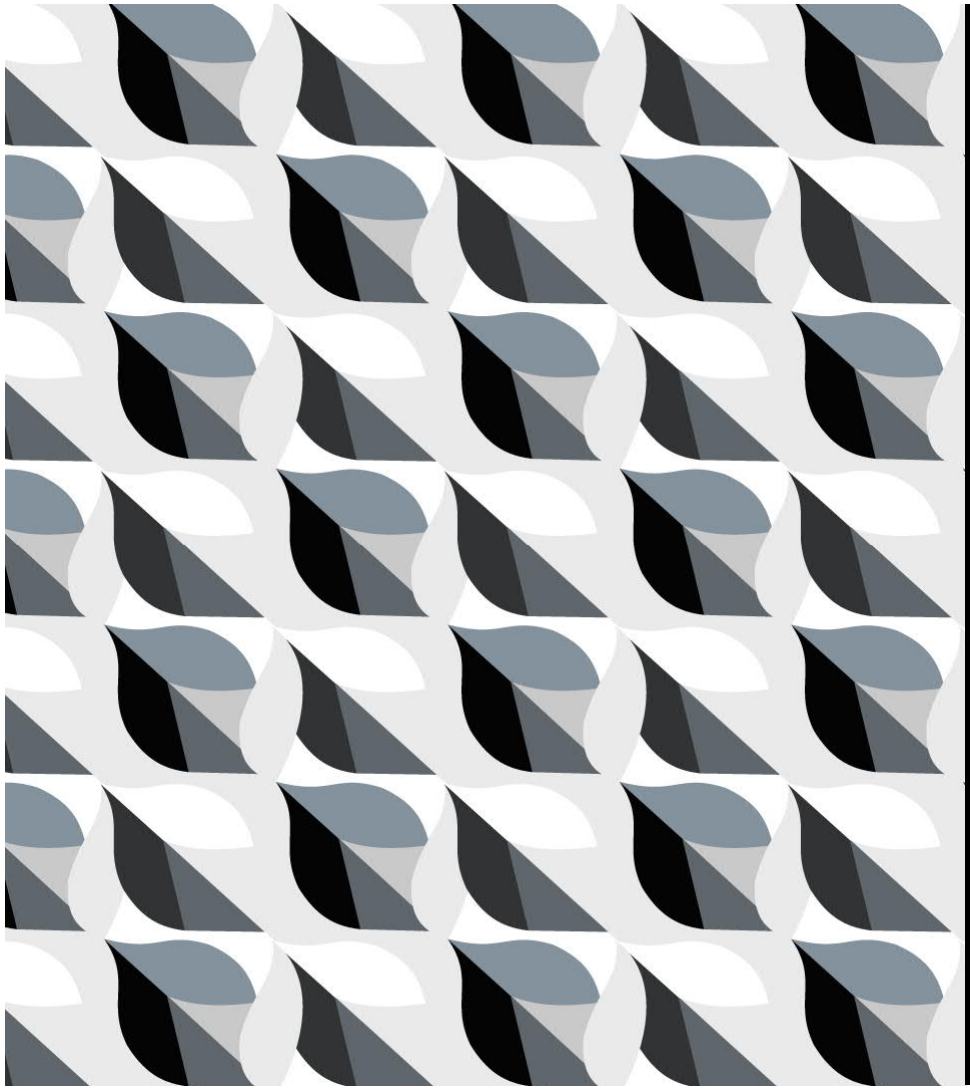


Metabolic modelling in synthetic biology

Paula Jouhten
VTT Technical Research Centre of Finland Ltd

This lecture will introduce

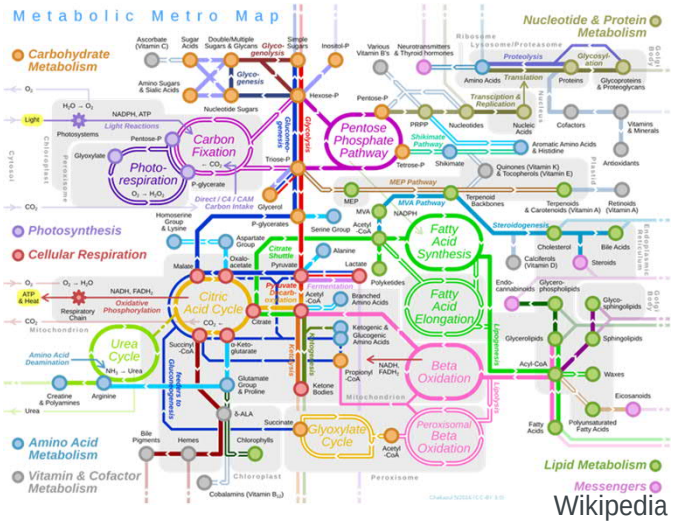
1. Metabolic phenotype prediction and estimation using genome-scale metabolic models
2. Design of synthetic metabolic pathways
3. Design of engineering strategies for optimizing production

1. Metabolic phenotype prediction and estimation using genome-scale metabolic models

Why is metabolism relevant for synthetic biology?

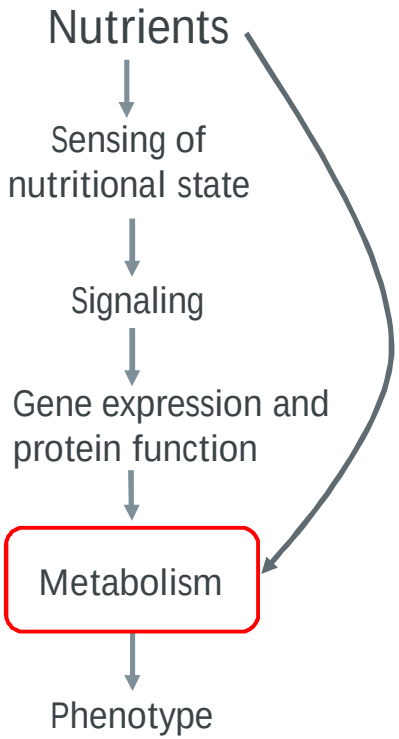
Metabolism = (bio)chemical reactions involved in sustaining a living state of cells and an organism

- Metabolism generates precursors for product compounds but also for circuit components
- Metabolism generates energy and redox power
- Metabolism is involved in the regulation of cells



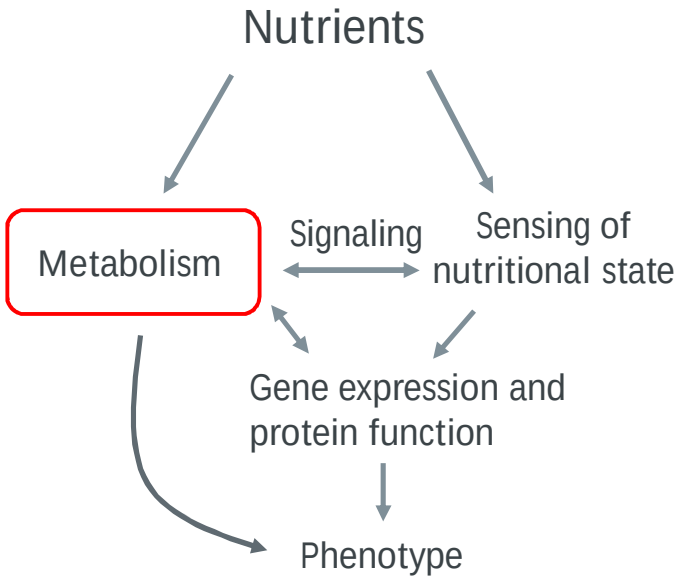
Active metabolism

Conventional view



”Passive metabolism”

