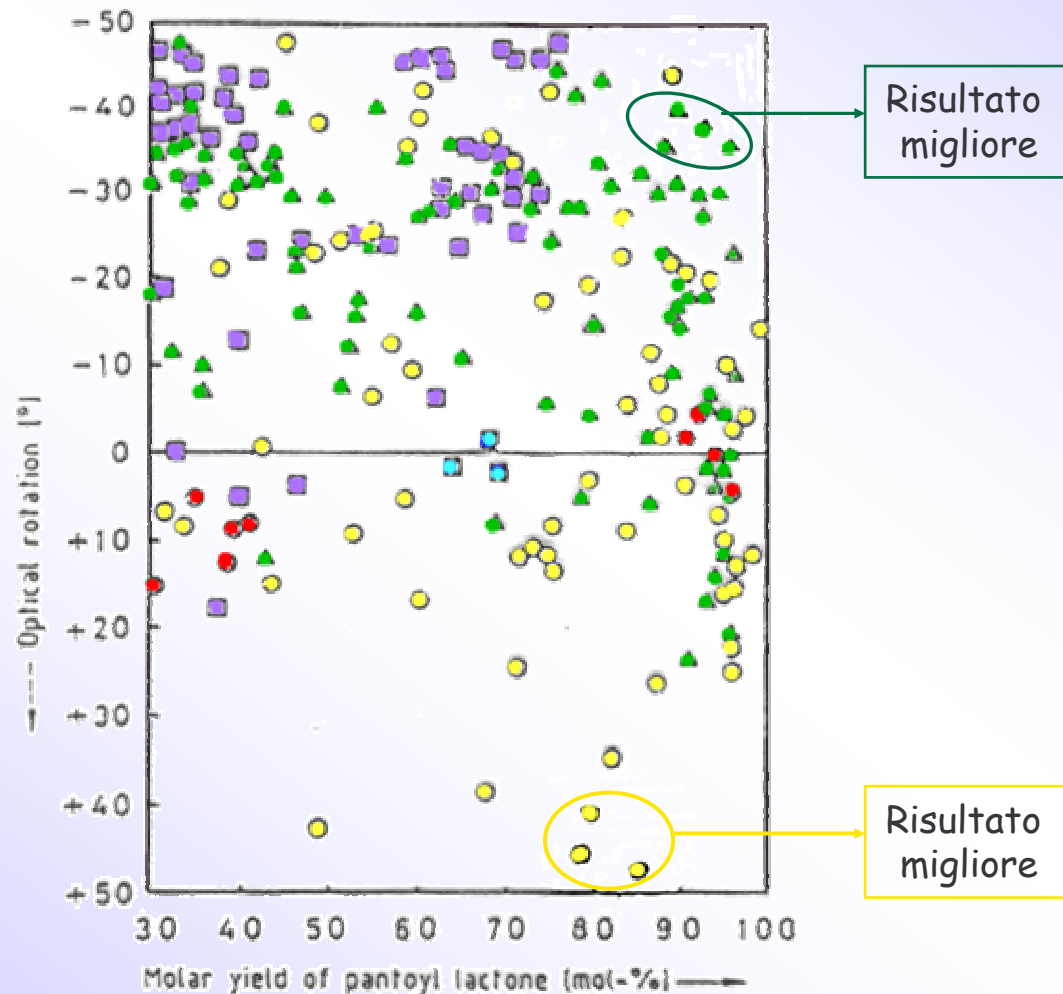
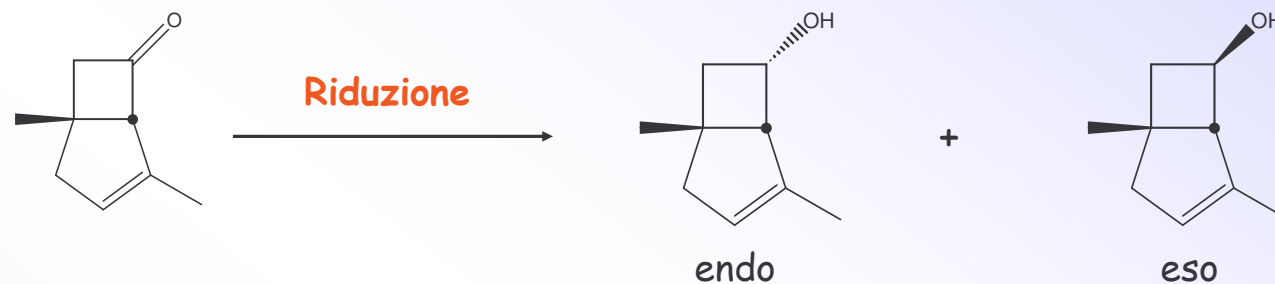


Fig. 6 - Diversità della riduzione microbica del chetopantol lattone a pantol lattone

- ▲ Lieviti
- Muffe
- Batteri
- Actinomiceti
- Basidiomiceti



Esempio - Riduzione semipreparativa con LIEVITI e MUFFE del 1,4-dimetilbiciclo[3.2.0]ept-3-en-6-one



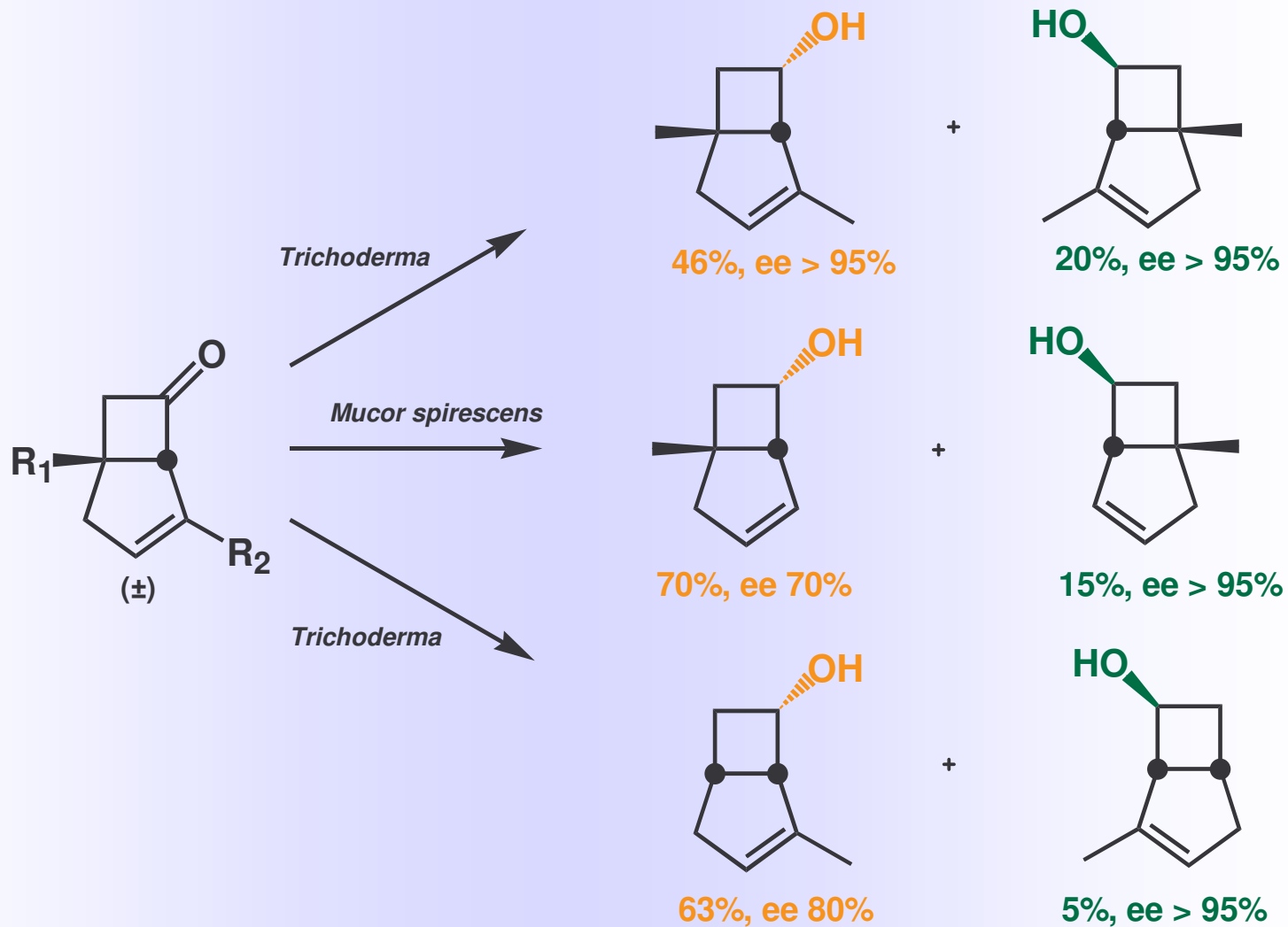
| Microorganismo | Resa chetone (ee%) ^a | | Resa endo (ee%) ^a | | Resa eso (ee%) ^a | |
|------------------------|---------------------------------|------------------|------------------------------|------------------|-----------------------------|------------------|
| | (-) ^b | (+) ^b | (-) ^b | (+) ^b | (-) ^b | (+) ^b |
| Saccharom. cerev. RM1 | -- | 13 (54) | 46 (100) | -- | 12 (100) | -- |
| Saccharom. cerev. RM74 | 36 (0) | | 3 (100) | -- | 31 (100) | -- |
| Yarrowia lipolytica Y2 | -- | 25 (100) | 48 (100) | -- | 7 (14) | -- |
| Yarrowia lipolytica Y9 | -- | 26 (100) | 43 (100) | -- | 6 (2) | -- |
| Fusarium | 25 (24) | -- | -- | 28 (38) | 7 (100) | -- |
| Trichoderma sp. | -- | 4 (100) | 46 (98) | -- | 20 (100) | -- |
| Penicillium roqueforti | -- | 40 (44) | 30 (100) | -- | 3 (100) | -- |

^a Le rese sono state calcolate con il metodo dello standard interno.

