S & C BEST S.r.l.
Biotecnologie Enzimi
Servizi Tossicologici

From Research to Production: the idea evolution in a chemical industry

Claudia Cusan
European Registered Toxicology
Independent Consultant in Toxicology, Pharmacology and Sustainable Products
Trieste, May 2016

CV

S & C BEST S.r.l.
Biotecnologie Enzimi
Servizi Tossicologici

- December 2017 – Current: Vicepresident of S & C BEST Srl (Biotecnologie, Enzimi, Servizi Tossicologici)
- April 2017-Current: Independent Consultant in Toxicology and Sustainable Products (self-employed)
- 2013 – 2017 (March): Corporate Expert in Product Stewardship and Toxicology at DSM Operations & Responsible Care (Heerlen, NL)
- 2010 - 2013: Project Manager at DSM Innovative Synthesis (Geleen, NL)
- 2005-2010: Research Scientist at DSM Pharmaceutical Products (Geleen, NL)
- 2004: PostDoc at università di Trieste
- 2004- PhD in Pharmaceutical Science at università di Trieste
- 2000- Master Degree cum laudae in CTF (magistrale), università di Trieste

Introduction

- Presentation goals:
  - Show how an enzymatic reaction is generated and developed within a chemical industry
  - Show some key elements of industrial approach

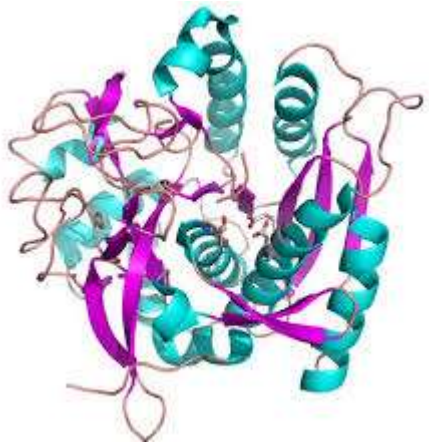
- It is not a goal of this presentation:
  - Describe in details enzymes and the enzymatic transformations plus and minus

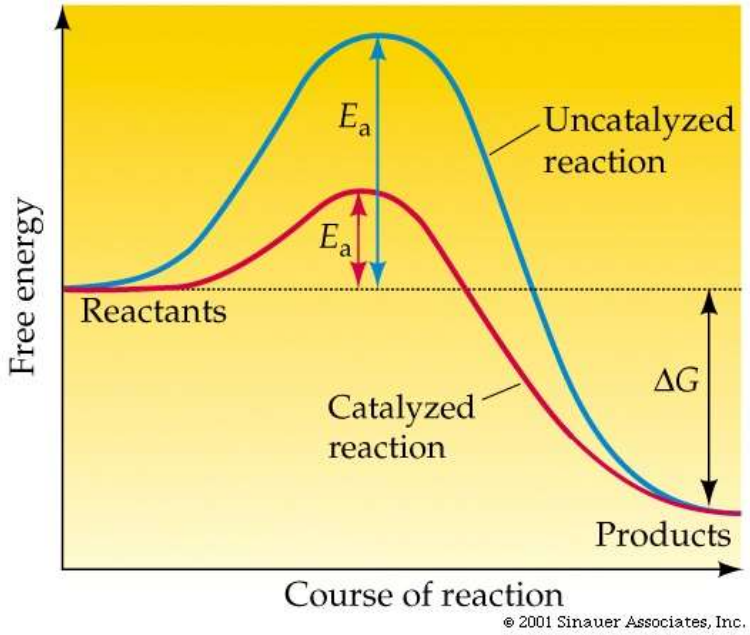
- La presentazione e`disponibile c/o Prof. Gardossi
- L`interazione e`apprezzata ☺

Enzymes

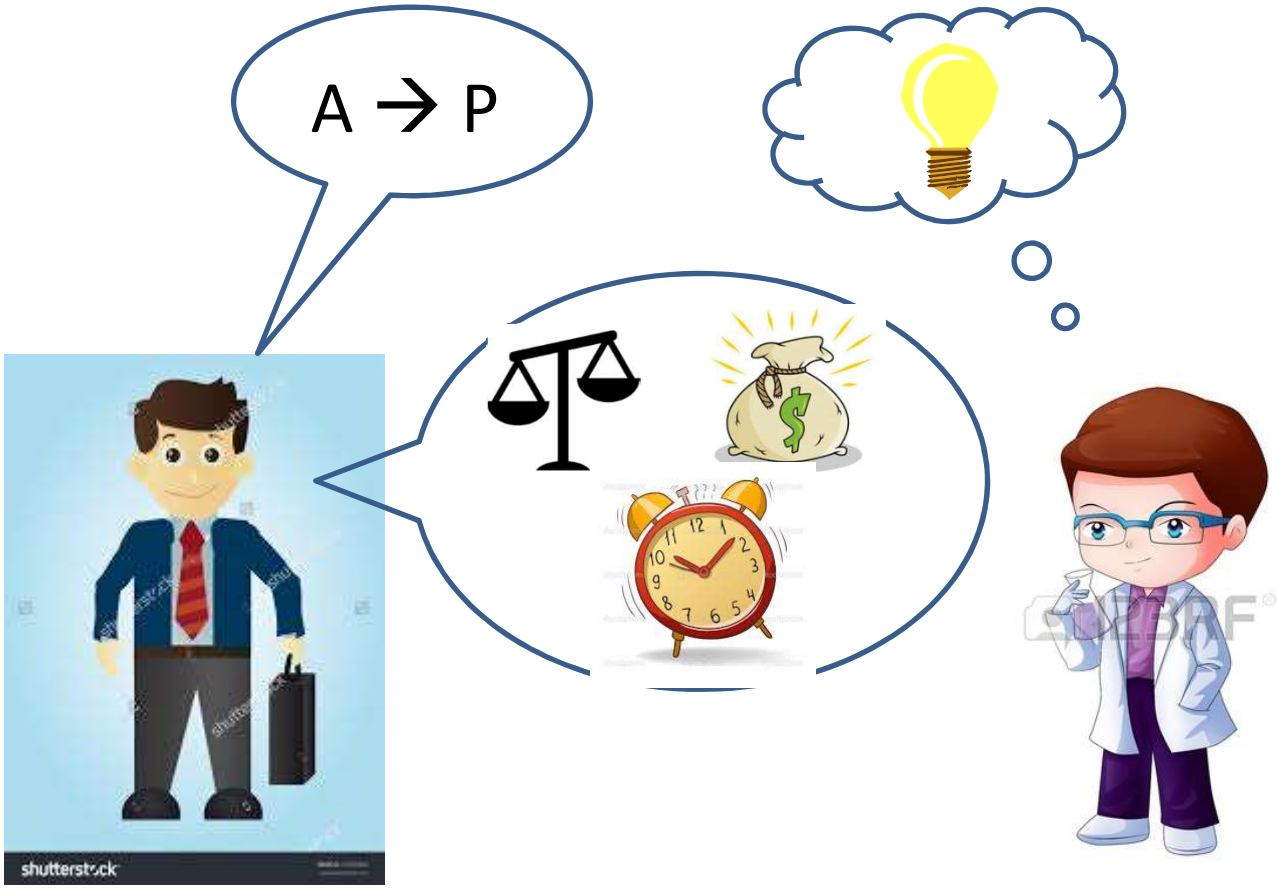
Enzymes are proteins, which act as catalists

(→ increase the reaction speed, without changing the reaction equilibrium, and acting on the activation energy)





Development of an enzymatic reaction: Step 0: Contact with the client



Customers

Project leader

Idea evaluation = “brain storm”

Project leader

Analytical expert

Chemist

Sustainability expert

Technologist

Patent attorney

R&D Team manager

Process safety specialist

-Do we agree on an enzymatic transformation

-Yes, we do

-Great, let's go

Goal of this meeting is the identification of a synthetic route, evaluating risks and opportunities of different options (technology, costs, involved competences

-Do we agree on an enzymatic transformation
-Yes, we do
-Great, let‘s go

If you are the chemist, where do you go?