Current understanding

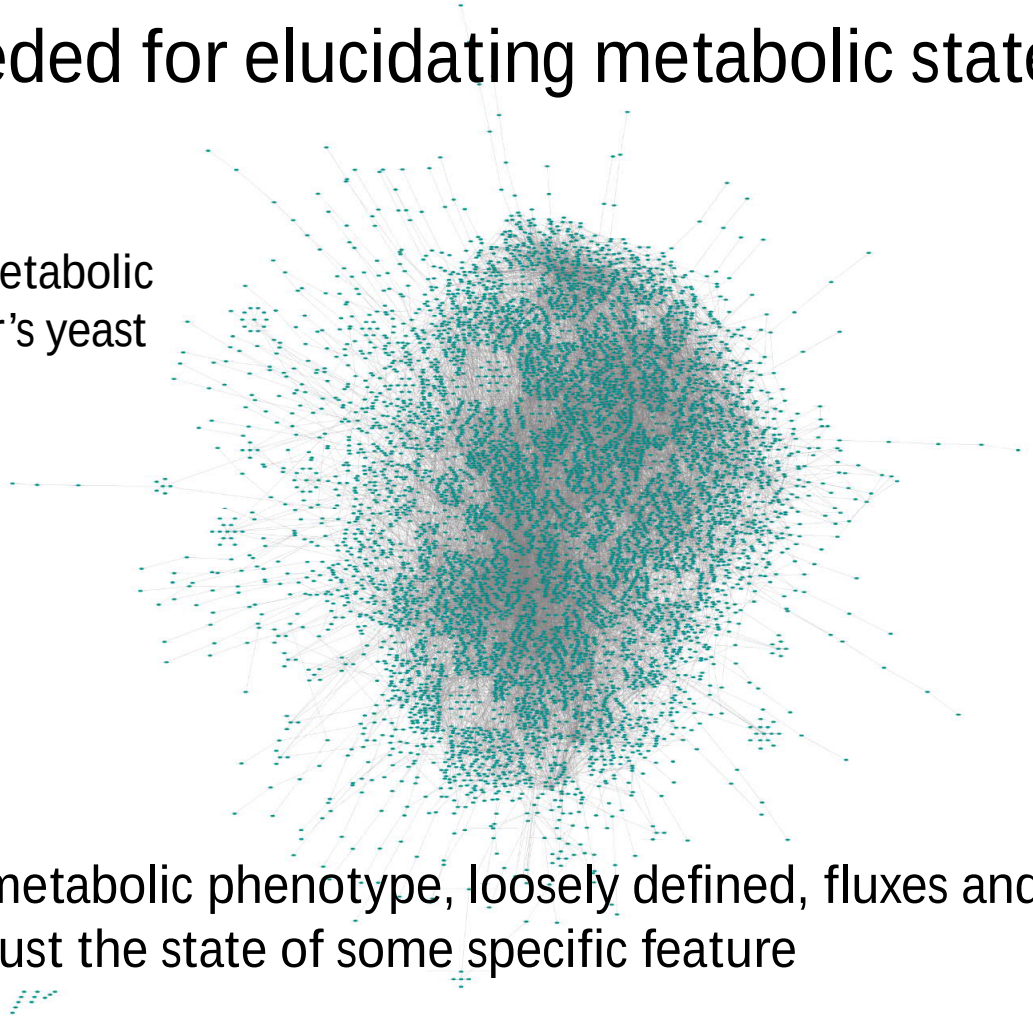


”Active metabolism”

Slide from Jaakko Mattila

Modelling needed for elucidating metabolic states

Genome-scale metabolic network of Baker's yeast



Metabolic state = metabolic phenotype, loosely defined, fluxes and metabolite concentrations or just the state of some specific feature

Assembly of genome-wide metabolism

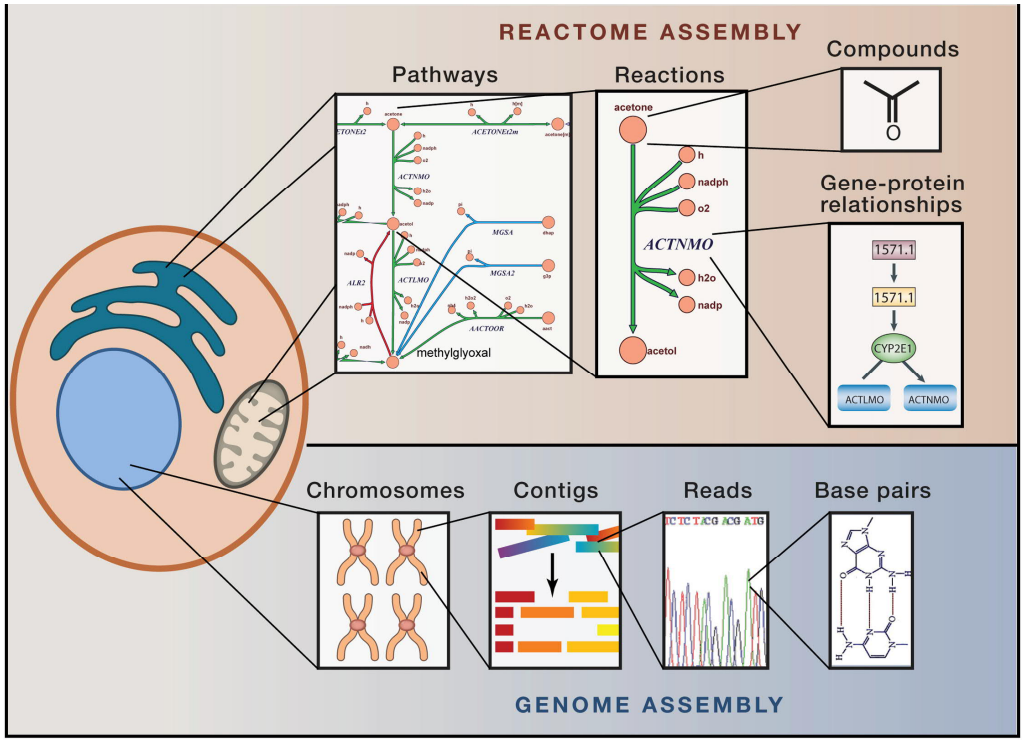
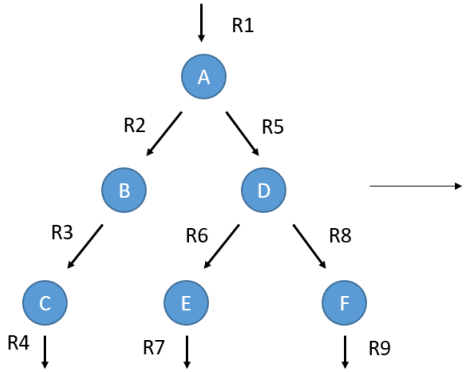


Figure from O'Brien et al. 2015
https://doi.org/10.1016/j.cell.2015.05.019

Conversion to a mathematical representation



Stoichiometric matrix

Metabolites	R1	R2	R3	R4	R5	R6	R7	R8	R9
A	1	-1			-1				
B		1	-1						
C			1	-1					
D					1	-1		-1	
E						1	-1		
F								1	-1