^b I poteri ottici rotatori delle coppie di enantiomeri sono stati attribuiti isolando i prodotti ottenuti da reazioni fatte su scala preparativa.

screening

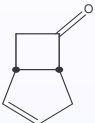
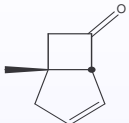
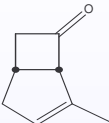
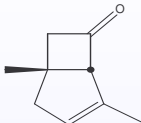
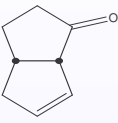
Altri substrati per la verifica del tipo di specificità dei microrganismi



Tetrahedron Asymmetry 7, 277 (1996)

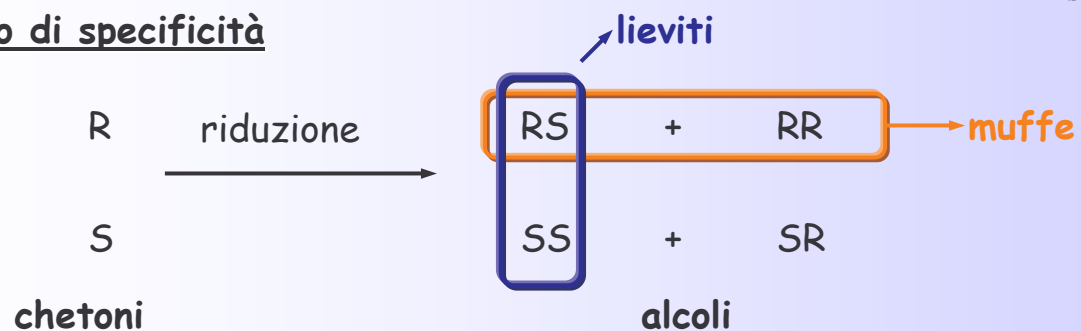
Informazioni ottenibili

"Specificità" dei microrganismi.

| |  |  |  |  |  |
|---------------------------|---|--|---|---|---|
| Saccharomyces cerev. RM1 | ● | ● | ● | ● | ● |
| Saccharomyces cerev. RM9 | ● | | | | |
| Saccharomyces cerev. RM74 | ● | | | ● | |
| Saccharomyces cerev. ML31 | ● | ● | ● | | ● |
| Yarrowia lipolytica Y2 | | | | ● | ● |
| Yarrowia lipolytica Y9 | | | | ● | |
| Mucor spirescens | ● | ● | ● | | ● |
| Trichoderma sp. | ● | ● | ● | ● | ● |
| Penicillium digitatum | ● | | | | |
| Penicillium roqueforti | | | | ● | |
| Fusarium | | | | ● | |
| Rhizopus nigricans | ● | | | | |

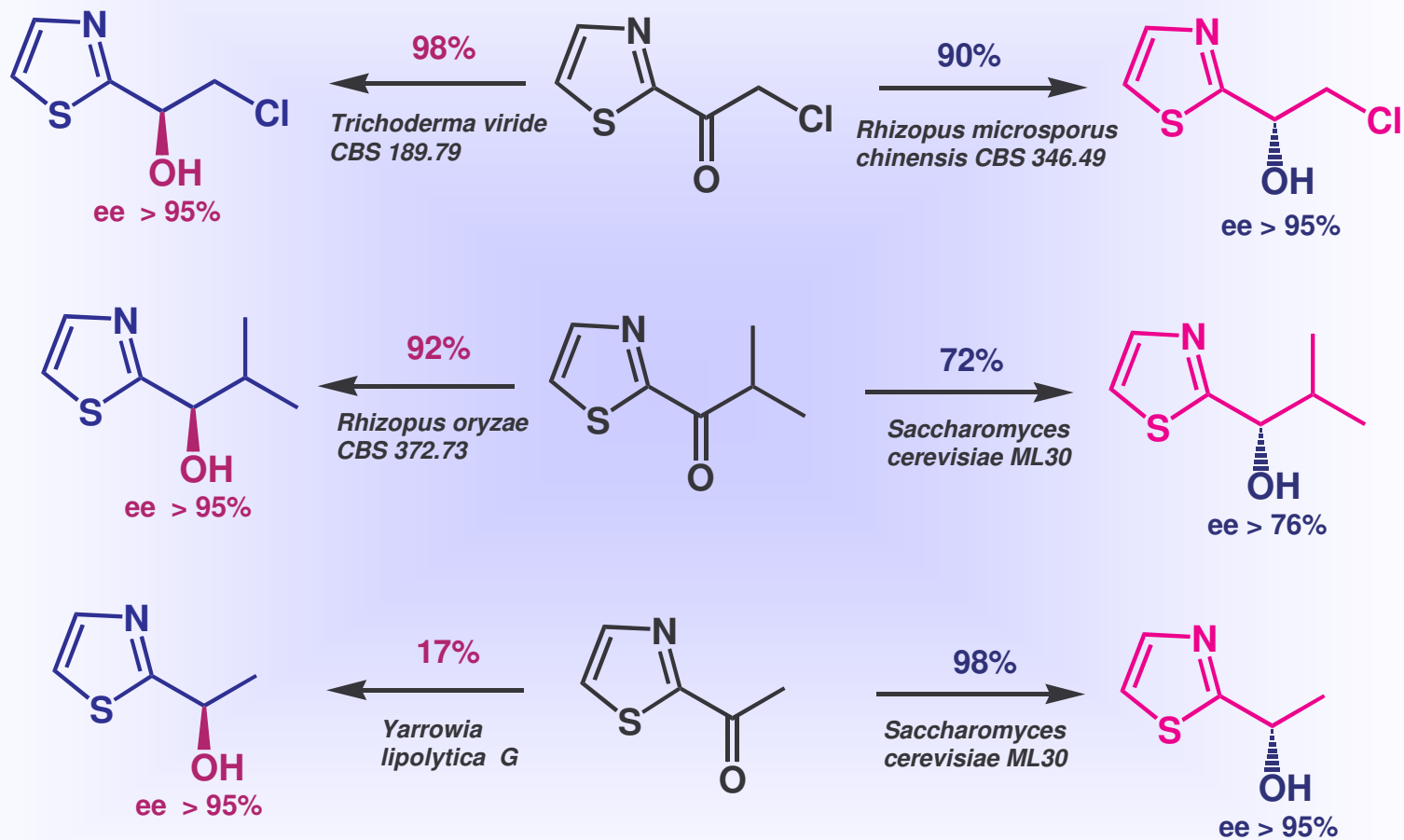
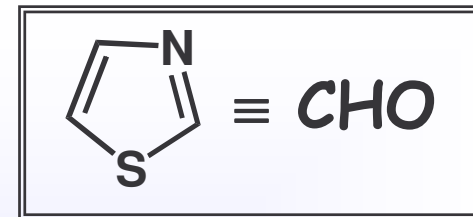
● = biotrasformazione avvenuta

Tipo di specificità



screening

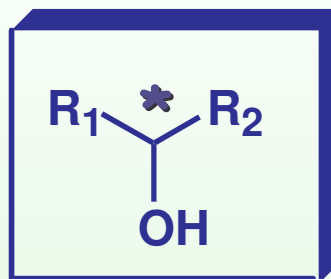
substrati mascherati



Tetrahedron Asymmetry 2, 243 (1991)



Tipo di reazione



Lieviti ed enzimi isolati



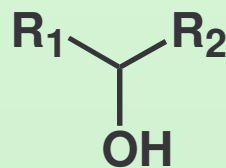
Lieviti e muffe
Generalmente
S-stereospecifica

ossidazione



Batteri





Generalmente
R-stereospecifica

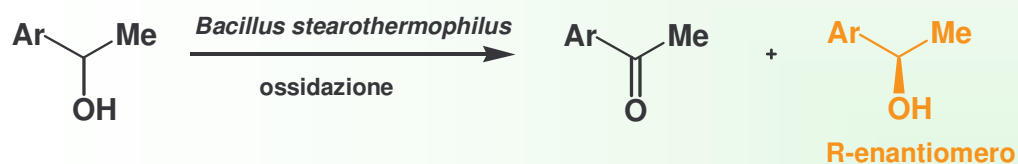


Tipo di reazione



Prima evidenza della capacità ossidativa di un lievito

| | | | |
|--|----------------------------|-----|----------------|
| Ar =  | 7 giorni/ tampone fosfato | 68% | 32% (ee 90%) |
|  | 9 giorni/ tampone fosfato | 90% | 10% (ee > 95%) |
|  | 13 giorni/ tampone fosfato | 64% | 36% (ee 86%) |
|  | 8 giorni/ tampone fosfato | 46% | 54% (ee 40%) |