5 steps from idea to market

1. Idea evaluation
2. Feasibility studies
3. Research & Development
4. Protocol validation & Scale up
5. Production

5 steps from idea to market

1. Idea evaluation
2. Feasibility studies
3. Research and Development
4. Protocol validation & Scale up
5. Production

Literature search

- Literature search, to identify enzymes and reaction conditions

- Result (example): 2 o 3 enzymes, each with a different experimental protocol

Literature data reproduction

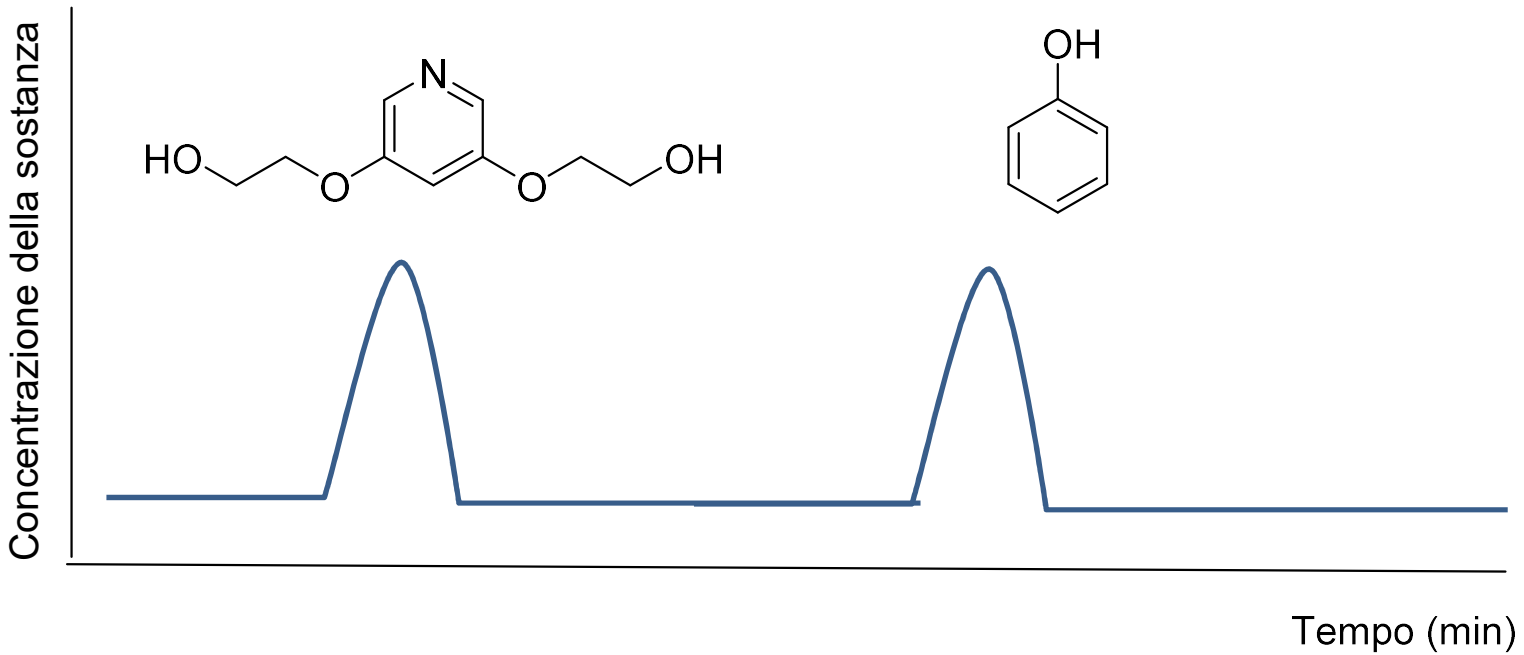
- The enzymes found in literature are tested according to literature conditions, on the client substrate “A”
  (the literature protocol is probably applied on other substrates, which chemical structure similar to A)

What is necessary to organize, before starting the enzymatic reactions?