Reactions

Figure modified by Tuula Tenkanen from O'Brien et al. 2015

Obeying the law of conservation of mass,
metabolite mass balances constrain metabolic phenotypes

$$\frac{d\boldsymbol{X}}{dt} = \boldsymbol{S} \cdot \boldsymbol{v} = \boldsymbol{S} \cdot \boldsymbol{f}(\boldsymbol{e}(\boldsymbol{t}), \boldsymbol{s}(\boldsymbol{t}), \boldsymbol{p}) \qquad \text{(Equation 1)}$$

Steady state assumption linearizes the mass balances

$$\frac{dX}{dt} = \boldsymbol{S} \cdot \boldsymbol{v} = \boldsymbol{S} \cdot \boldsymbol{f}(\boldsymbol{e}(\boldsymbol{t}), \boldsymbol{s}(\boldsymbol{t}), \boldsymbol{p}) = 0 \qquad \text{(Equation 2)}$$

Constraints:
1) $\boldsymbol{Sv} = 0$
2) $\boldsymbol{v,lb} < \boldsymbol{v} < \boldsymbol{v,ub}$

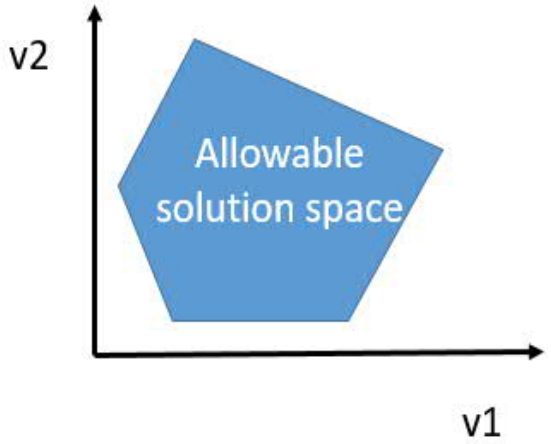


Figure modified by Tuula Tenkanen from O'Brien et al. 2015

The linear system is lighter to solve and free of kinetic equations and parameters
Additional constraints introduced to obey the second law of thermodynamics

Linear optimization can be used to identify optimal metabolic states

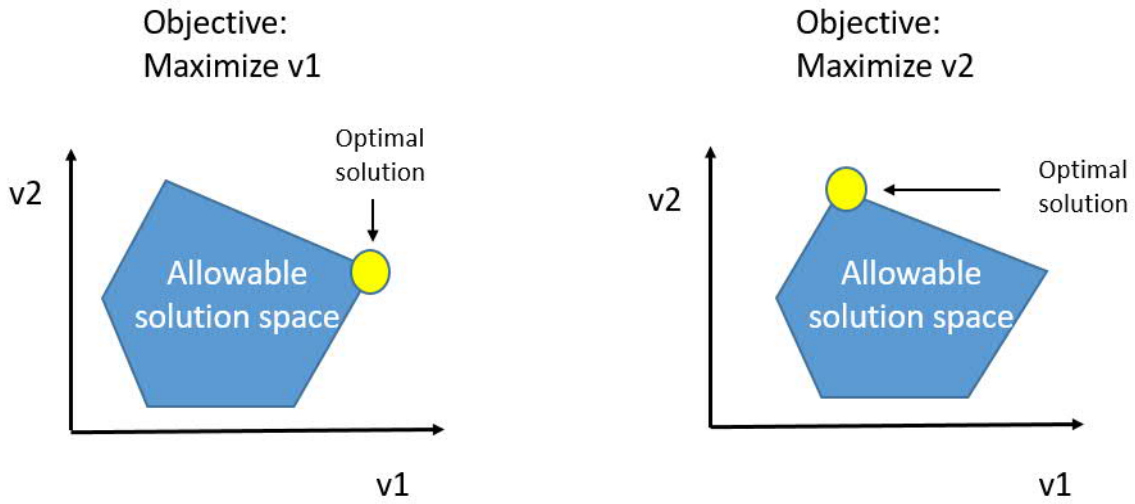


Figure modified by Tuula Tenkanen from O'Brien et al. 2015

Flux Balance Analysis (FBA)

Varma and Palsson, 1993; Varma and Palsson, 1994

maximize (or minimize) $\boldsymbol{c}' \cdot \boldsymbol{v}$

subject to $\boldsymbol{S} \cdot \boldsymbol{v} = 0$ (Equation 3)

$v,lb < v < v,ub$

Task:

What are the benefits arising from the steady state assumption?

a) kinetic parameters are not needed

b) linear problems are easier to solve

c) metabolism is not dynamic

d) metabolite concentrations are not variables

Artificial reaction(s) forming biomass allows growth simulations

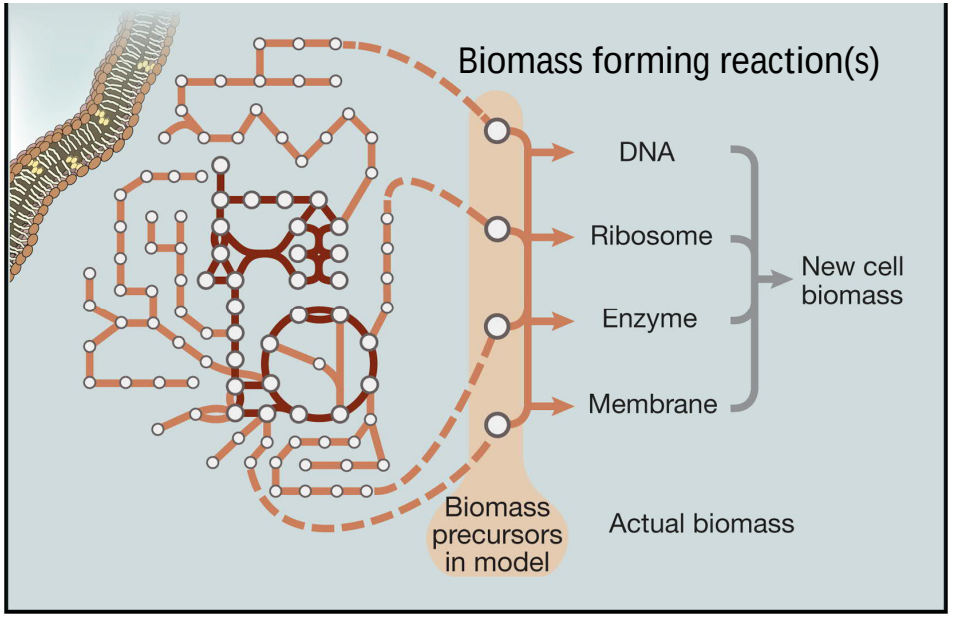
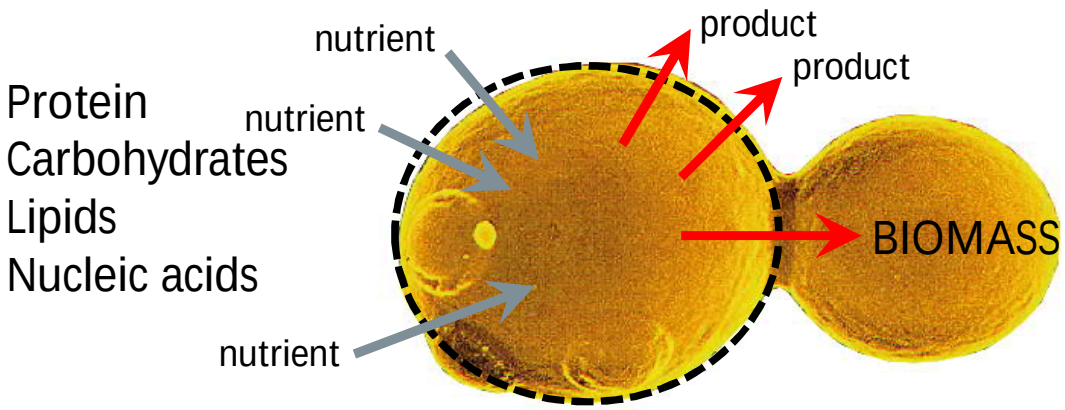


Figure adopted from O'Brien et al. 2015

What should the artificial biomass reaction(s) include?