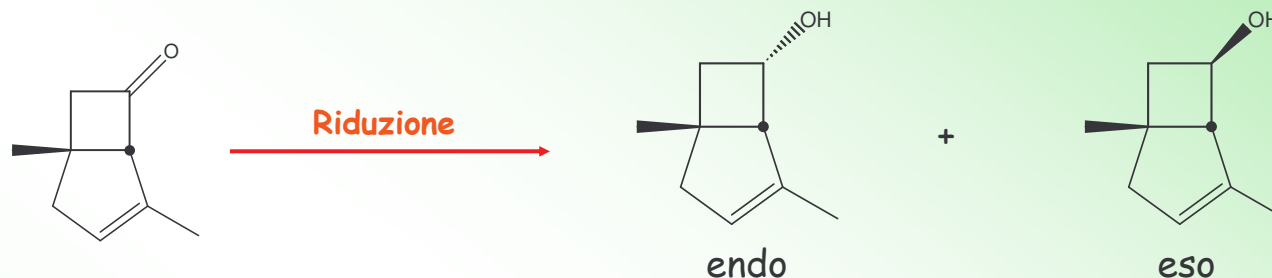
Tetrahedron Letters 34, 883 (1993)

| | | | |
|--|----------|-----|----------------|
| Ar =  | 6 giorni | 47% | 53% (ee > 95%) |
|  | 4 giorni | 50% | 50% (ee > 95%) |
|  | 6 giorni | 42% | 58% (ee 92%) |
|  | 6 giorni | 46% | 54% (ee 40%) |
|  | 6 giorni | 49% | 51% (ee > 95%) |
|  | 5 giorni | 40% | 60% (ee 90%) |

Ottima capacità ossidativa di un batterio

Tipo di reazione

Esempio - Confronto tra ossido-riduzioni microbiologiche.



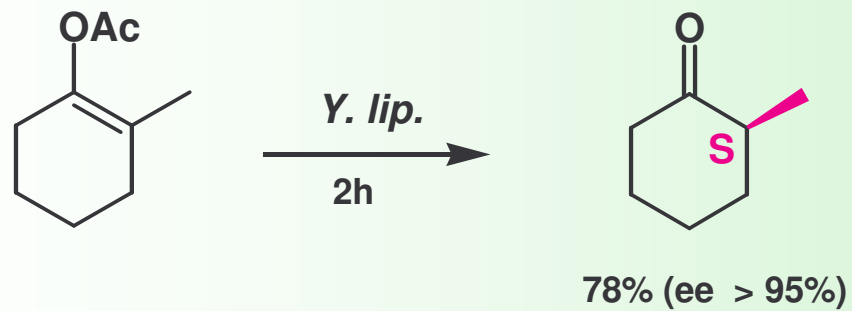
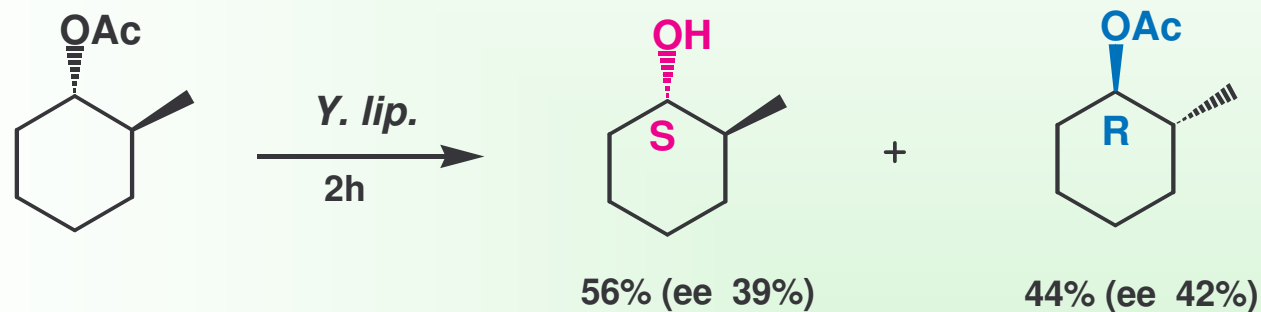
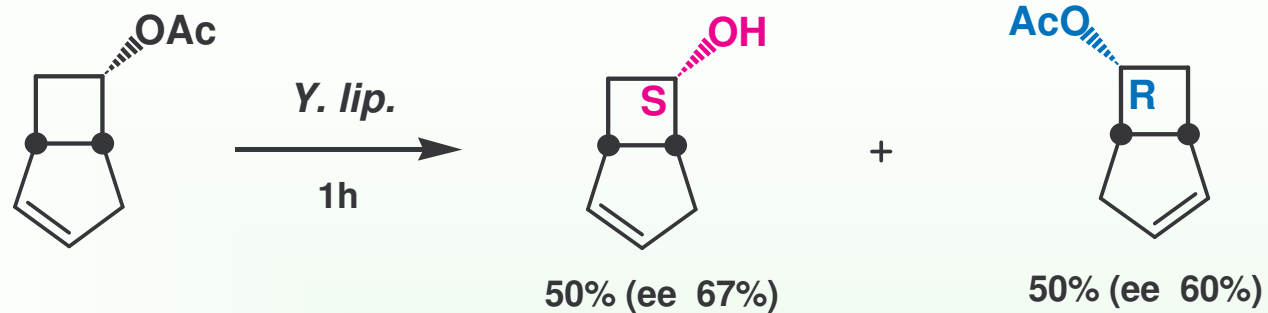
| | | | | | | |
|---|---------------------------------------|------------------------|------------------------------------|------------------------|-----------------------------------|------------------------|
| Penicillium roqueforti | -- | 66 (44) | 30 (100) | -- | 4 (100) | -- |
| Microrganismo | Resa chetone (ee%)^a | | Resa endo (ee%)^a | | Resa eso (ee%)^a | |
| | (-)^b | (+)^b | (-)^b | (+)^b | (-)^b | (+)^b |
| Bacillus stearotherm. ATCC2027 | 51 (86) | -- | -- | 49 (95) | | |



^a Le rese non sono state calcolate sui composti isolati, ma come rapporti tra le aree dei picchi gascromatografici considerando che dalla reazione si ottengano solo i prodotti desiderati e che si recuperi il 100% in peso dei prodotti.

^b I poteri ottici rotatori delle coppie di enantiomeri sono stati attribuiti isolando i prodotti ottenuti da reazioni fatte su scala preparativa.

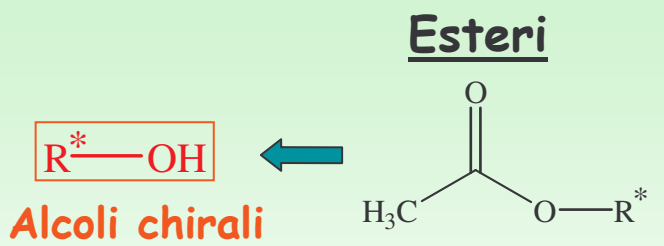
Tipo di reazione



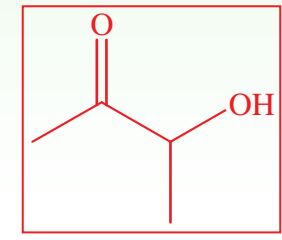
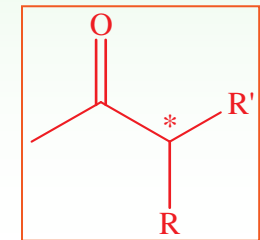
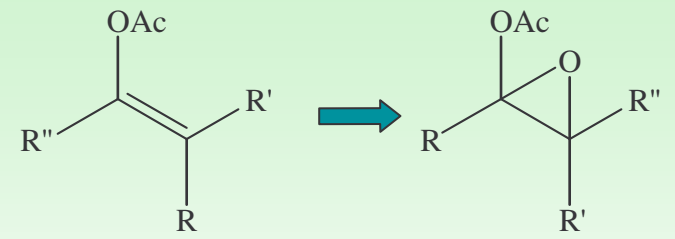
Metodologia:

- cellule fresche
- cellule liofilizzate
- solvente organico

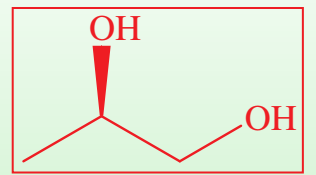
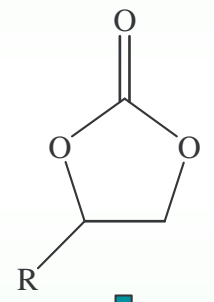
Idrolisi con YL



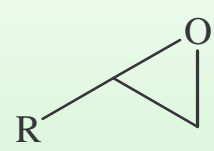
Enol Acetati



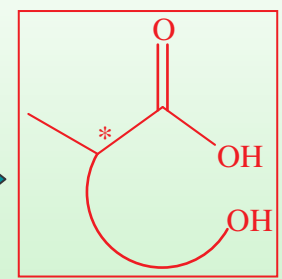
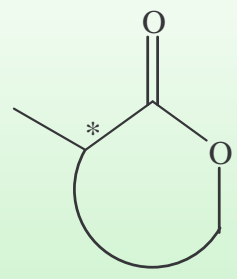
Carbonati



Epossidi

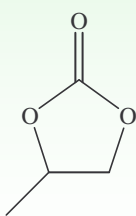


Lattoni

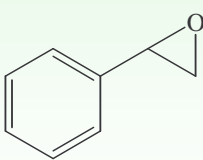


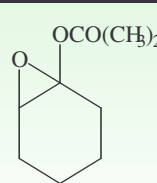
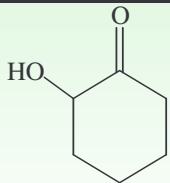
Idrolisi di substrati non convenzionali: risultati recentissimi

