Industrial Fermentation and Strain Improvement of Secondary Metabolite Producing Microorganisms
Strain Collection

Extract Bank

Assay

Target

Enviromental samples

Microbial population

Selected strains

Flask fermentation
Strain preservation

PRODUCING STRAIN

Analytical or biological quantification

FERMENTATION PROCESS

DOWNSTREAM & PURIFICATION
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 maintenance

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*
Fingerprinting of the producing organism

Identity, stability, vitality, reproducibility, safety and security

Patent, IPR protection, compliance with the regulatory authorities, drug master files, FDA approval etc.
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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<td>51.5</td>
<td>64.0</td>
<td>7.05</td>
<td>47.4</td>
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Average: 25.5 14.4 49.8 62.0 60.4
Black box model of cell metabolism:
secondary metabolite producers are generally chemoorganotrophes
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)

Volontè, Protein Expression and Purification, 2008
BATCH o FED-BATCH fermentation process to produce an antibiotic from an actinomycete

<table>
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<tr>
<th>E26</th>
<th>g/L</th>
<th>AF3</th>
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<td>SBM</td>
<td>20</td>
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<td>Dextrose</td>
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<tr>
<td>CaCO3 (B)</td>
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<td>Antifoam (Hodag)</td>
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<td>H2O demi</td>
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Feeding Glucose and precursors

WCB -80°C

1% (v/v) Reactivation culture E26

3% (v/v) Vegetative seed E26

10% (v/v) Production

72-96h, 28°C, 200 rpm

72-96h, 28°C

Vegetative seed E26
**SIROLIMUS**

**Chemical structure**
C\textsubscript{51}H\textsubscript{79}NO\textsubscript{13}

**Molecular Weight**
914.172 g/mol

**Synonyms**
Rapamycin

**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 advanced renal cell carcinoma.

March 2009 - **Everolimus** (trade name Afinitor ® - Novartis) was approved by the US FDA for the treatment of patients with advanced kidney cancer.
SIROLIMUS FERMENTATION PROCESS

Increase in Sirolimus production

Yield (mg/L)

Flask, Minif.fermenter, Flask add glucose, Minif. feeding glucose, Flask + L-lysine, Minif. + L-lysine, Carbon source trials, Minif. control pH
pH controlled fed-batch fermentation time course
**Precursors:**
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

**Effectors:**
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

Soybean oil
Lard oil
L-valine
**M. Brunati et al. (2005)** Influence of leucine and valine on ramoplanin production by Actinoplanes sp. ATCC 33076. *Journal of Antibiotics* 58: 473-478


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.
## Production scale

<table>
<thead>
<tr>
<th>Products</th>
<th>Volume ($m^3$)</th>
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<tbody>
<tr>
<td>DNA rec.proteins</td>
<td>0.5-50</td>
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<tr>
<td>Yeast</td>
<td>100-250</td>
</tr>
<tr>
<td>Amino acids</td>
<td>100-250</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>80-200</td>
</tr>
<tr>
<td>Enzymes</td>
<td>80-250</td>
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</table>
Strain taxonomy and maintenance

Isolation of spontaneous mutants & strain purification

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Process Scale-up
Strain “purification” is needed to reduce fermentation variability:

selection of morphological/pigmentation variants

in conditions of nutritional stress or in presence of subinhibitory concentrations of toxic agents
“Ribosome engineering” from Kozo Ochi group:

modulation of ribosomal components (proteins and rRNA) by the introduction of mutations conferring drug resistance

- Aminoglycosides
- Tetracyclines
- Macrolides
- Liconsamides
- Streptogramins
- Everninomycins
- Oxazolidinones
- Cloramphenicol
Ochi et al., Fig. 11. Scheme of “ribosome engineering” to activate cell’s ability.
Cumulative drug resistance mutations

Selection of spontaneous mutants resistant to streptomycin, gentamicin, rifamycin etc.