- Proportions and exact compositions are species, strain, and condition dependent
- Biomass equation commonly describes the energy and redox balancing requirements of synthesizing macromolecules
- Dilution of other intracellular metabolites due to cell division is neglectable and omitted in simulations

Universally Essential Cofactors in Prokaryotes
Xavier JC et al. (2017) Metab Eng.

Organic cofactor(s)	A. BOFs of manually-curated GEMs (1)	Biosynthesis genes are essential (2)	B. Participates in essential reactions (3)	C. Reviewed Evidence: ModelSEED (4)	C. Reviewed Evidence: Literature (5)	Essentiality	Functional role
NAD(H)						Universal	Transport and transfer of hydride groups.
NADP(H)						Universal	Transport and transfer of hydride groups.
S-adenosyl-methionine						Universal	Universal methyl donor; generator of deoxyadenosyl radicals.
FAD						Universal	Electron transfer, radical and photoreceptor-induced reactions.
Pyridoxal 5p						Universal	Electrophilic catalyst
Coenzyme A						Universal	Transport and transfer of acyl groups
C1 carriers (derivatives of H(4)-MPT or H(4)folate)						Universal	Transport and donation of C1 units
Thiamin diphosphate						Universal	Making and breaking bonds between C and S, O, H and N atoms, and most notably C-C bonds
FMN						Universal	Electron transfer, radical and photoreceptor-induced reactions.

Task:

How is microbial growth described in genome-scale metabolic models?

a) number of cells increase

b) biomass is a product as any other compound produced out of the cells

c) energy and redox costs of growth predicted using model simulations

Metabolic states depend on environment

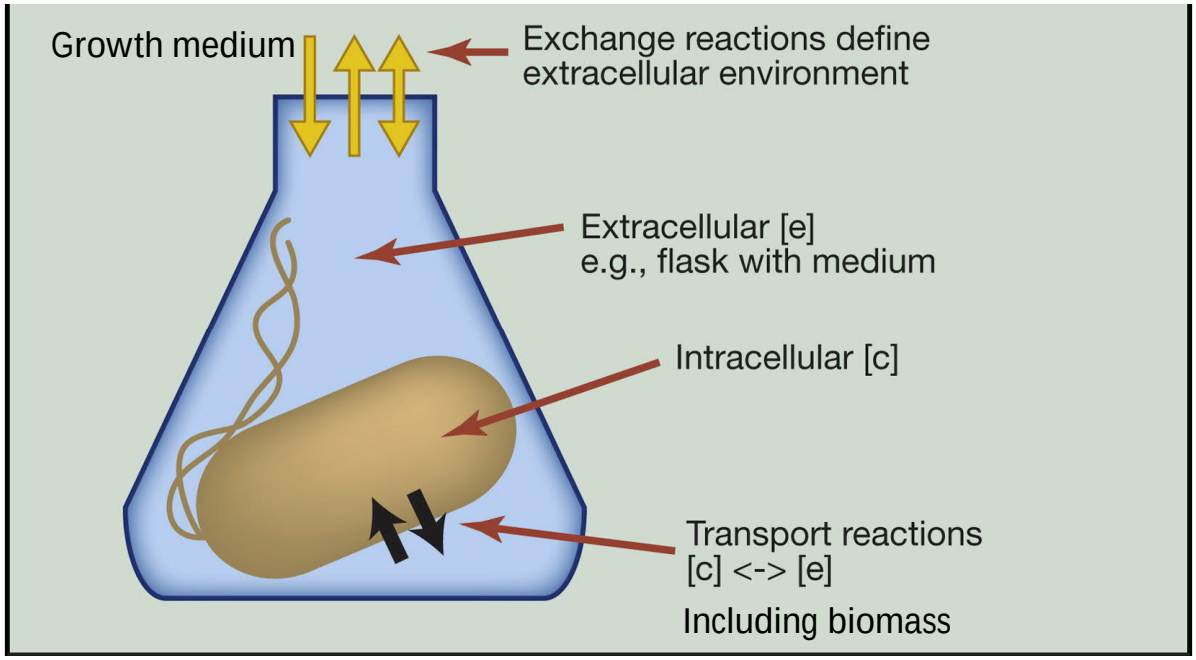
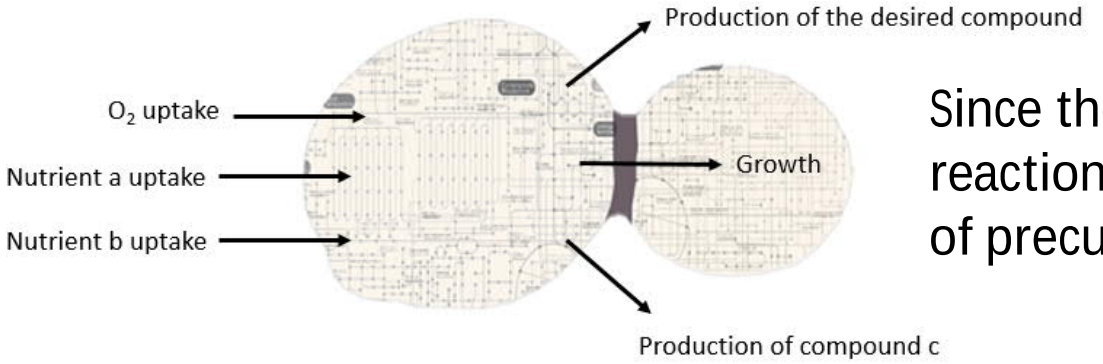


Figure adopted from O'Brien et al. 2015

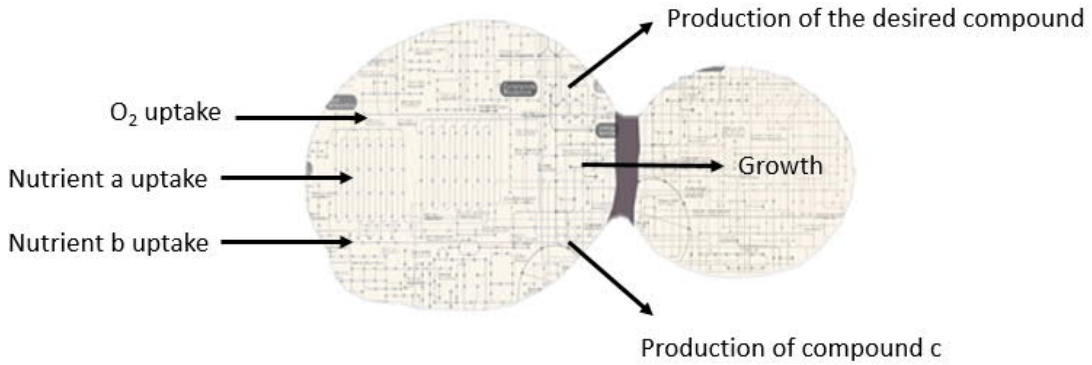
Specific fluxes

Flux units mmol/(g CDW h)



Since the artificial biomass reaction is defined as mmoles of precursors for 1 g CDW

Prediction vs estimation of metabolic state?