Analytical method

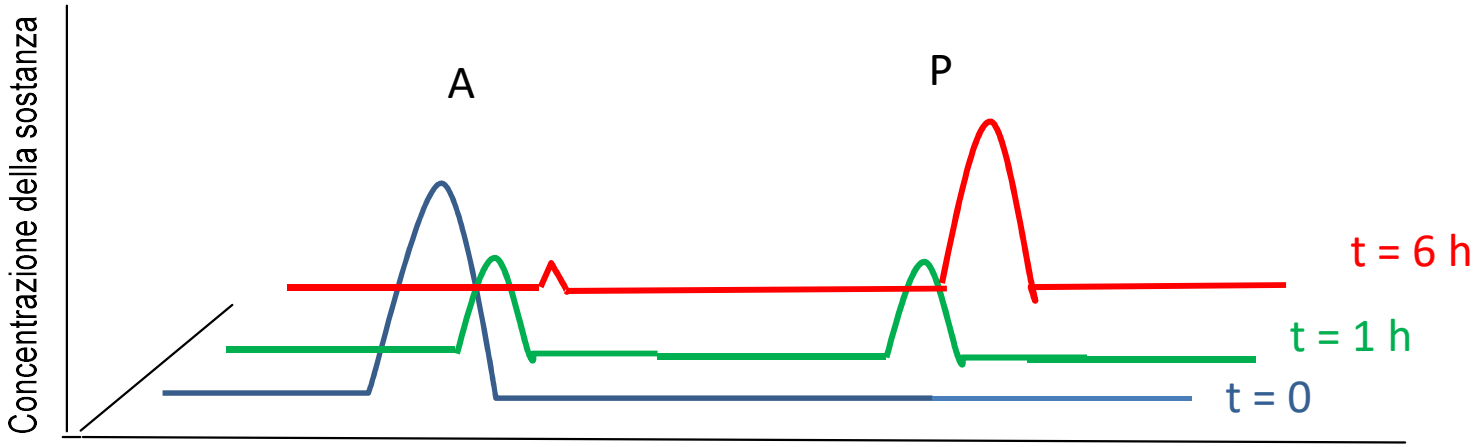
Analisi

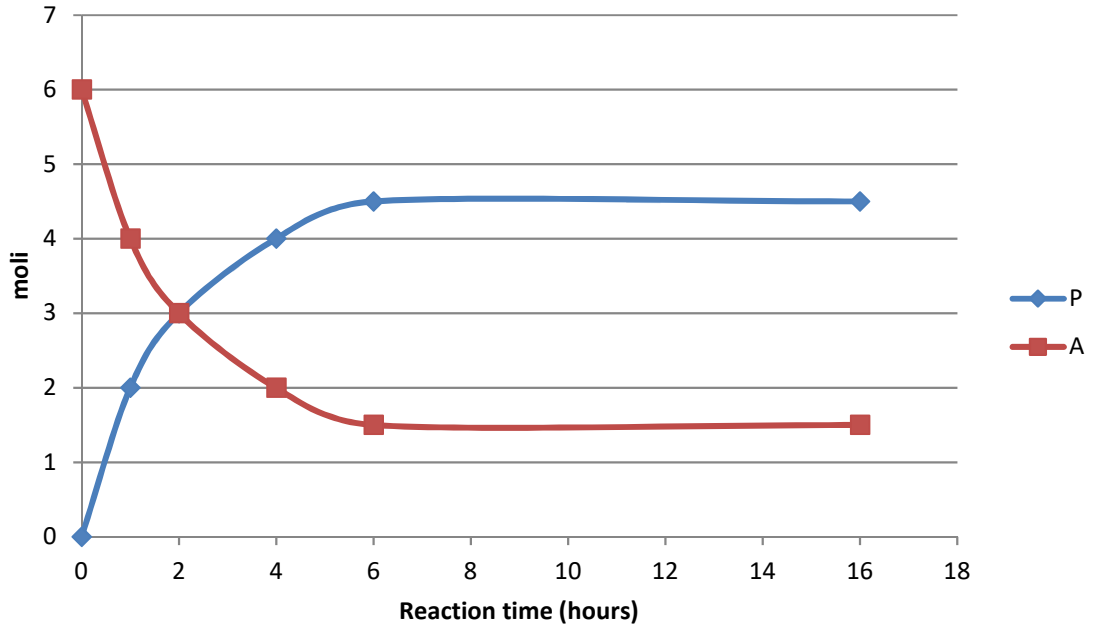
- HPLC- Reverse Phase (alta affinita`per molecole lipofile)



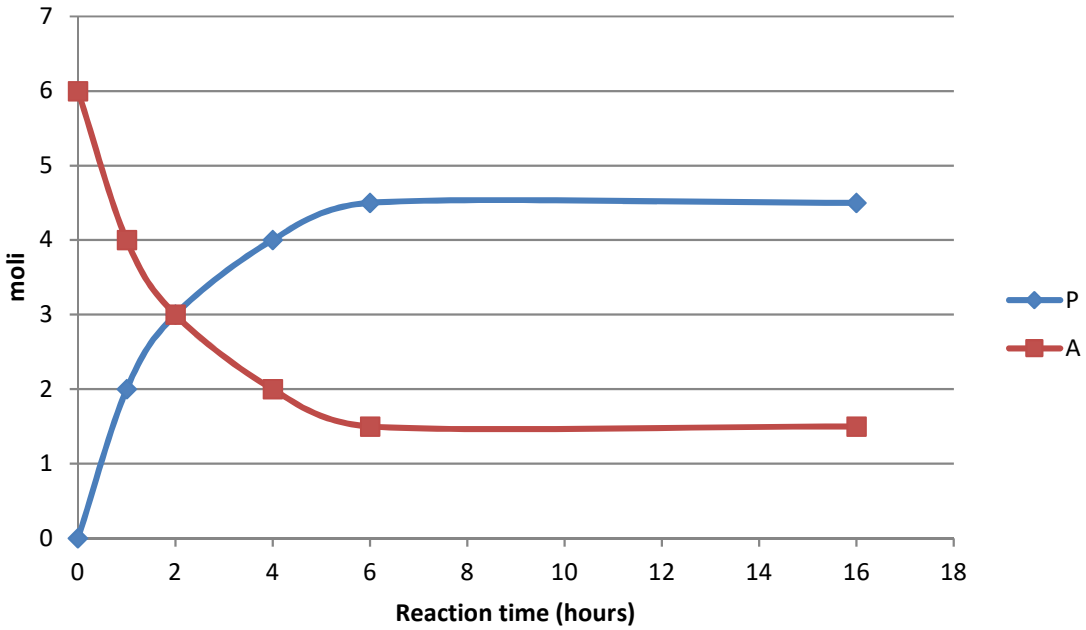
Domanda: in che parte del cromatogramma sara`visibile in benzene?

HPLC della miscela di reazione A → P





Reaction A → P



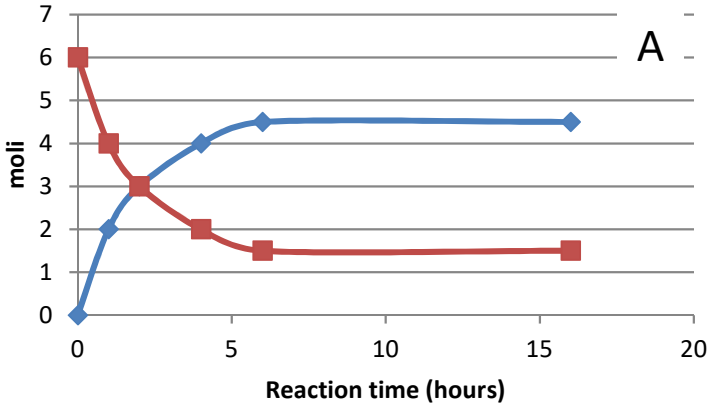
Check the mass balance
mol A + mol P = constant

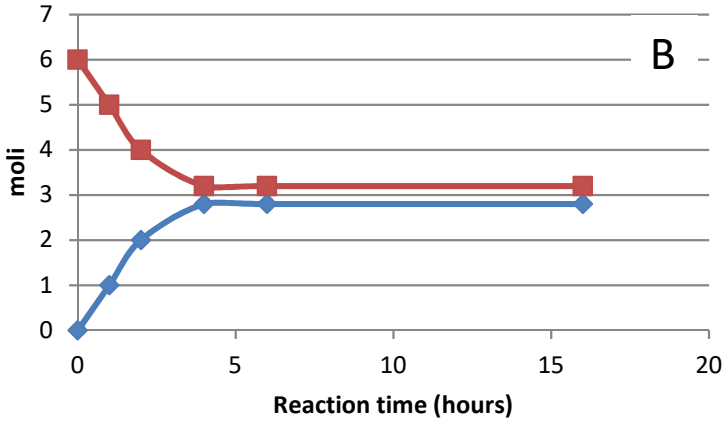
If not constant:

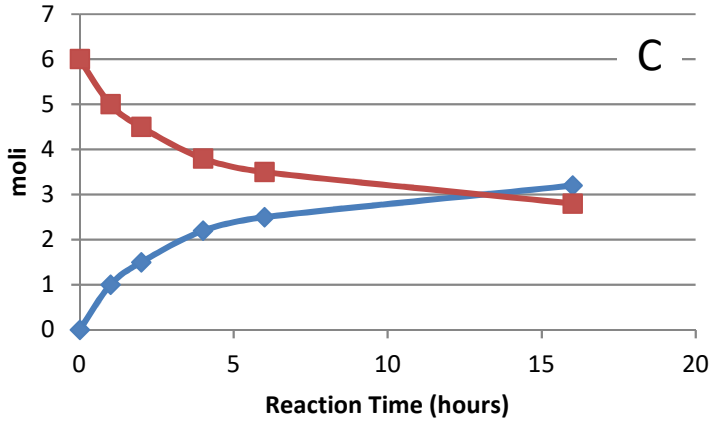
- impurities
- Evaporation of A o P o both
- Precipitation (on the enzyme)

Reaction mixture A → P

Of those three reactions, which one you do prefer?







Conclusion step 2 – feasibility studies

- Enzyme is identified, with indications for a starting reaction protocol

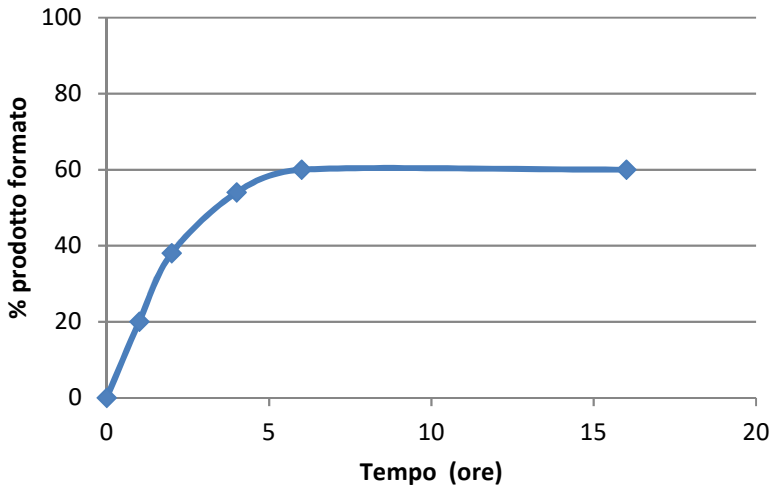
- Before proceeding, it is important to understand plus and minus

  → Is the reaction feasible?