→ Improved production of antibiotics in Streptomyces, Nonomuraea, Saccharopolyspora, Bacillus

→ Activated synthesis of dormant genes in actinomycetes
Antibiotic production improvement in the rare actinomycete *Planobispora rosea* by selection of mutants resistant to the Aminoglycosides Streptomycin and Gentamycin and to Rifamycin

**GE2270:**

Bacterial protein synthesis inhibitor active on Gram-positives
Mutant Screening & Selection

Mutagenesis
- N-Methyl-N’-nitro-N-nitrosoguanidine (NTG)
- Ethylmethane sulfonate (EMS)
- UV
- Nitrous acid
- Hydroxyamine
- 4-nitroquinoline-1-oxide (NQO)

Strategies
- Develop quick prescreening assay
- Random screening
- Rational selection
- Media development
- Stable strain/fermentation
Selection Strategies

- Mutants resistant to precursors or end products
- Mutants resistant to the inhibitor of production in the biosynthetic pathway
- Deregulation on: glucose, NH$_4^+$, PO$_4^{3-}$
- Enzyme system (protease, lipase)
- Permeability mutants
- Morphological mutants
- Rate mutants
- Lower oxygen requiring mutants
- High carbon conversion efficient mutants
- Low viscosity mutants
- Shearing stress resistance
Progress of Cyclosporin A

Fermentation Technologies

Tolypocladium inflatum

cyclosporin
### Example of Screening Numbers for One Quarter

<table>
<thead>
<tr>
<th>Mutagens</th>
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<tbody>
<tr>
<td>Rounds of mutagenesis</td>
<td>40</td>
</tr>
<tr>
<td>Selection strategies</td>
<td>15</td>
</tr>
<tr>
<td>Rounds of selection</td>
<td>42</td>
</tr>
<tr>
<td>Colonies screened</td>
<td>$1.03 \times 10^9$</td>
</tr>
<tr>
<td>Colonies fermented in flask</td>
<td>6279</td>
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</table>
Classical Strain Improvement

• Significant enhancements in production parameters without a detailed knowledge of the biosynthesis/genetics/regulation and without the need of genetic manipulation tools.

• Methods are time consuming, labour intensive, lack precision and introduce unwanted mutations that can hamper further improvements.
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Genome shuffling leads to rapid phenotypic improvement in bacteria (2002)
Y. Zang et al. Nature 415:644-646

*Streptomyces fradiae*, tylosin producer

![Tylosin molecule]

Genome shuffling (repeated protoplast fusion)

vs.

classical strain improvement (mutagenesis and selection)
SF1 \( (1\text{g/l}) \)

\[ \text{GS1 (8.2g/l), GS2 (6.2 g/l)} \]

20 rounds of mutagenesis and selection

\( (\text{UV, NTG, HNO}_2) \)

20 years, 10 \( \text{6} \) screens

GS (7)

1 year, 24,000 screens

NTG

(22,000)
Figure 2 Genome shuffling versus classical strain improvement. a, Comparison of classical strain improvement to genome shuffling for production of tylosin from *S. fradiae*. SF21 was generated through 20 rounds of mutagenesis and screening from SF1. GS1 and GS2 were generated from two rounds of genome shuffling of a population of classically improved strains derived from SF1. The classical approach required twenty years and approximately a million screens, whereas genome shuffling required only a year and 24,000 screens. HNO$_2$, nitric acid; UV, ultraviolet irradiation; NTG, nitrosoquainidine; NS, natural selection; GS, genome shuffling. b, Relative production of tylosin in 300-μl, 96-well fermentations of SF1, SF21 (dotted line marks the production level of SF21); NTG, 11 NTG-improved mutants derived from 22,000 screened; genome shuffling 1, 7 strains identified from the shuffling of the NTG population, 1,000 were screened; and genome shuffling 2, the 7 strains identified from the shuffling of genome shuffling 1, 1,000 were screened. c, Relative production of tylosin from SF1, SF21, GS1 and GS2 from 250-ml shake flask fermentations.
**Figure 1** Asexual versus sexual evolution. Asexual evolution is the sequential process of accumulating individual mutations. Selection of the fittest results in the capture of only a single mutant. It is slow, as individuals within a population evolve alone as opposed to sharing information and evolving as a group. Genetic diversity is lost and deleterious mutations that are difficult to lose accumulate. Sex allows the information within a population to be shared. Mating within a selected population consolidates genetic information by providing a mechanism for the combination of useful mutations and the loss of deleterious mutations. Sexual evolution thus produces populations containing individuals that have a far greater fitness than their parents.
Recent applications:

Zheng et al. 2010 J Ind Microbiol Biotech
Drug resistance marker-aided genome shuffling to improve acetic acid tolerance in Saccharomyces cerevisiae.