When arbitrary constraints are used, yields can be predicted
When empirical rates are used as constraints, other rates can be estimated or predicted

Task:

How is growth environment considered in the genome-scale metabolic model simulations?

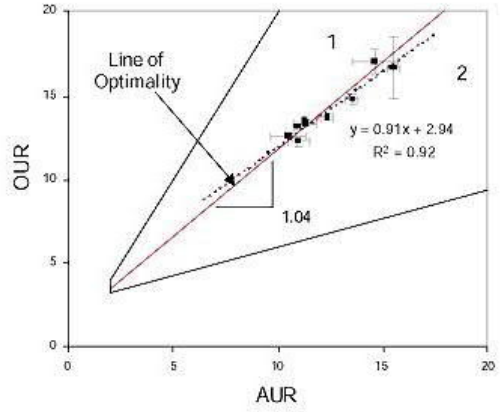
a) it is described in the manuscript

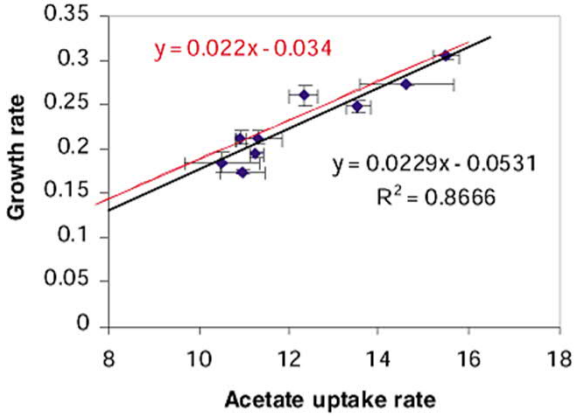
b) it is encoded in the exchange flux bounds

c) if experimental uptake rates are known, fluxes can be estimated

d) if experimental uptake rates are not known, nothing can be predicted

FBA simulations optimizing growth predict well experimental phenotypes





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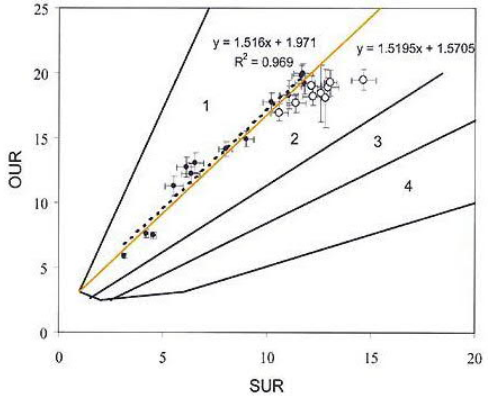
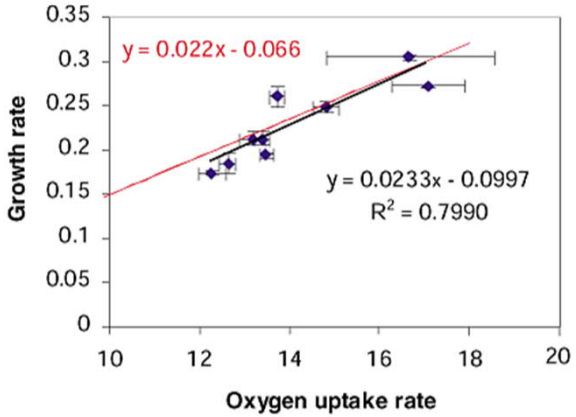
RESEARCH ARTICLES

In silico predictions of *Escherichia coli* metabolic capabilities are consistent with experimental data

Jeremy S. Edwards[1,2], Rafael U. Ibarra[1], and Bernhard O. Palsson[1*]

[1]Department of Bioengineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0412. [2]Current address: Department of Chemical Engineering, University of Delaware, Newark, DE 19716. *Corresponding author (palsson@ucsd.edu).