Carbonati

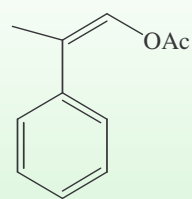
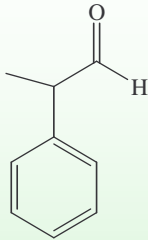
| | | |
|---|-------------|-------------|
|  | % | |
| | Resa | e.e. |
| | 37 | 39 GC1 |

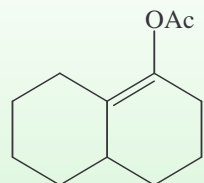
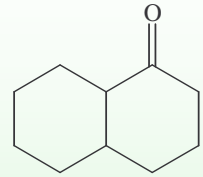
Epossidi

| | | |
|--|-------------|-------------|
|  | % | |
| | Resa | e.e. |
| | 40 | GC1 |

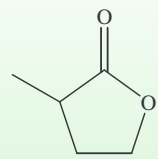
| | | | | | |
|---|-------------|-------------|---|-------------|-------------|
|  | % | |  | % | |
| | Resa | e.e. | | Resa | e.e. |
| 57 | 36 R,R | 43 | 23 | GC1 | |

Enol Acetati

| | | | |
|---|--|-------------|-------------|
|  |  | % | |
| | | Resa | e.e. |
| | | 28 | 72 GC2 |

| | | | |
|--|--|-------------|-------------|
|  |  | % | |
| | | Resa | e.e. |
| 65 | 40 | GC1 | |

Lattoni

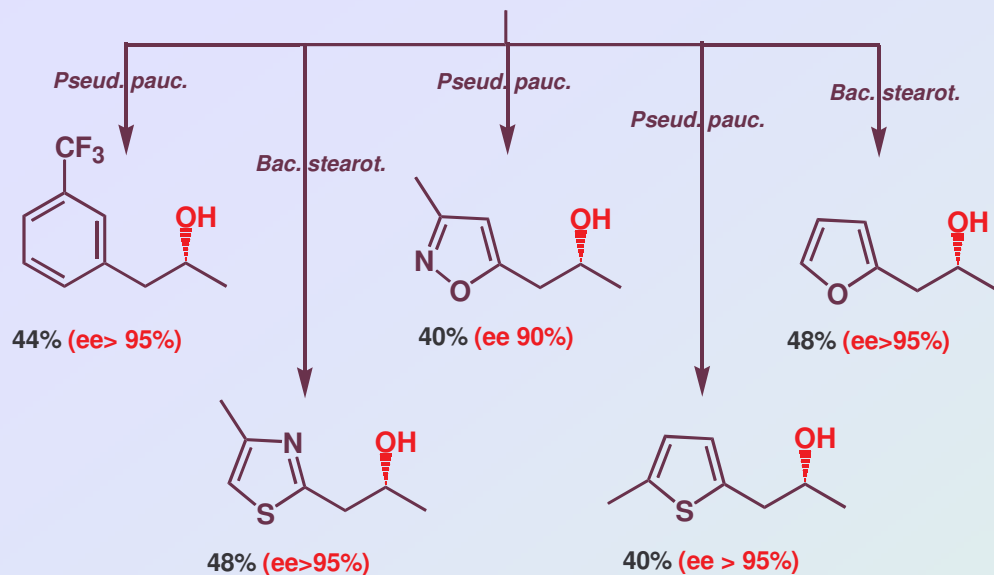
| | | |
|---|-------------|-------------|
|  | % | |
| | Resa | e.e. |
| | 48 | 72 GC2 |



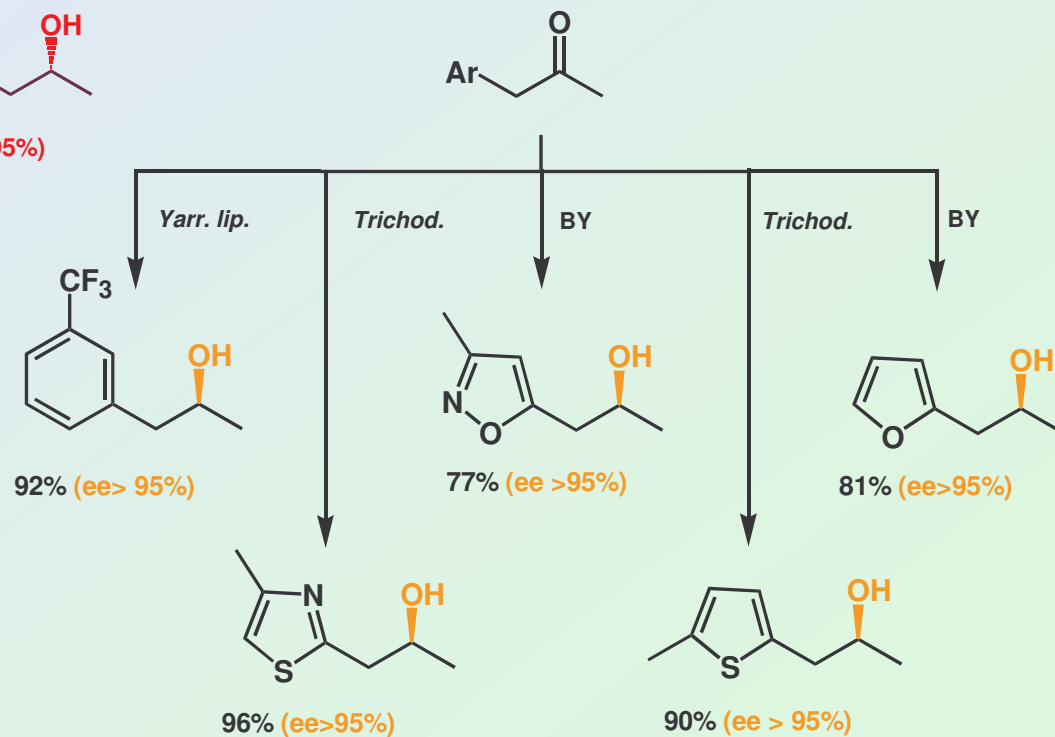
screening



tipo di reazione



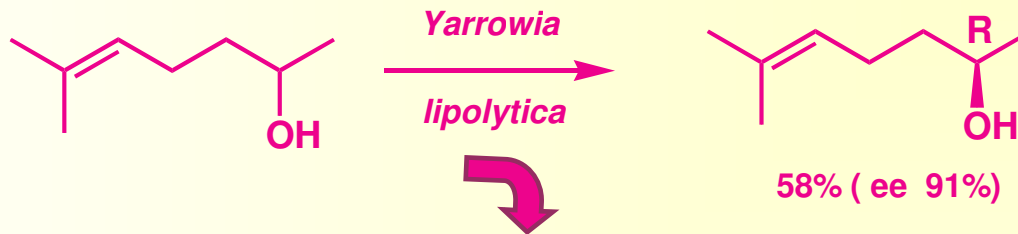
Tetrahedron Asymmetry 9, 2317 (1998)



Tetrahedron Asymmetry 9, 2317 (1998)

screening + tipo di reazione

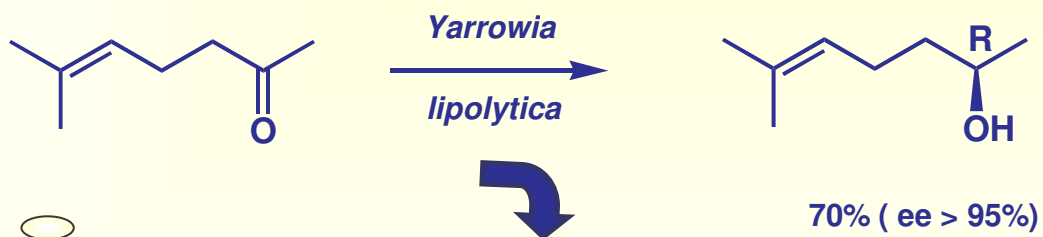
Ossidazione



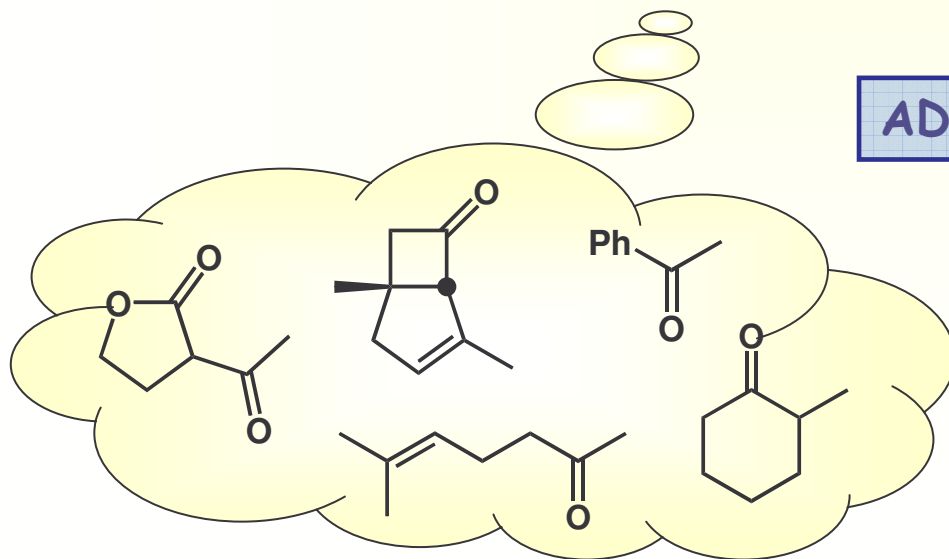
Incidente di percorso ?

