Industrial Fermentation and Strain Improvement of Producing Microorganisms

Flavia Marinelli

Dubrovnik Summer School 31-8-2012
Strain Collection

Extract Bank

Assay

Target

Environental samples

Microbial population

Selected strains

Flask fermentation
Strain Collection

Extract Bank

Assay

Target

Screening

HIT

Lead

Biological & Chemical Profiling, Patent

Supply by fermentation

In vivo efficacy, PK, Tox

Optimized Lead

Clinical candidate
STRAIN & FERMENTATION IMPROVEMENT

TO INCREASE PRODUCTIVITY
from few mg/l to more than 1 g/l

TO BETTER THE PRODUCT QUALITY
i.e.: to orientate productivity toward the more active component in a complex

- REDUCTION OF PRODUCTION VOLUME AND TIME
- FACILITATION OF RECOVERY AND PURIFICATION

COST SAVING
Strain taxonomy and maintenance

Isolation of spontaneous mutants & strain purification

Mutagenesis and selection

Recombination by protoplast fusion

Metabolic engineering

Vegetative and fermentation medium optimization

Fermentation process definition

Process Scale-up
Filamentous actinomycetes and fungi survive as lyophilized for 25 years (MCB)

Good viability and stability at -80°C (WCB)

Myxobacteria very often do not survive as lyophilized and/or cryopreserved

Cyanobacteria have specific requests of light and air bubbling and have to be subcloned each month
Fingerprinting of the producing organism and quality of culture collection

Identity, stability, vitality, reproducibility, safety and security

Patent, IPR protection, compliance with the regulatory authorities, drug master files, FDA approval etc.

Concept of Working Cell Bank and Master Cell Bank
Strain taxonomy and maintenance

Isolation of spontaneous mutants & strain purification

Mutagenesis and selection

Recombination by protoplast fusion

Metabolic engineering

Vegetative and fermentation medium optimization

Fermentation process definition

Process Scale-up
First Screening of Culture Collection Strains and Literature Media

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Black box model of cell metabolism: “secondary metabolite” producers are generally chemoorganotrophes
Our pannel of media for actinomycetes: rich complex industrial media

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Selection of Nutrient Sources

- Cost per unit of nutrient
- Transportation cost
- Cost stability
- Availability
- Storage costs
- Stability characteristics
- Safety factors
- Handling and distribution requirements
- Flexibility in application

- Pretreatment costs
- Sterilization costs
- Consistency of nutritional quality
- Rheological properties
- Surface tension factors
- Product recovery and purification impacts
- Process yield
- Product concentration
- Plant productivity
Methods to optimize medium composition in fermentation processes

- One by one change
- Experimental design with the help of statistical softwares
- Biochemical methods: precursors or effectors
Simplified Plackett-Burman factorial design experiments: the set up and data analysis was performed using Statgraphics plus 4.1 software

(Statistical Graphics Corp, Herndon, USA)
BATCH o FED-BATCH fermentation process to produce an antibiotic from an actinomycete

Reactivation culture E26

Vegetative seed E26

Production

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SIROLIMUS

**Chemical structure**

\[ C_{51}H_{79}NO_{13} \]

**Molecular Weight**

914.172 g/mol

**Synonyms**

Rapamycine

**Brand Names**

Rapamune

**Producing strains**

*S. hygroscopicus*

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**Clinical Uses**

**Sirolimus in transplantation**

- Kidney

**Sirolimus non-transplantation uses**

- Anti cancer activity (kidney, brain)
- Antifungal agent
- Starting compound for derivatives

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May 2007 - **Temsirolimus** (trade name Torisel ® - Wyeth Pharmaceutical) was approved by the US FDA for the treatment of patients with advanced kidney cancer.