Genome shuffling of Propionibacterium shermanii for improving vitamin B12 production and comparative proteome analysis.

Genome shuffling in Clostridium diolis DSM 15410 for improved 1,3-propanediol production.

Generation of high-yield rapamycin-producing strains through protoplasts-related techniques.

Xu et al. 2008 Appl Microbiol Biotechnol
Evolution of Streptomyces pristinaespiralis for resistance and production of pristinamycin by genome shuffling

Hida et al. 2007 Appl Microbiol Biotechnol
Genome shuffling of Streptomyces sp.u121 for improved production of hydroxycitric acid

Beltrametti et al. 2007 J Antibiotics
Protoplast fusion and gene recombination in the uncommonn actinomycete Planobispora rosea producing GE2270

Wang et al. 2007 J Biotechnol
Genome shuffling improved acid tolerance and L-lactic acid rhamnosus volumetric productivity in Lactobacillus
Protoplast production and manipulation

Molecular tools to manipulate recalcitrant strains

Intergeneric conjugation
E. coli-Nonomuraea
Metabolic engineering: targeted approaches such as knocking out or overexpressing genes and introducing alterations in enzyme activity.

Which are the most relevant targets?

Is the generation of recombinant strains achievable?
Transposon mutant library to identify target genes

Random insertion transposon mutant libraries in *Streptomyces* spp.

- HTS screening for growth and antibiotic production in microtiter wells
- Identification of genes involved in antibiotic production
Improving hosts by overexpression of specific genes:

S-Adenosylmethionine synthetase (metK)

Aminoglycoside resistance (mutant rpsL, frr)

Mutant-type RNA polymerase (rpoB(R))

Self-resistance genes

Pathway specific regulators
Links between antibiotic resistance and antibiotic production

**R. Crameri and J. E. Davies, 1986 J Antibiotics**

Increased production of aminoglycosides associated with amplified antibiotic resistance genes

Aminoglycoside 6’-N- acetyltransferase

(Kotra et al., 2000)
Comparative analysis of industrial high producer strain and parental strains

Amplification of the entire kanamycin biosynthetic gene cluster during empirical strain improvement of Streptomyces kanamyceticus

k.Yanai, T.Murakami and M.Bibb
PNAS (2006)
<table>
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<td>Methionine</td>
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<td><em>Clostridium acetobutylicum</em></td>
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<td>Comparative analysis of wild-type and recombinant strains for understanding regulatory mechanisms</td>
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<td>Proteome</td>
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<td>Human leptin</td>
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<td><em>Corynebacterium glutamicum</em></td>
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<td>Understanding phenotypic behavior of recombinant strains by changing carbon source</td>
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<td>Comparative analysis of wild-type and recombinant strains for understanding regulatory mechanisms</td>
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<td>L-threonine</td>
<td>Understanding regulatory mechanisms of mutant strain</td>
<td>[24]</td>
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Heterologous expression in a suitable host

The selection of the host is crucial:

Some examples:

*Streptomyces albus, Streptomyces coelicolor, Streptomyces lividans, Streptomyces venezualae:* robust actinomycetes

*Streptomyces toyocaensis* for glycopeptides

*Streptomyces fradiae* for macrolide

*E.coli:* best choice for rapid engineering

*Pseudomonas putida:* for gram-negative metabolic pathways
Strain and fermentation improvement of heterologous hosts is the following step!

Examples:

epothilone production in *Streptomyces venezuelae*, *Streptomyces coelicolor* and *Myxococcus xanthus*

Polyketide production in *Escherichia coli*