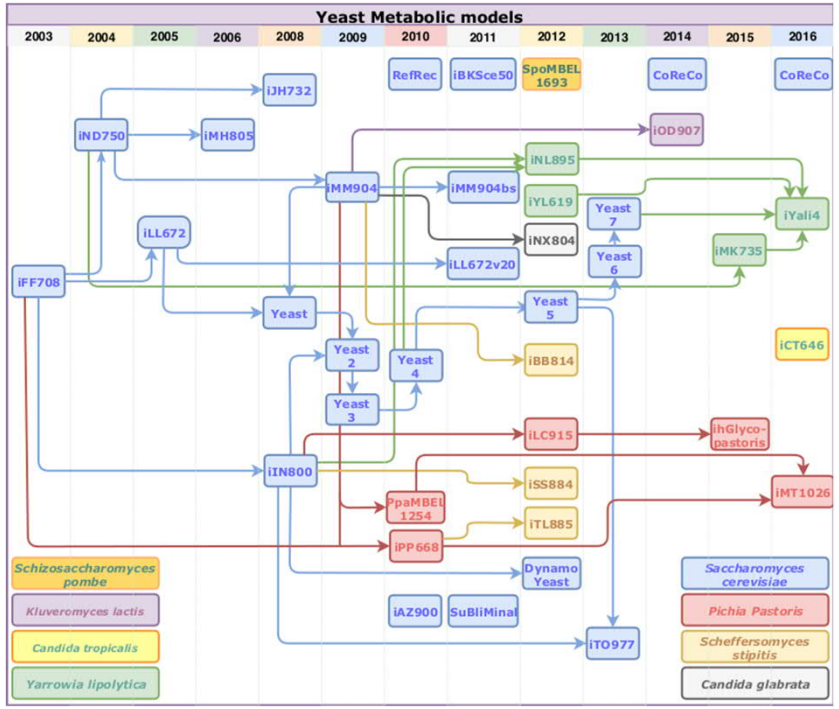




Manually curated models for model organisms

For example

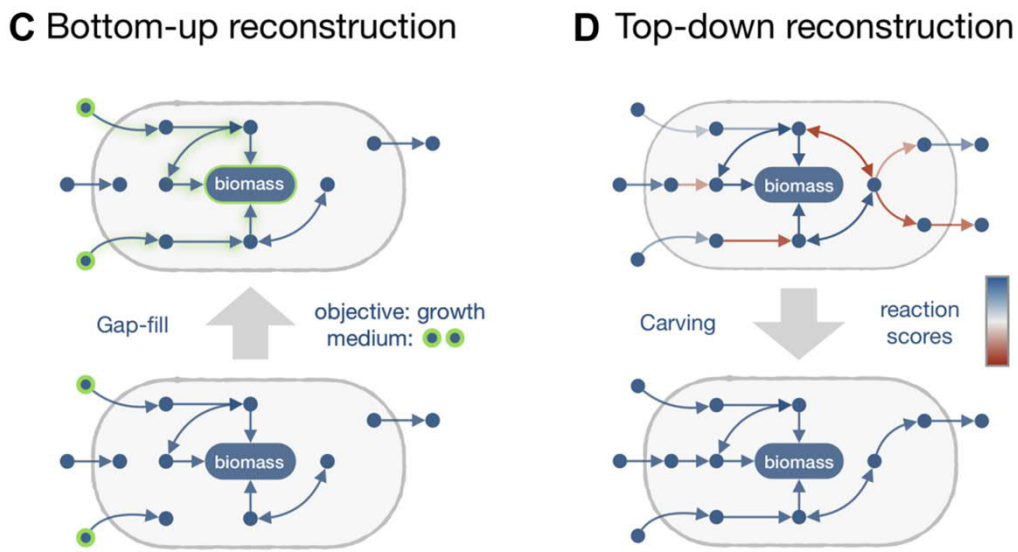
- *Escherichia coli*
- *Bacillus subtilis*
- *Corynebacterium glutamicum*
- *Saccharomyces cerevisiae*
- Other yeasts (figure)
- Human
- Mouse



Castillo S, Patil KR, Jouhten P. Yeast Genome-Scale Metabolic Models for Simulating Genotype-Phenotype Relations. DOI:10.1007/978-3-030-13035-0_5

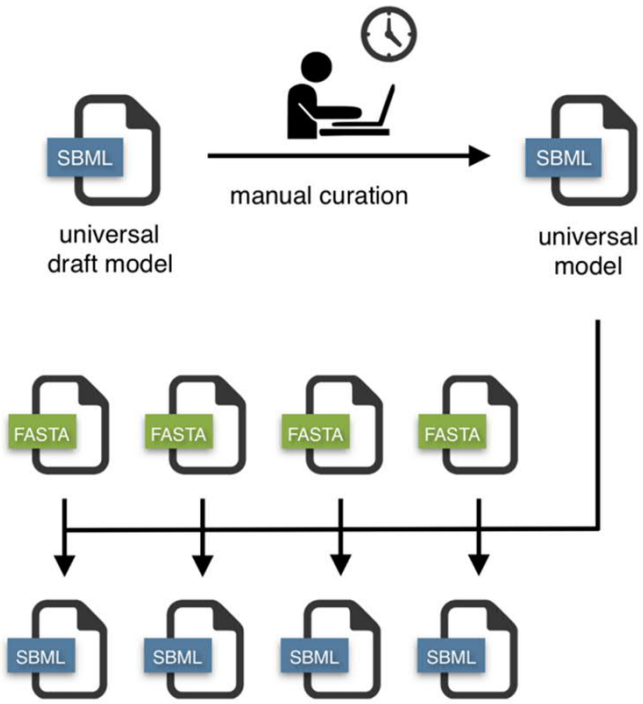
Model reconstruction automatically from genome either bottom-up or top-down

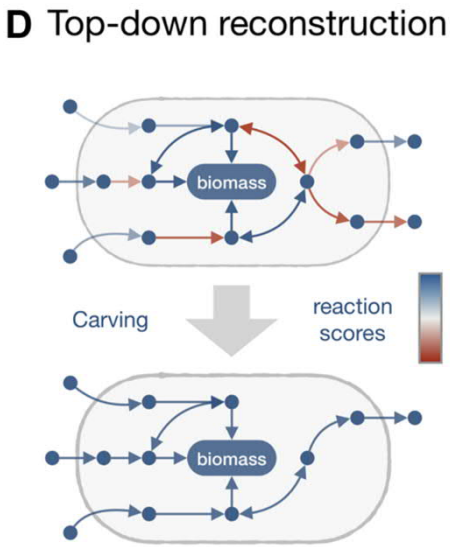
Functional genome annotation for reaction scores



Machado et al. Fast automated reconstruction of genome-scale metabolic models for microbial species and communities. Nucleic Acids Res. (2018) **46**:7542-7553. doi:10.1093/nar/gky537.

CarveMe for top-down reconstruction of bacterial models

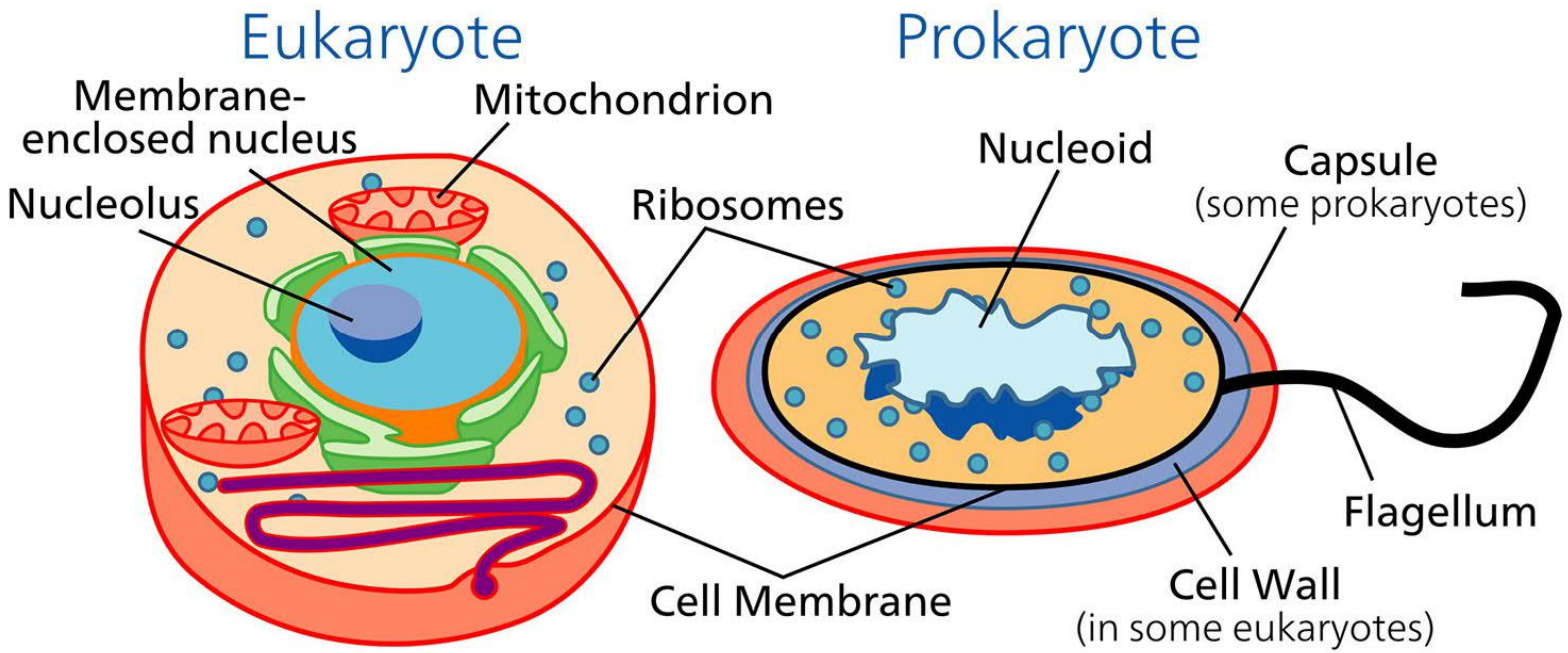




CarveMe: Machado et al. Fast automated reconstruction of genome-scale metabolic models for microbial species and communities. Nucleic Acids Res. (2018) **46**:7542-7553. doi:10.1093/nar/gky537.