March 2009 - **Everolimus** (trade name Afinitor ® - Novartis) was approved by the US FDA for the treatment of patients with advanced kidney cancer.
Increase in Sirolimus production

C. Taurino et al. unpublished results
pH controlled fed-batch fermentation time course
Precursors & effectors:

Phenyl acetic acid in Penicillin G
Phenoxy acetic acid in Penicillin V
Aminoacids in peptide antibiotics
Fatty acids or their aminoacidic precursors in lipopeptides such as teicoplanin, A40926 and ramoplanin
Propanol in erythromycin and spinosyn
Methionine in cephalosporin
Benzyl thiocyanate in tetracycline
Diethylbarbituric acid in rifamycin
Glycopeptide Antibiotic
Teicoplanin active against
multiresistant gram positive
infections

Actinoplanes teichomyceticus
Teicoplanin TA2 complex

Targosid: T-A2-1, T-A2-2, T-A2-3, T-A2-4, and T-A2-5 represent 6, 58.3, 7.3, 14.4 and 14% of the total T-A2.
European Pharmacopoeia

**Targosid 250mg/L**

- T-A2-1
- T-A2-2
- T-A2-3
- T-A2-4
- T-A2-5
Growth and teicoplanin production in 3 L-batch fermentations of A. teichomyceticus ATCC 31121 in TM1

The pH value was naturally self-regulated, whereas the pO$_2$ was controlled over the 20% of saturation by adjusting agitation speed.

In (A), time courses of pH (●, solid line), pO$_2$ (□, dashed line), glucose (▲, solid line), and growth curve measured as dry weight (▲, dashed line) and PMV (■, solid line). In (B), production of T-A$_2$ measured by HPLC analysis as mg/L (filled bars).

C.Taurino et al. Microbial Cell Factories 2011
Teicoplanin fermentation is reproducible from miniaturized Duetz System to bioreactor scale.
HPLC profile of 120 hour sample showing the following complex composition:

T-A_{2-1}, T-A_{2-2}, T-A_{2-3}, T-A_{2-4} and T-A_{2-5} represent 7.3, 60.2, 13.1, 9.1 and 10.3% of the total T-A_{2}.

Control fermentation

2.5 g/L corn oil
2.5 g/L olive oil
2.5 g/L sesame oil
I. Biosynthetic tool: addition of fatty acid as precursors of linear acyl moieties in teicoplanin complex

Linoleic acid $C_{18}H_{32}O_2$ → $\beta$-oxidation → T-A2-1 n C:10:1

Oleic acid $C_{18}H_{34}O_2$ → $\beta$-oxidation → T-A2-3 n C:10:0
II. Biosynthetic tool: addition of amino acids as precursors of branched acyl moieties in teicoplanin complex

- **Valine** (Amino Acid) → (α-Ketoisovalerate) → (Isobutyryl-CoA) → (Iso-C₁₀ or C₁₀ acid)
- **Isoleucine** (Amino Acid) → (α-Keto-β-methylvalerate) → (α-Methylbutyryl-CoA) → (Anteiso-C₁₀ or C₁₀ acid)
- **Leucine** (Amino Acid) → (α-Ketoisocaproate) → (Isovaleryl CoA) → (Iso-C₁₁ or C₁₁ acid)

- **T-A2-2** iso C:10:0
- **T-A2-4** anteiso C:11:0
- **T-A2-5** iso C:11:0
Growth and teicoplanin production in 3 L-batch fermentations of A. teichomyceticus ATCC 31121 in TM1 added with L-valine

The pH value was naturally self-regulated, whereas the pO$_2$ was controlled over the 20% of saturation by adjusting agitation speed.

In (A), time courses of pH (●, solid line), pO$_2$ (□, dashed line), glucose (▲, solid line), and growth curve measured as dry weight (△, dashed line) and PMV (■, solid line). In (B), production of T-A$_2$ measured by HPLC analysis as mg/L (filled bars).

C. Taurino et al. Microbial Cell Factories 2011
Addition of L-valine increases $T-A_{2-2}$ and total productivity.

HPLC profile of 120 hour sample showing the following complex composition:

$T-A_{2-1}$, $T-A_{2-2}$, $T-A_{2-3}$, $T-A_{2-4}$ and $T-A_{2-5}$ represent 7.3, 73.4, 10.5, 2.0 and 6.8% of the total $T-A_2$. 
Combination of oil and L-valine effect to modulate complex composition

C. Taurino et al. Microbial Cell Factories 2011
Elicitors:

Heavy metals

Rare earth elements
(17 elements including scandium, yttrium and lanthanides)

Oils

Microbial cell wall components

Dimethylsulfoxide

S-Adenosylmethionine

N-acetylglucosamine
Stirred tank fermentors up to 200 cubic meters for antibiotic production
On-line and off-line analyses: analytical tools are extremely important for the “metabolome” and for the flux analysis.
Lab scale

Pilot plant scale