  (cost, enzyme availability, solvent, … etc)

5 steps from the idea to the market

1. Idea evaluation
2. Feasibility studies
3. Research and Development
4. Protocol validation & Scale up
5. Production

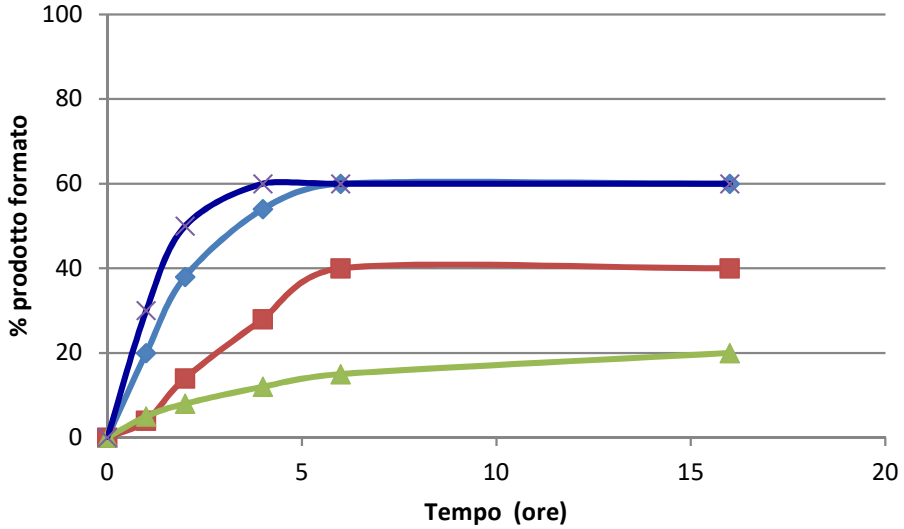


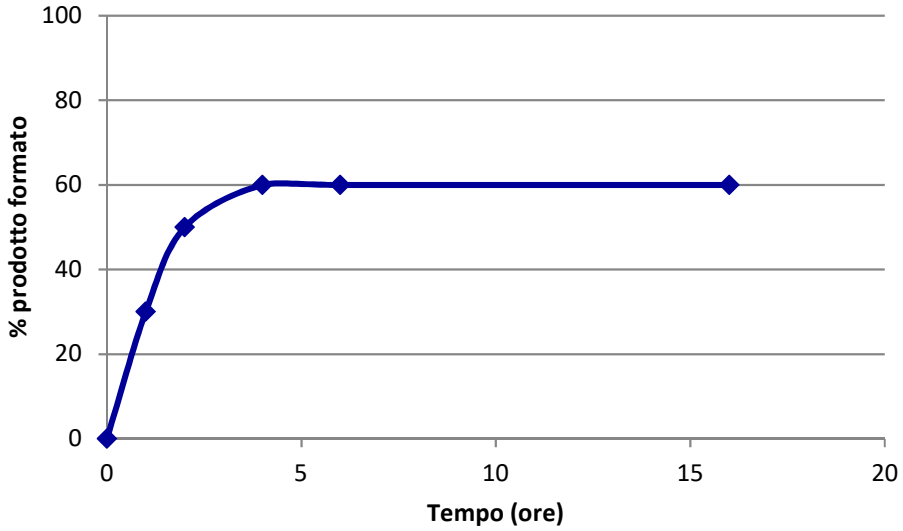
Screening of different reaction conditions

:Temperature, solvent*, stirring spees , kind of stirrer, pH, base if needed (i.e. pka, steric hindrance, speed addition)…

Experimental matrix
(only few reactions are performed)

	Solvent 1	Solvent 2	Solvent 3
Temperature 1			
Temperature 2			
Temperature 3			





What can we do to improve the yield?

WHY DOES THE REACTION STOP?

Observation is important →
Look at the reaction mixture,
not only at HPLC

- Enzyme is inhibited by:
  1. Product
  2. Change reaction conditions (T, pH, solvent, dilution…)

→ Remove the product
→ Keep the reaction conditions as much constant as possible

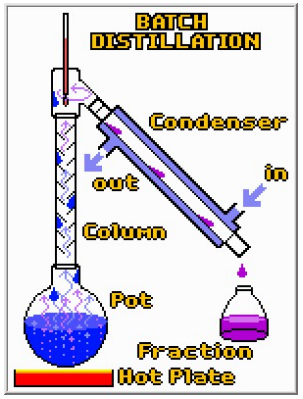
- Enzyme is denaturated

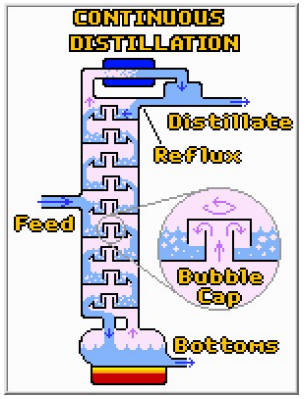
→ In the reaction mixture there are some flocculated or precipitated.
Solution → avoid denaturation

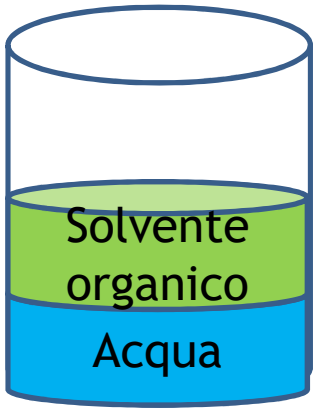
Product Inibition

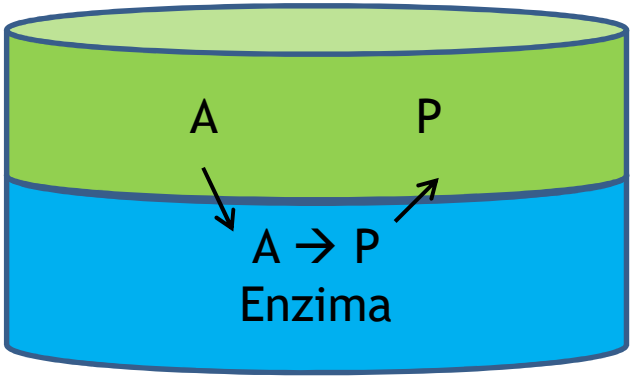
Remove product by:

- Distillation
- Precipitation*
- Biphasic reaction









Solvent for enzymatic reaction -1

1. water
2. Organic solvent
3. Water / organic solvent mixture

1. water

+ best choice (green chemistry, cost, process safety, no toxic)
+ Product is isolated by pH change or extraction with organic solvent
- Some substrate do not dissolve in water
? hydrolysis / synthesis