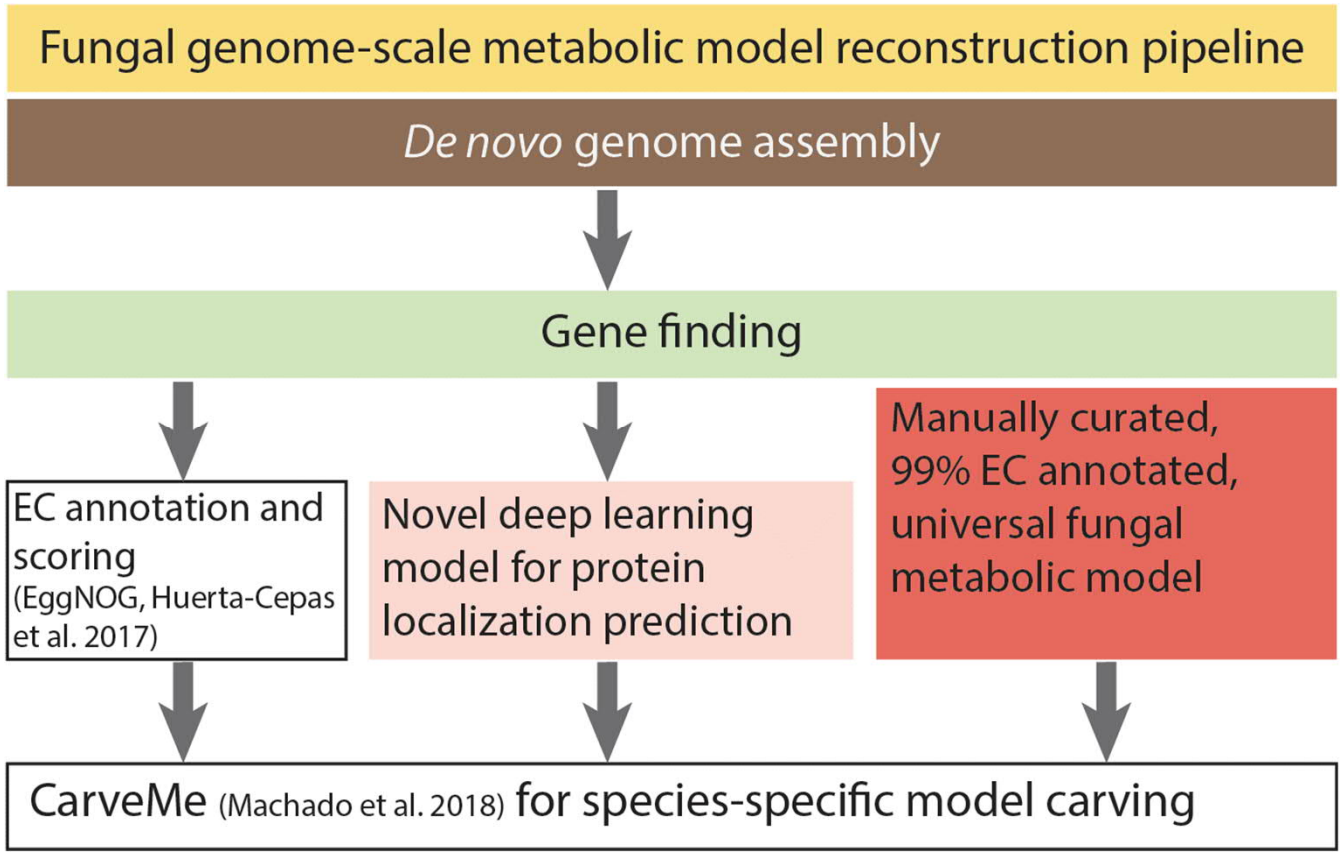
Available as python package: https://github.com/cdanielmachado/carveme

Eukaryotic metabolism is compartmentalized



National Center for Biotechnology Information, https://www.thoughtco.com/types-of-cells-1224602

Novel CarveFungi for eukaryotic model reconstruction by predicting enzyme subcellular localizations



~ 5000 reactions

Unpublished work

Task:

Why is model reconstruction more challenging for eukaryotic than for prokaryotic species?

a) it cannot be done top-down

b) subcellular compartment membranes are not permeable to many compounds

c) enzyme subcellular localization varies between species

Recent literature

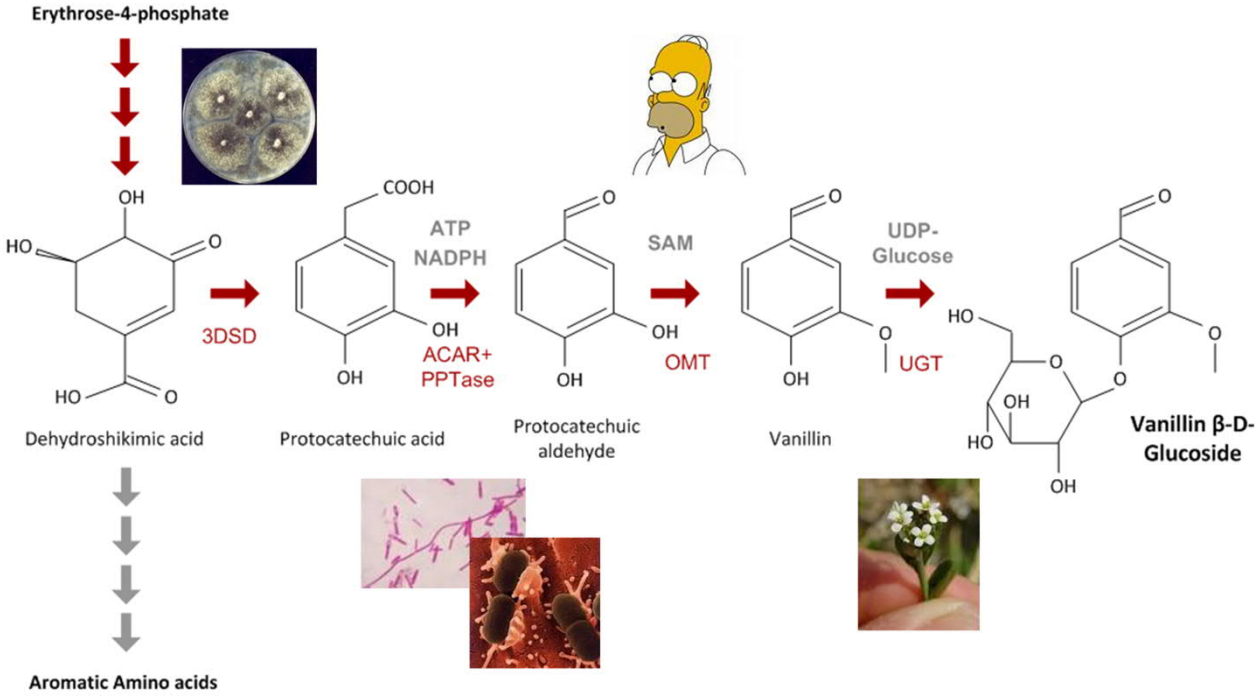
Reconstructing organisms in silico: genome-scale models and their emerging applications