ADH S-stereospecifica

Riduzione



ADH R-stereospecifica





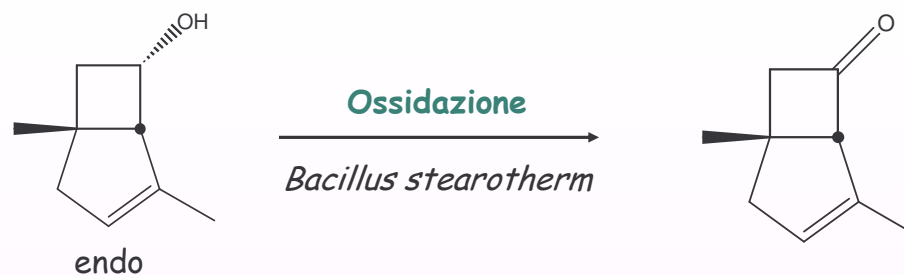
Fonte di carbonio: cambio del terreno



| Ar chetone | Terreno | Giorni | Resa % | ee % |
|------------|----------------------|--------|--------|---------|
| 2-furyl | Glucosio | 5 | 7 | 20 (S) |
| | Tampone fosfato pH=5 | 5 | 7 | 80 (S) |
| | Sodio citrato | 7 | 15 | 60 (S) |
| | Sodio lattato | 7 | 10 | 50 (S) |
| 2-thienyl | Glucosio | 5 | 14 | 22 (S) |
| | Sodio succinato | 3 | 10 | 86 (S) |
| | Sodio lattato | 3 | 10 | 82 (S) |
| Phenyl | Glucosio | 4 | 90 | 100 (S) |
| 3-pyridyl | Glucosio | 6 | 100 | 76(S) |

| Ar alcol | Terreno | Giorni | Resa % | ee % |
|-----------|------------------------------------|--------|--------|---------|
| 2-furyl | Tampone fosfato pH=5 | 7 | 39 | 90 (R) |
| | CH ₃ COONH ₄ | 7 | 17 | 100 (S) |
| | Glucosio | 5 | 51 | 66 (R) |
| 2-thienyl | CH ₃ COONa | 5 | 59 | 38 (S) |
| | CH ₃ COONH ₄ | 7 | 21 | 100 (R) |
| | Sodio citrato | 5 | 60 | 18 (S) |
| Phenyl | CH ₃ COONa | 6 | 70 | 18 (S) |
| | CH ₃ COONH ₄ | 8 | 41 | 100 (R) |
| | Sodio citrato | 8 | 47 | 72 (S) |
| 3-pyridyl | Sodio succinato | 8 | 39 | 74 (R) |

Esempio - Ossidazione con terreni modificati.



| Terreno ^b | Resa alcol (ee%) ^a | | Resa chetone (ee%) | |
|----------------------------------|-------------------------------|---------------|--------------------|-----|
| | (-) | (+) | (-) | (+) |
| Glucosio | -- | -- | -- | -- |
| Saccarosio ^c | -- | 86(76) | 14(100) | -- |
| Acido citrico^d | -- | 49(95) | 51(86) | -- |
| Mg ^{++e} | -- | 86(14) | 14(100) | -- |
| Mn ^{++e} | -- | 94(8) | 6(100) | -- |
| Fe ^{++e} | -- | 97(2) | 3(100) | -- |

^a Le rese non sono state calcolate sui composti isolati, ma come rapporti tra le aree dei picchi gascromatografici considerando che dalla reazione si ottengano solo i prodotti desiderati e che si recuperi il 100% in peso dei prodotti. (ee%) sta per eccesso enantiomerico.

^b Il terreno di coltura è stato variato rispetto allo standard utilizzato per *Bacillus stearothermophilus* sia come fonte di carbonio sia per la presenza di sali.

^c Terreno modificato: fonte di carbonio è il saccarosio.

^d Terreno modificato: fonte di carbonio è l'acido citrico.

^e Terreno modificato con la presenza di un unico sale.

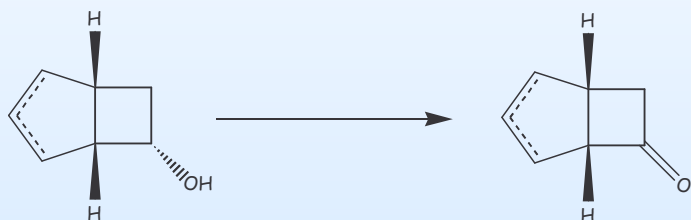


Effetto del solvente

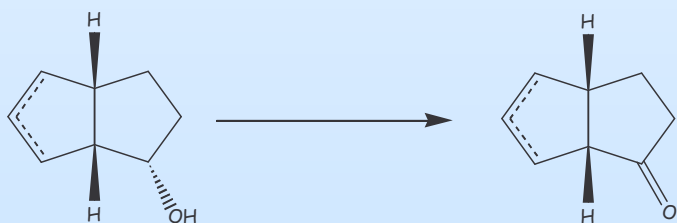
Le ossidazioni con *Bacillus stearothermophilus* in eptano costituiscono il primo esempio di reazioni con batteri in ambiente organico.

VANTAGGI

stessa efficienza delle reazioni in acqua
volumi di reazione tre volte minori
procedura più semplice



| Condizioni | Tempo (h) | Resa % | ee % |
|---------------|-----------|--------|-------------|
| Acqua | 6 | 41 | 99 (1S,5R) |
| Eptano | 6 | 49 | 100 (1S,5R) |
| Immobilizzato | 3 | 41 | 100 (1S,5R) |

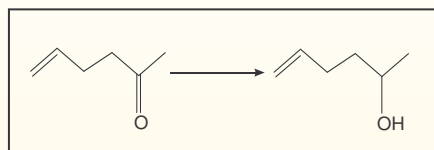
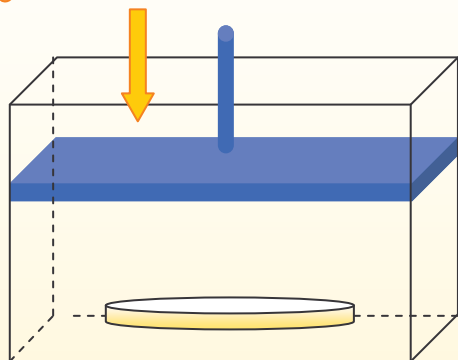


| Condizioni | Tempo (h) | Resa % | ee % |
|------------|-----------|--------|-------------|
| Acqua | 4 | 37 | 100 (1S,5S) |
| Eptano | 4 | 48 | 98 (1S,5S) |

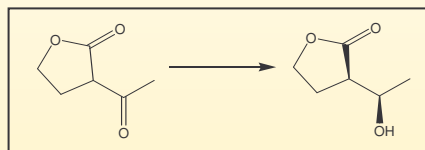


Variazione di Pressione

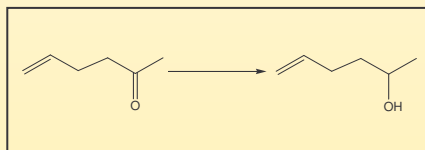
Pressione statica



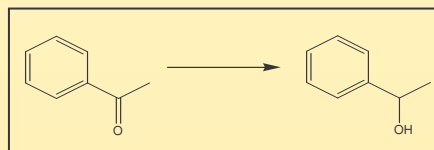
| <i>Saccharomyces cerevisiae</i> RM1 | | |
|-------------------------------------|--------|---------|
| Pressione | Resa % | ee % |
| P. atm. | 77 | 84 (S) |
| 400 bar | 21 | 100 (S) |



| <i>Yarrowia lipolytica</i> Y5 | | |
|-------------------------------|--------|-------------|
| Pressione | Resa % | ee % |
| P. atm. | 6 | 100 (3R,1R) |
| 1500 bar | 91 | 100 (3R,1R) |

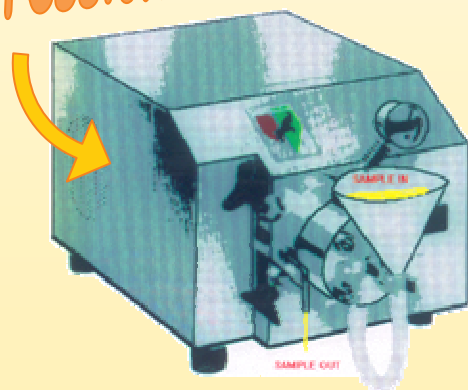


| <i>Yarrowia lipolytica</i> PO5 | | |
|--------------------------------|--------|--------|
| Pressione | Resa % | ee % |
| P. atm. | 22 | 14 (R) |
| 1000 bar | 25 | 33 (S) |



| <i>Saccharomyces cerevisiae</i> ML29 | | |
|--------------------------------------|--------|--------|
| Pressione | Resa % | ee % |
| P. atm. | 24 | 53 (S) |
| 1500 bar | 50 | 92 (S) |