Solvent for enzymatic reaction -2

2. Organic solvent

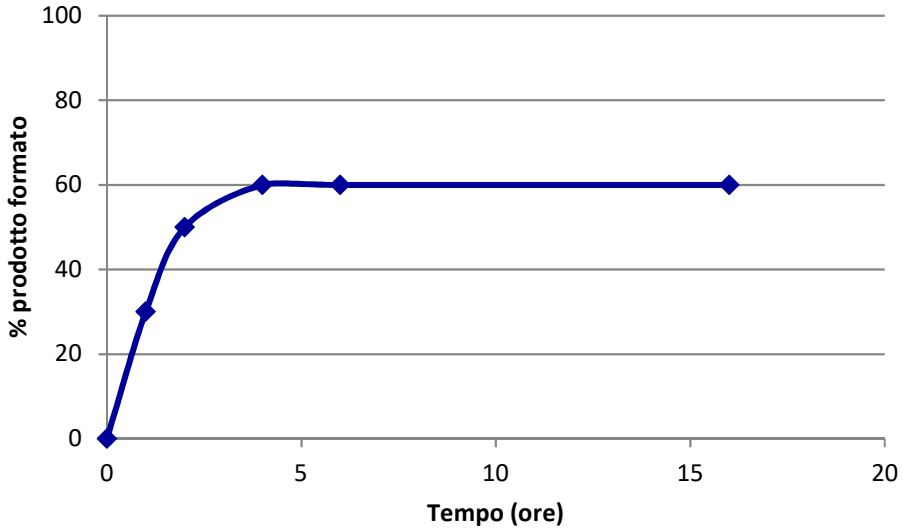
+ Solvent for most of the substrates and products
+ if boiling point is < 100C, its removal could be even easier (= cheaper) than water removal
- Most of the enzymes do denaturate in organic solvents
- Most of the enzymes are less active in organic solvents than in water
- Potential toxicity issue (residues in the final product) and potential issue of process safety (explosive, …?)

Solvent for the enzymatic reaction - 3

3. Water / organic solvent mixture

+ often a good compromise, esp. with organic solvent non mixable with water
? Enzyme denaturation
? Hydrolysis / sythesis

ATT: water / organic solvent ration must be well evaluated:
for instance 30:70 butanol / water the reaction is complete in 6h,
70:30 butanol / water the reaction needs 24 h to be finalized→ impurities formation



What can we do to improve the yield?

WHY DOES THE REACTION STOP?

Observation is important →
Look at the reaction mixture,
not only at HPLC

- Enzyme is inhibited by:
  1. Product
  2. Change reaction conditions (T, pH, solvent, dilution…)

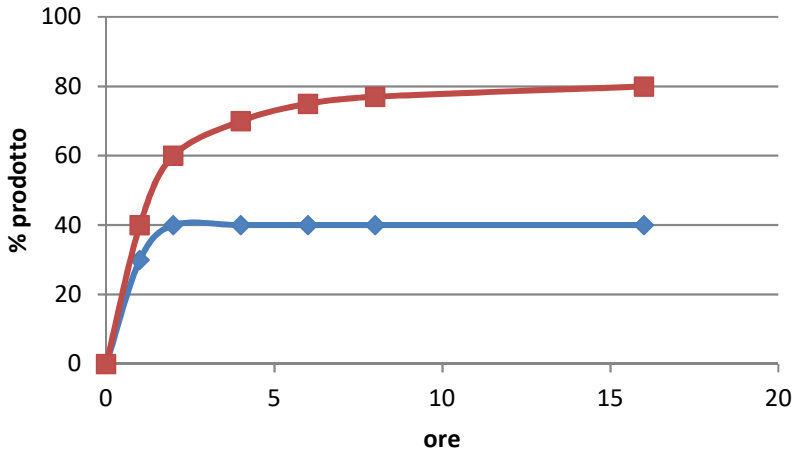
→ Remove the product
→ Keep the reaction conditions as much constant as possible

- Enzyme is denaturated

→ In the reaction mixture there are some flocculated or precipitated.
Solution → avoid denaturation

Monitoring of reaction conditions

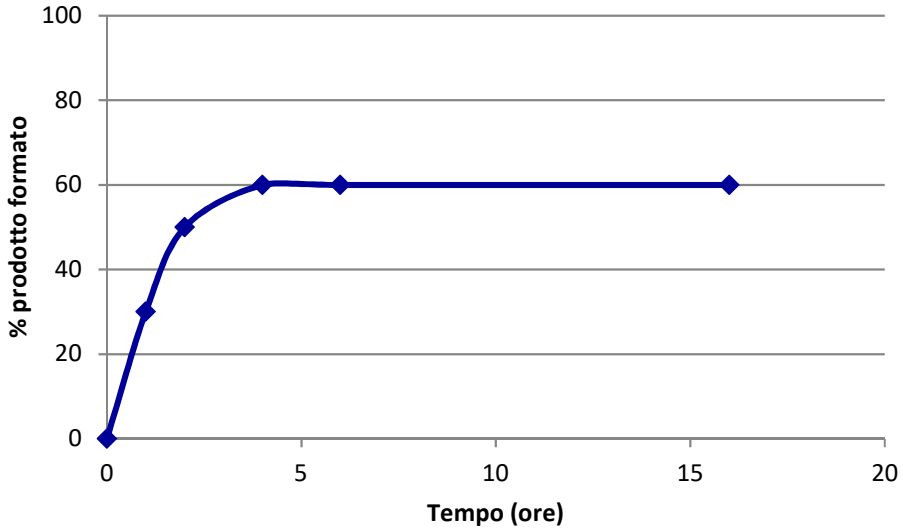
- pH: examples: reaction R-COOMe → RCOOH



pH costante a 7 (tampone migliore o aggiungendo base alla reazione)

pH non controllato → da 7 a 5 in 3 ore

Pictures From google



What can we do to improve the yield?

WHY DOES THE REACTION STOP?

Observation is important →
Look at the reaction mixture,
not only at HPLC

- Enzyme is inhibited by:
  1. Product
  2. Change reaction conditions (T, pH, solvent, dilution…)

→ Remove the product
→ Keep the reaction conditions as much constant as possible

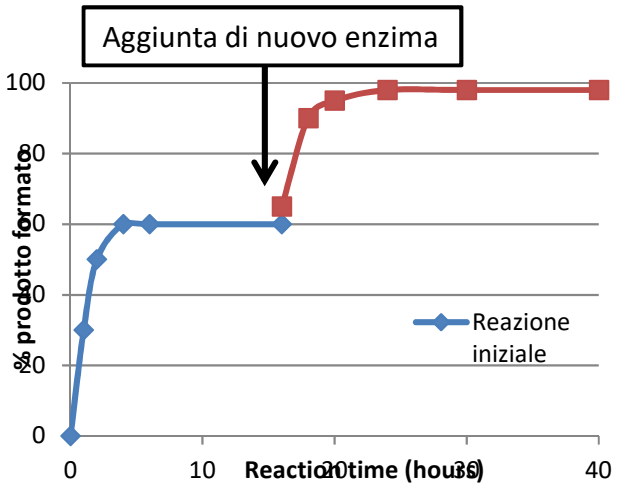
- Enzyme is denaturated

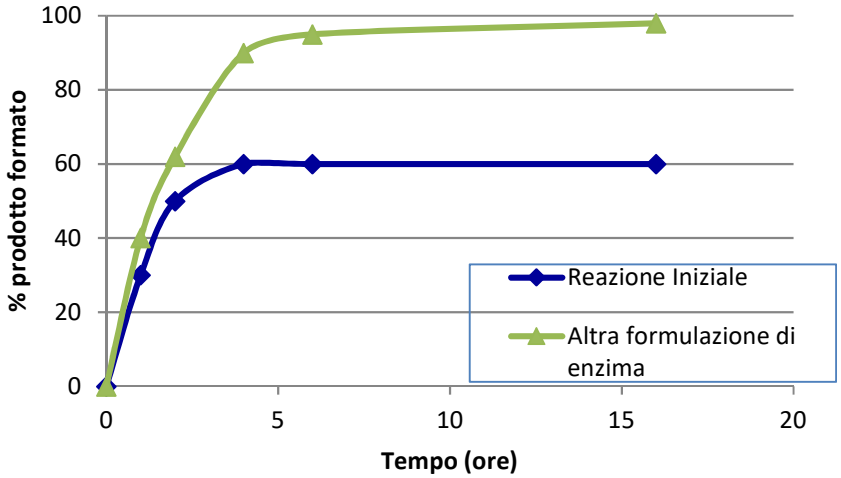
→ In the reaction mixture there are some flocculated or precipitated.
Solution → avoid denaturation

Enzyme is denaturated

To verify it→ other enzyme is asseded
To avoid it→

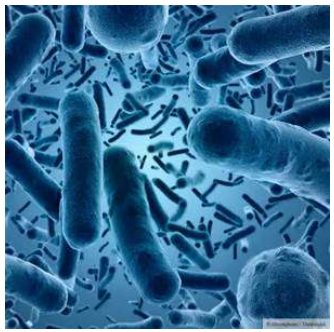
- Reaction conditions need to be changes to avoid denaturation (T o pH o base pKa or solvent of ionic strenght)
- Change the reaction conditions to speed up the reaction
- Change formulation (i.e. immobilization)





Formulazioni enzimatiche: vantaggi e svantaggi

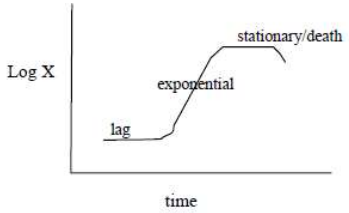
La scelta della formulazione enzimatica determina:
- il progresso e successo di una reazione enzimatica
- la fattibilita`del processo di isolamento e purificazione del prodotto finale



Whole cells
Enzima puo
essere secreto o meno

Enzima in soluzione
Veloce purificazione
Del brodo di fermentazione
(i.e. rimozione biomassa)

Enzima purificato liofilizzato
Solo enzima (e Sali
Di liofilizzatione)



Enzima immobilizzato

	Whole cells	Enzima soluzione	Enzima liofilizzato	Enzima immobilizzato
Costo	☺	😐	☹	☹
Introduce acqua, Sali, stabilizzanti	Yes	A bit	Almost nothing	No
Richiede aggiunta di cofattore a part e anche cofattore rigenerazione	No	Yes	Yes	Yes
Facile calcolare quantita da aggiungere e attivita	☹	😐	☺	☺
Facile da standardizzare in ogni fase	☹	😐	☺	☺
Puo`creare allergie	No	Rischio molto limitato	Richio molto alto	Rischio molto limitato
Isolamento del prodotto	☹	😐	☺	☺☺
Reazioni parallele (da presenza di altri enzimi)	It could be	Really rare	Not at all	Not at all

Formulazioni enzimatiche: vantaggi e svantaggi

- Enzima puro -liophilizzato
  - \+ Stabile, Non introduce acqua o altri stabilizzanti (ecc. sali)
  - \+ Facile da aggiungere, Facile da calcolare la quantita`
  - – Crea allergie agli operatori
  - – Cofattore (i.e. NADH) non incluso nell enzima
- Enzima puro in soluzione
  - \+ Non allergenico, Piu economico del liofilizzato
  - – Introduce acqua e altre sostanze (i.e. stabilizzanti)
  - – Cofattore (i.e. NADH) non incluso nell enzima
- Enzima immobilizzato
  - \+ Facilmente riciclabile, selettivo per una reazione specifica, piu alta stabilita`
  - – Costoso (+/-)
  - – Perdita di attivita`durante immobilizzazione
  - – Cofattore (i.e. NADH) non incluso nell enzima
- “Whole cell”
  - \+ In caso di enzimi che non sono facilemente disponibili o isolabili
  - \+ Spesso per enzimi che necessitano cofattore (non hanno bisogno di aggiungerlo)
  - \+ Costo piu basso
  - – Difficile standardizzare, isolamento del prodotto difficile (phospholipidi di membrana, etc)
  - – Isolamento del prodotto spesso difficile
  - +/- Intectual property (IP = brevetti)
  - +/- reazioni parallele

Qualche informazione in piu su “whole cell” enzymes

Advantages/Pros	Disadvantages/Cons
• Only limited by the number of organisms that can be cultured, while enzymes are limited by the number that are commercially available.	• Processes are more complicated (so dev’t takes longer), since you must design for cell growth (*ie,* enzyme manufacturing phase) and substrate conversion (*ie,* production phase)
• “Biomass is cheap”, *ie*, the cost of fermentation is usually not too high, and you’re not paying the purification costs associated with the purchase of an enzyme.	• Processes are “messier” – the vessel may contain cells and spent growth medium in addition to products and residual substrates (affects downstream purification)
• You don’t need to provide exogenous co-factor for re-dox reactions since the cells will recycle co-factor with existing machinery (true even if cells aren’t growing).	• Much more likely to have unwanted by-products since many other enzymes in addition to your desired enzyme will be present in the “catalyst”
• Also for re-dox reactions, you don’t need to provide a complimentary reaction to recycle the co-factor that is converted (example below).	• If the enzyme is not secreted, it requires that the cells be lysed prior to the conversion, or that substrate be transported across the cell wall.

Isolamento del prodotto

In Olandese “Chimica” significa “arte della separazione”

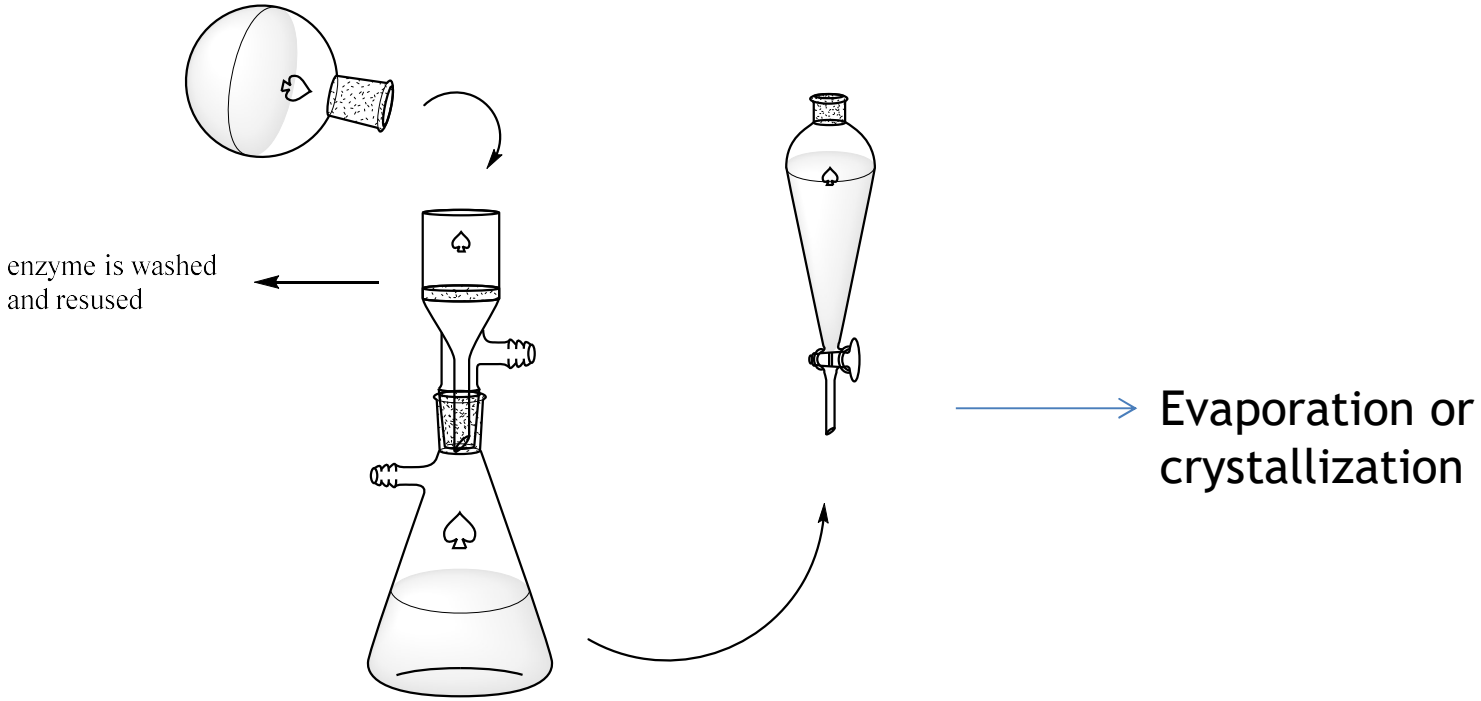
- Puo essere il passaggio piu complicato
- Spesso e`la parte piu costosa di un processo
- Non c`è un unico protocollo che va sempre bene
- Colonne cromatografiche sono bandite perche troppo costose → solo estrazione o cristallizatione o precipitazione
- Spesso filtrazioni su materiale inerte che adsorbe impurezze