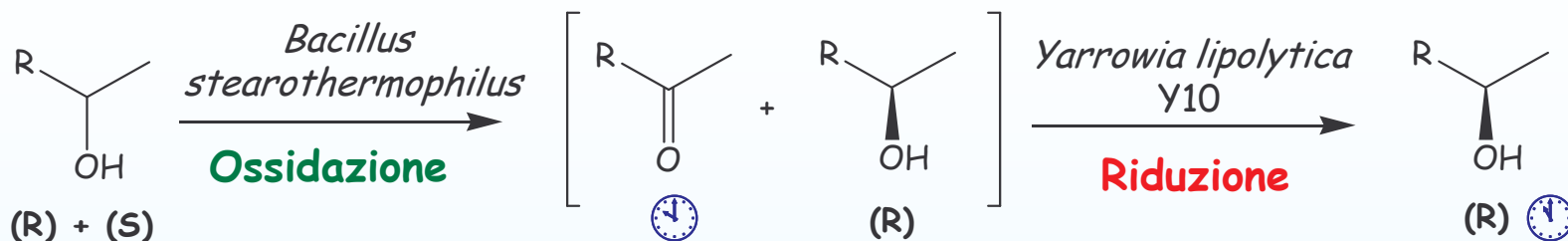
Pressione dinamica

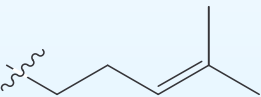
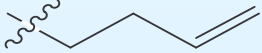
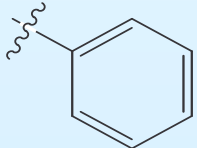


Omogeneizzatore

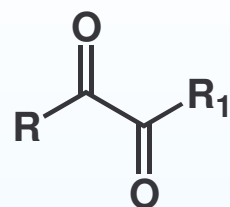
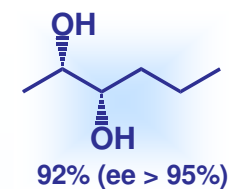
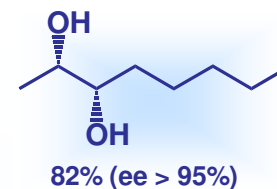
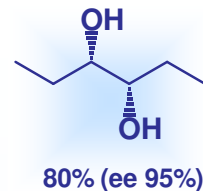


Reazioni combinate



| R | Metodo | ⌚ Resa % | ⌚ Resa % | ee % |
|---|--------|----------|----------|---------|
|  | A | 13 | 87 | 100 (R) |
| | B | 9 | 91 | 100 (R) |
| | C | 18 | 82 | 90 (R) |
|  | A | 0 | 100 | 100 (R) |
| | B | 9 | 91 | 100 (R) |
| | C | 15 | 85 | 40 (R) |
|  | A | 12 | 88 | 40 (R) |
| | B | 20 | 80 | 85 (R) |
| | C | 0 | 100 | 0 |

Reazioni enzimatiche combinate

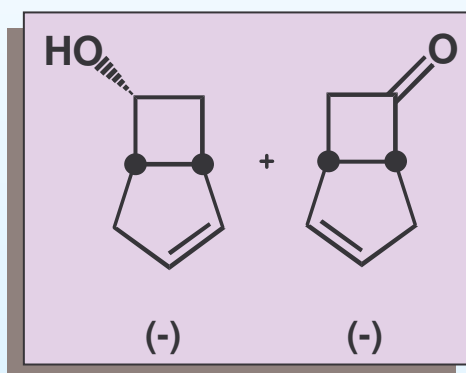
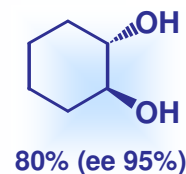
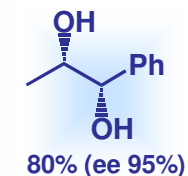
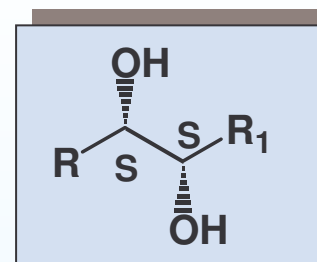


BSDR

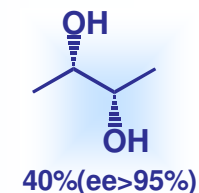
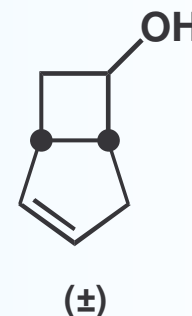


NADH

NAD⁺



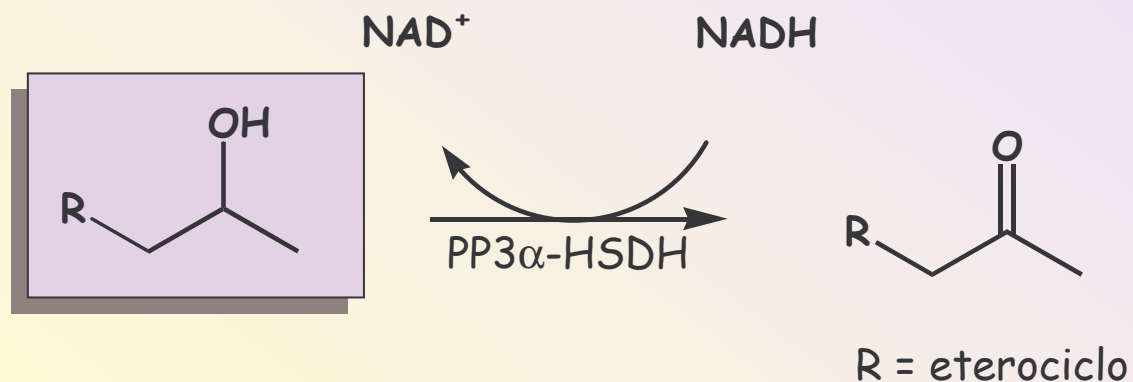
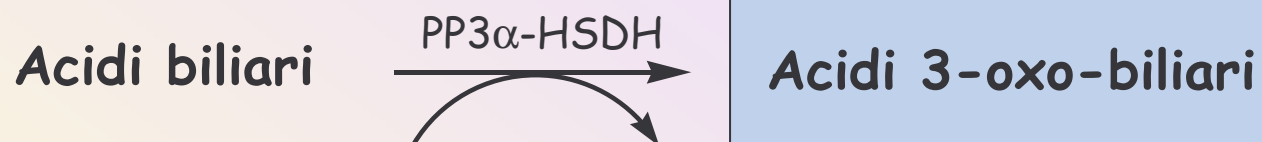
BSDR



BSDR: Diacetile Reduttasi da *Bacillus Stearothermophilus*

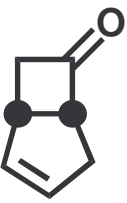

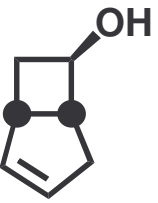
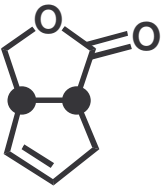
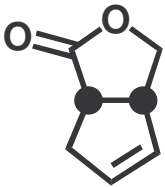


L'enzima è stato impiegato in un sistema monoenzimatico che accoppiando due reazioni ad alto valore sintetico garantisce il riciclo del cofattore.

Reazioni enzimatiche combinate



L'enzima da *Pseudomonas paucimobilis* (PP3 α -HSDH), oltre a suggerire l'uso per scopi sintetici accoppiando due reazioni ad alto valore aggiunto, può essere usato per applicazioni volte a determinare la presenza di tracce di 3 α -idrossi steroidi in analisi biomediche.

Screening e ottimizzazione di una biotrasformazione di interesse applicativo

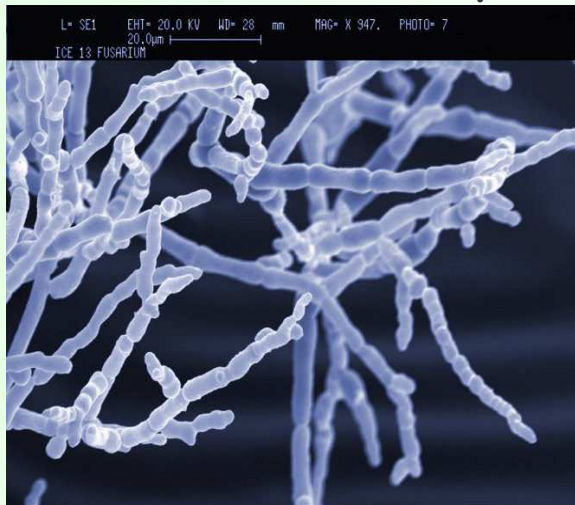
| Ceppo | Resa % | | | Resa% (ee%) | | | |
|-------|--|---|--|--|---|--|---|
| |  (±)-1 |  |  |  6 |  |  7 |  |
| | | | (-)-(1S,5R) | (+)-(1R,5S) | (-)-(1S,5R) | (+)-(1R,5S) | |
| E1 | 41 | 41 | 9 | | 6 (33) | | 10 (>98) |
| E5 | 21 | 34 | 32 | | 3 (33) | | 10 (19) |
| E6 | 47 | 32 | 15 | | 6 (9) | --- | |
| E19C | 31 | 26 | 11 | 5 (13) | | | 28 (72) |
| E19D | 47 | 13 | 6 | | 8 (<1) | | 26 (90) |
| ICE3 | 62 | 14 | 22 | | --- | | 5 (33) |
| ICE7 | 43 | 26 | 13 | | --- | 5 (20) | |
| ICE13 | 24 | 33 | 14 | | 6 (33) | | 23 (82) |
| ICE15 | 26 | 47 | 10 | | 7 (14) | | 10 (20) |
| ICE16 | 25 | 28 | 20 | | --- | | 27 (68) |