Problemi tipici, dopo reazione enzimatica

- Fosfolipidi di membrana che fanno emulsioni
- Piccole catene proteiche da enzima che contaminano il prodotto
- Sali o additivi presenti nella formulazione iniziale dell`enzima
- Zuccheri, presenti nella formulazione iniziale dell`enzima e spesso non visibili all HPLC o H-NMR
- Impurezze con caratteristiche chimico fisiche molto simili a prodotto desiderato e per tanto difficili da eliminare

Product isolation

- With immobilized enzyme:
  1. Enzyme is removed by filtration (often some impurities are released by the enzyme formulation)
  2. Product purified by extraction or precipitation
  3. Solvent removal by evaporation or crystallization (and filtration)



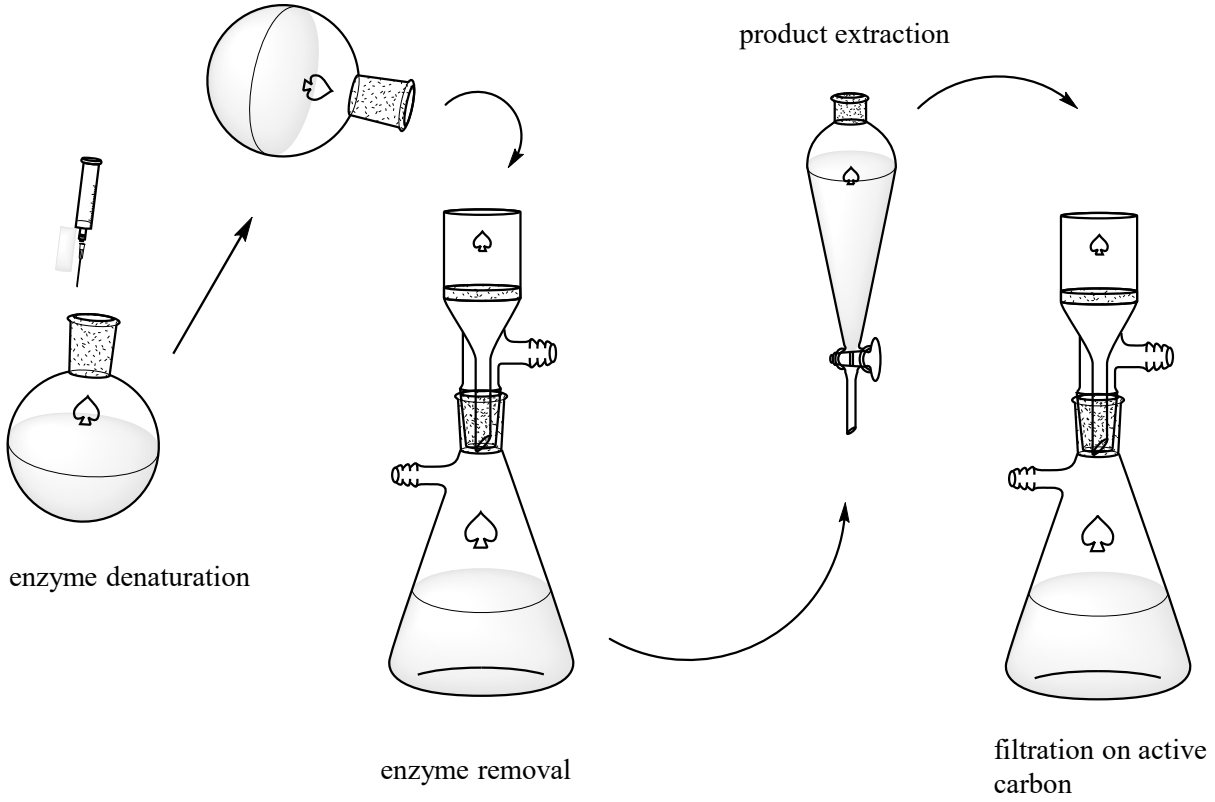
Product isolation

- With pure enzyme
  1. Enzyme denaturation (i.e. by pH change)
     - Att. Enzyme can become sticky or create emulsions
     - Att. The product could precipitate with the enzyme and it get lost
  2. Filtration
  - Difficult, it depends on the complexity of the denaturation and of the enzyme

    → Specificy in the protocol: filter kind, filter diameter, cake dimension
  1. Product isolation could be done by extraction or by precipitation
  2. Product re-dissolved in solvent
  3. Filtration on silica or active carbon to remove the impurities and colors
  4. Solvent removal for evaporation or crystallization (and then filtration)

Product isolation

- With pure enzyme:

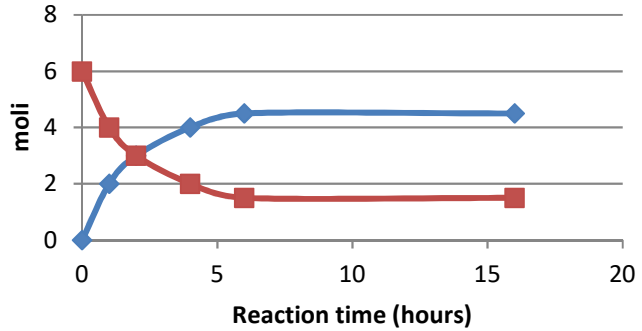


Product isolation

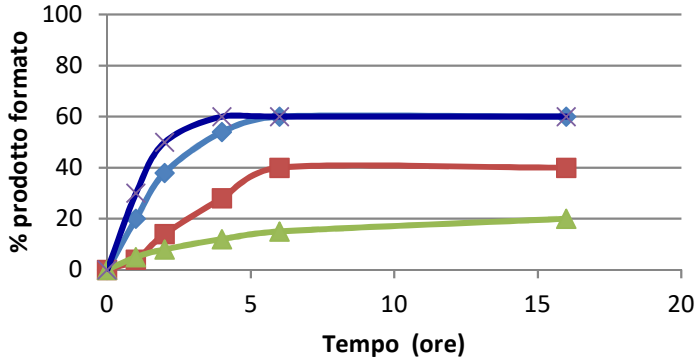
- From whole cell
  Similar to protocol with pure enzyme, but additionally it is necessary to kill the cells → see for example the addition of a denaturating enzyme as Methanol or isopropanol
  → Methanol or isopropanol could negatively influence the phase separation during next extraction phase
  → the mass amount of the whole cell is bigger(3x → 10x) tehn the pure enzyme
  → whole cells contains more substances, which could interefer with the product isolation (i.e. sugar → caramel)

Summary steps 2 and 3

- Literature search and enzymes screening (2)



- Screening of reaction conditions



- Product isolation

Other things that could occur during phase three

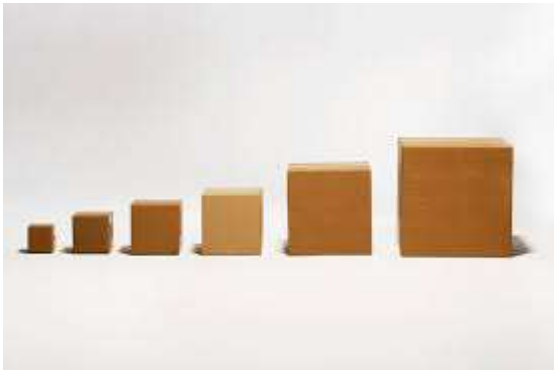
- Patent → if idea is new and innovative

5 steps from the idea to the market

1. Idea Evaluation
2. Feasibility Studies
3. Research and Developement
4. Protocol validation & Scale up
5. Production

Protocol validation & Scale up

- A protocol is defined “ robust” when it can be applied as such in bigger scale (i.e. 5x, 10x…) obtaining the best results
- Scale up is the most difficult part: often impurities – never observed in small scale – appear in larger scale anc could determine the end of the ful project
- Moreover, everything in bigger scale takes longer times, which might lead to denaturation of the enzyme, or substrate and/or product degradation



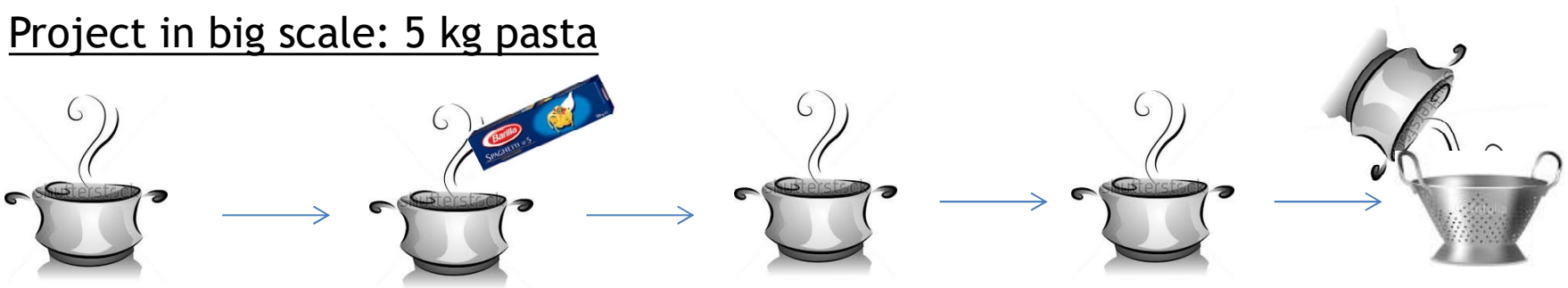
Scale up problem: example: project “preparation 5 kg pasta”

Initially in small scale: 100 g pasta

Portare a ebollizione 200 mL acqua	Aggiungere sale E spaghetti:	Aspettare che acqua Riprenda il bollore	Cucinare gli spaghetti	Scolare gli spaghetti
5 min	0.5 min	2 min	5 min	0.5 min

Total time of spaghetti in water → 8 min

Project in big scale: 5 kg pasta



Portare a ebollizione 10 L acqua	Aggiungere sale E spaghetti:	Aspettare che acqua Riprenda il bollore	Cucinare gli spaghetti	Scolare gli spaghetti
15 min	5 min	10 min	5 min	2 min

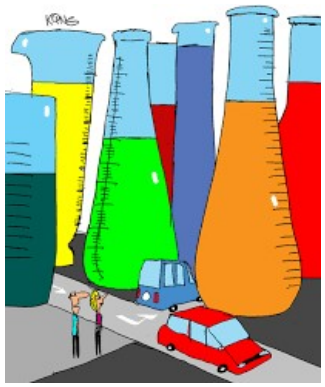
Total time of spaghetti in water → 22 min

How to solve the scale up problem in the project “ preparing 5 kg pasta”

- The customer wants “ pasta”. Why do we give to him spaghetti – which hace the most difficult boiling time vs. other pasta
- With high managers it is necessary tp speak a easy language to make him properly understanding the concepts

What to do to develop a “robust” process

- Consider the differences between the lab scale reactor and the plant reactor. Very important in enzymatic reactions are: speed and kind of stirrer. The enzymes can be denaturated by “forze di taglio”
- Lab reaction must be continuously monitored and every small effect must be reported (i.e. hexothermic reaction)
- Good communication between the experts developing the protocolsand the experts working in the plants
- Protocol must be clear and no risk of misunderstandings (i.e. abbreviations)



5 steps from the idea to the market

1. Idea evaluation
2. Feasibility studies
3. Research & Development
4. Protocol validation& Scale up
5. Production

Challenges in production-1

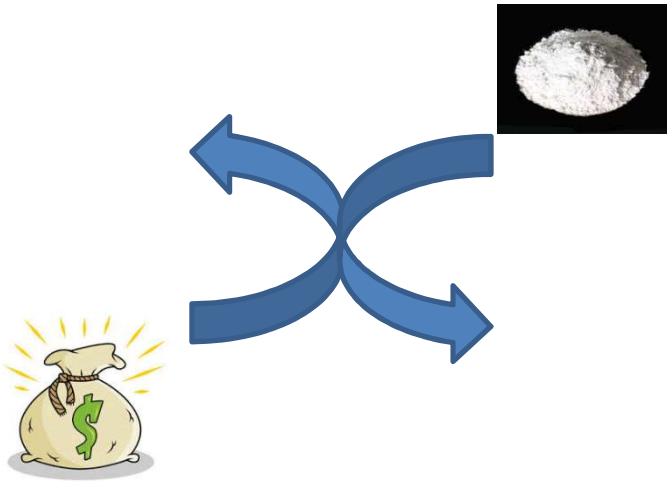
- Supply chain challenge
  - Raw materials supply
  - Enzyme storage (esp. whole cell)
  - Understaning the right aont of enzyme to be added to get the same reaction yields (different enzymes batches might have different activities)
  - If by mistake a parameter changes during the reaction (pH), the full production batch must be thrown away
  - Logistics → cost and transport feasibility of materials and finished products
  - Coordination of different competences and activities
- If sometimes there is not enough " interal capacity", there is the possibility to g to a toller → it can be also a strategic choice
- Intellectual properties
  - For instance, not real name in the plants
- Protection of plants data :
  - A good engener from competitor can understand a lot by looking at the plant pipelines
  - The plants data are secret

Challenges in production – 2

- Process safert
- Incidents
- Workers availability
- Regulations and permits (also environmental permits)
- Community leaving around the production plants

Development of a chemical reaction. Final step: customer contact

Customer



Project leader

Thanks for your attention !!!

How the customer explained it

How the Project Leader understood it

How the Business Consultant described it

How the Analyst designed it

How the Programmer wrote it

How the project was documented

What Operations installed

How it performed under load

How it was supported

What marketing advertised

How the customer was billed

What the customer really needed