Identificazione

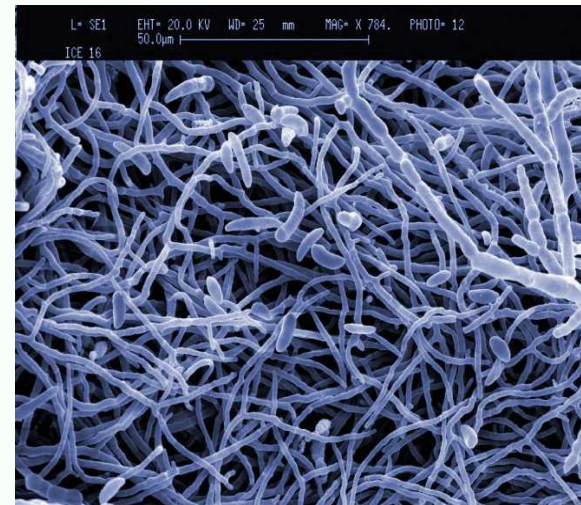
Microscopia:

- ottica
- elettronica
a scansione
- elettronica
a trasmissione

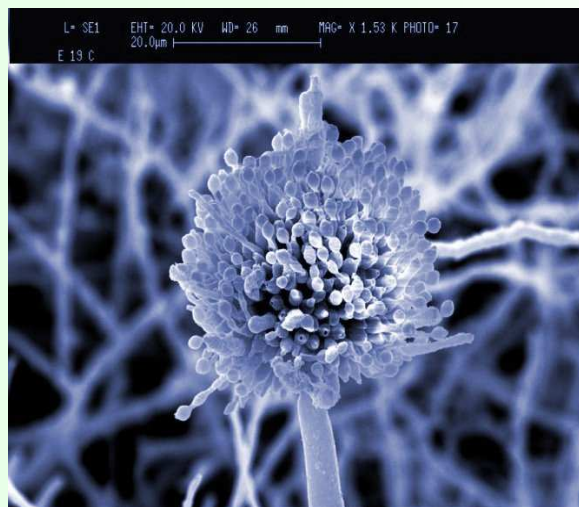
ICE13: *Fusarium* sp.



ICE16: *Fusarium solani*



E19C: *Aspergillus terricola*
var. *americana*

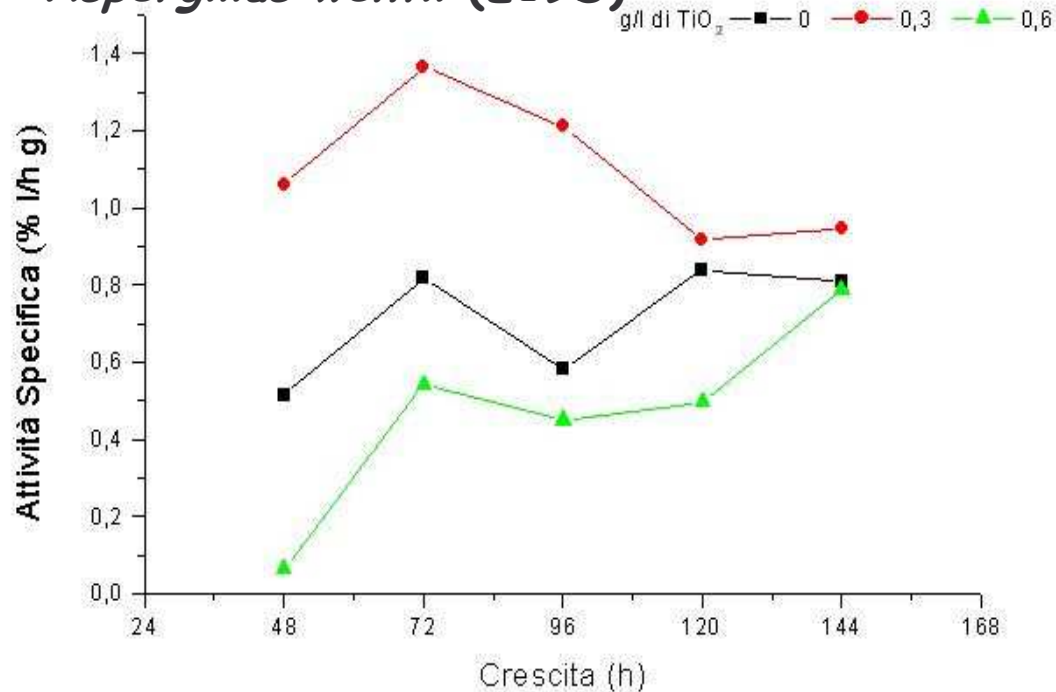


E19D: *Aspergillus wentii*

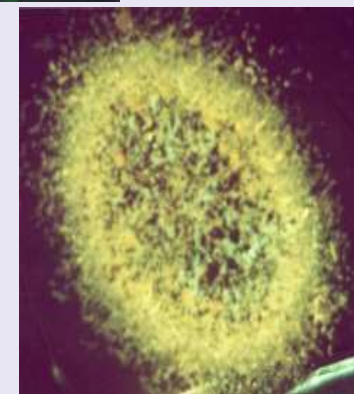
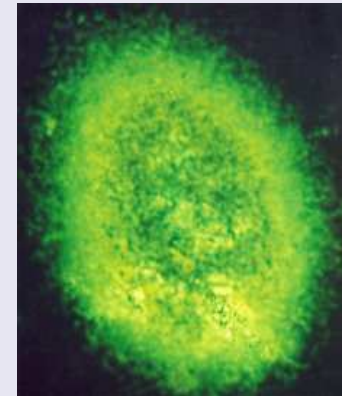


Impiego di TiO_2

Aspergillus wentii (E19D)



Aspergillus wentii
(E19D)

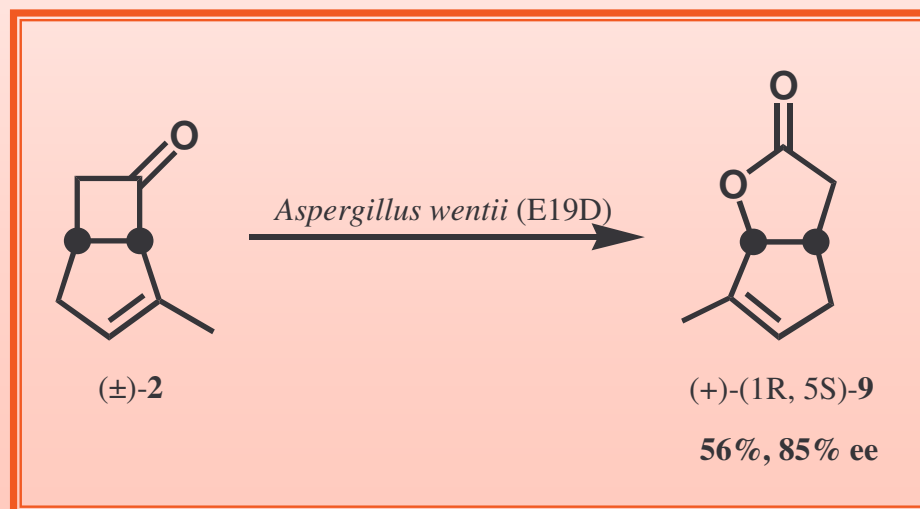
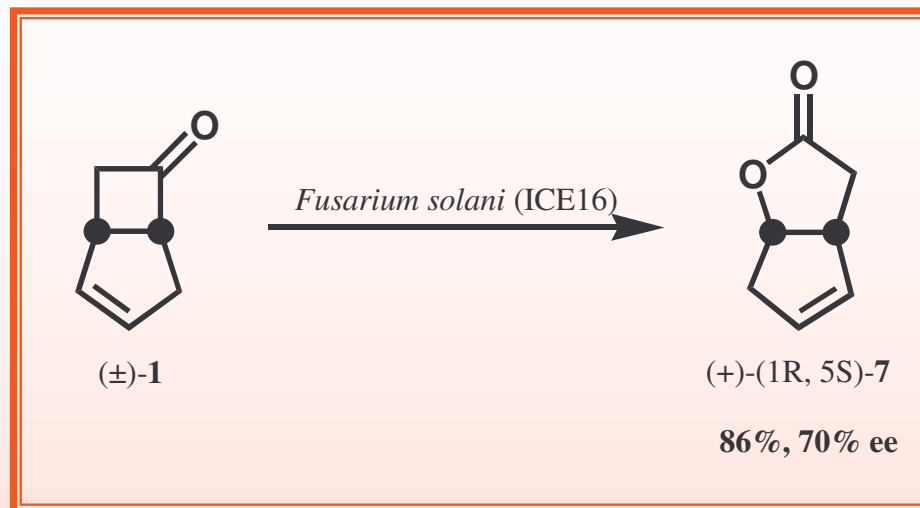


Aspergillus wentii
(E19D)
- 0.3 g/l TiO_2

Vantaggi:

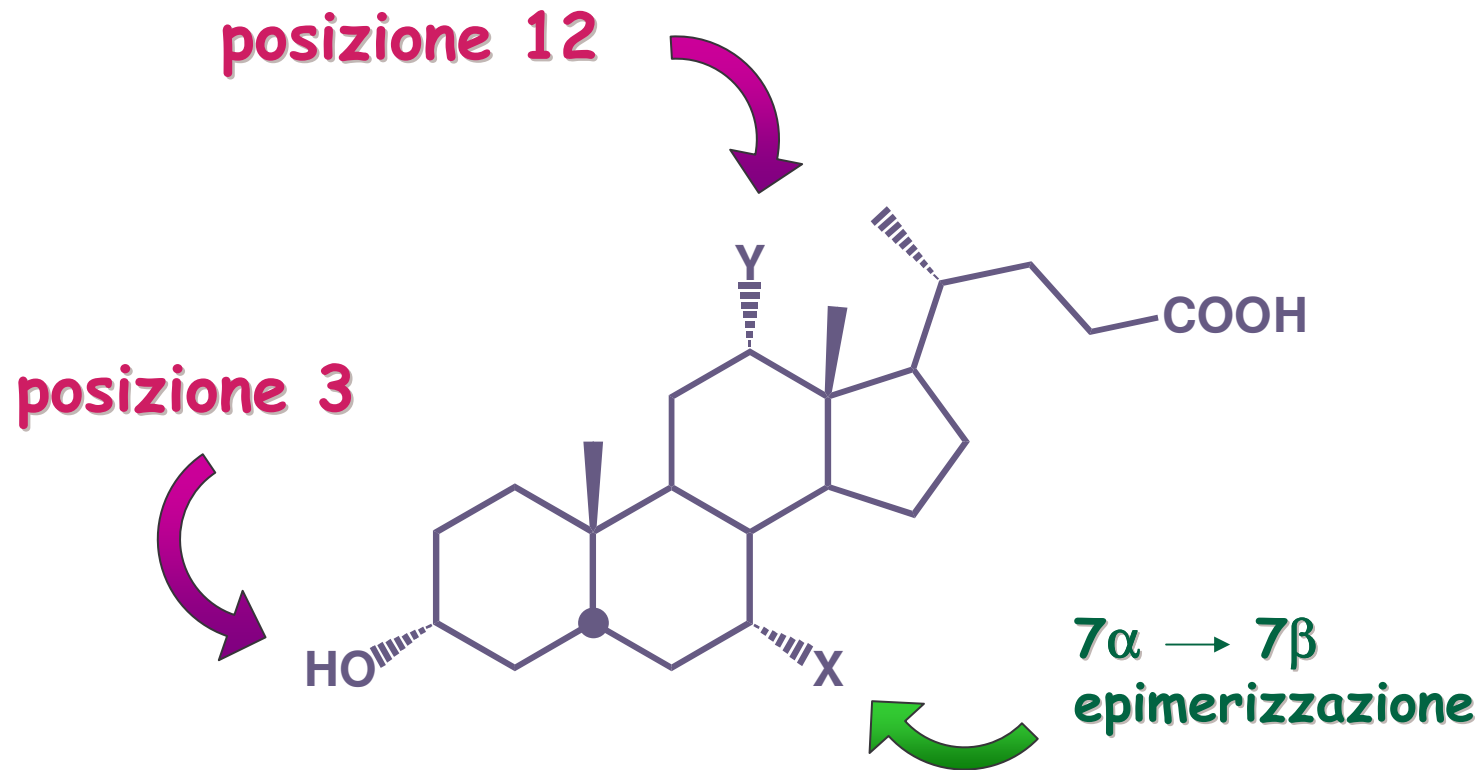
- favorisce la formazione di microcolonie
- evita la necrotizzazione delle cellule interne alla colonia
- aumenta la superficie di scambio
- potenziale smaltimento della biomassa per irradiazione

Risultati migliori



Biotrasformazioni di interesse industriale

Ossidazione ed epimerizzazione regioselettiva



Regioselettività dell'ossidazione

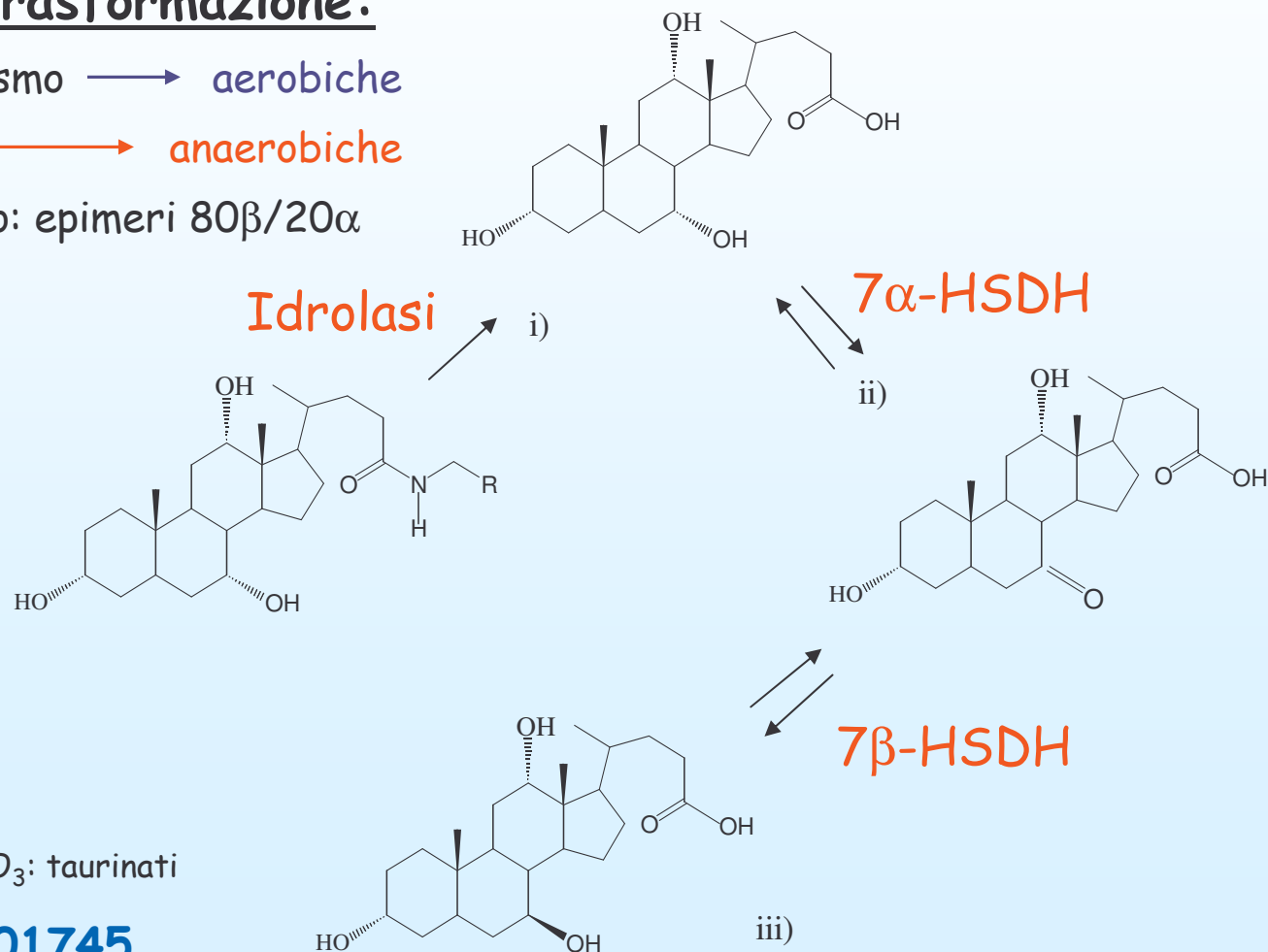
| C ₇ -ossidazione | C ₃ -ossidazione | C ₁₂ -ossidazione |
|--|---|---|
| <p><i>Xanthomonas malthophilia</i> BS6</p> <p><i>Pseudomonas fluorescens</i> BS7</p> <p><i>Pseudomonas fluorescens</i> B18</p> <p><i>Bacillus mycoides</i> B23</p> <p><i>Acinetobacter calcoaceticus</i> <i>lwoffii</i> BS11</p> <p><i>Acinetobacter calcoaceticus</i> <i>lwoffii</i> B37</p> <p><i>Acinetobacter calcoaceticus</i> <i>acidovorans</i> B49</p> <p><i>Pseudomonas fluorescens</i> B25</p> <p><i>Pseudomonas fluorescens</i> B26</p> | <p><i>Pseudomonas fluorescens</i> B18</p> <p><i>Bacillus mycoides</i> B23</p> <p><i>Pseudomonas fluorescens</i> B25</p> <p><i>Pseudomonas fluorescens</i> B26</p> | <p><i>Acinetobacter calcoaceticus</i> <i>lwoffii</i> BS11</p> <p><i>Acinetobacter calcoaceticus</i> <i>lwoffii</i> B37</p> <p><i>Acinetobacter calcoaceticus</i> <i>acidovorans</i> B49</p> <p><i>Pseudomonas fluorescens</i> B25</p> <p><i>Pseudomonas fluorescens</i> B26</p> |

Reazioni Catalizzate da

Xanthomonas maltophilia CBS 827.97

Condizioni della biotrasformazione:

- 1) crescita del microrganismo → **aerobiche**
- 2) epimerizzazione → **anaerobiche**
- 3) estrazione del prodotto: epimeri 80β/20α
- 4) utilizzo della biomassa per idrolisi della bile



Brevetto n° MI97A001745

Purificazione degli Enzimi di

Xanthomonas maltophilia