Xin Fang[1], Colton J. Lloyd[1] and Bernhard O. Palsson[1,2,3]

https://www.nature.com/articles/s41579-020-00440-4

1. Design of synthetic metabolic pathways

Synthetic pathway for vanillin production in yeast

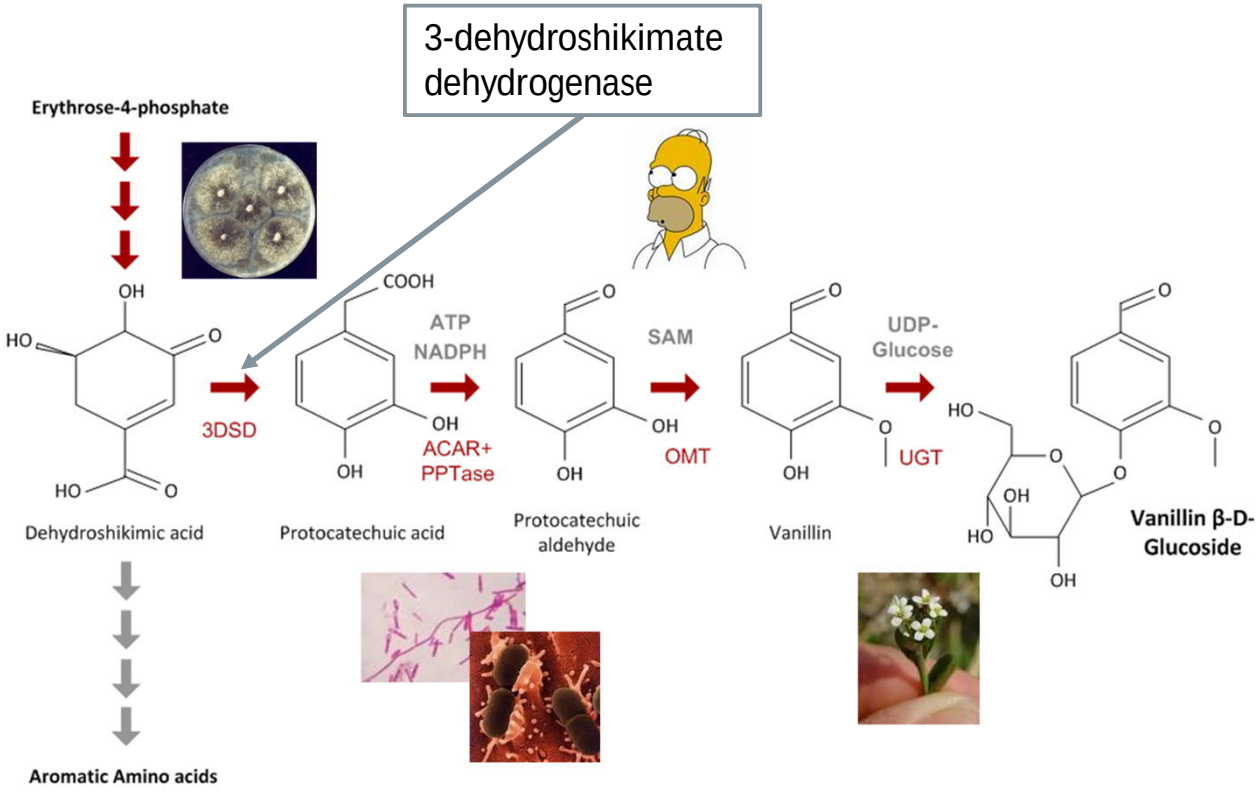


Hansen *et al.*, AEM, 2009
Brochado *et al.*, 2010

Slide from Dr. Kiran Patil

EMBL

Synthetic pathway for vanillin production in yeast



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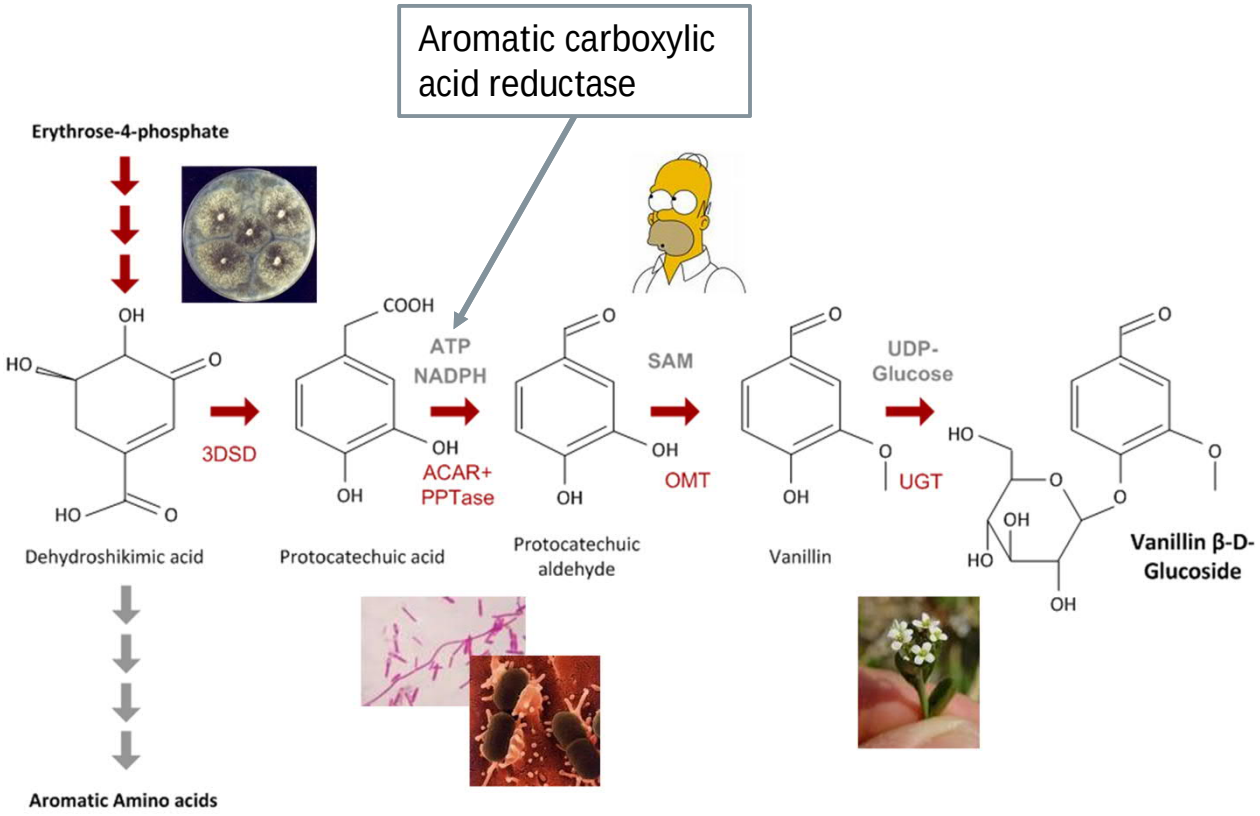

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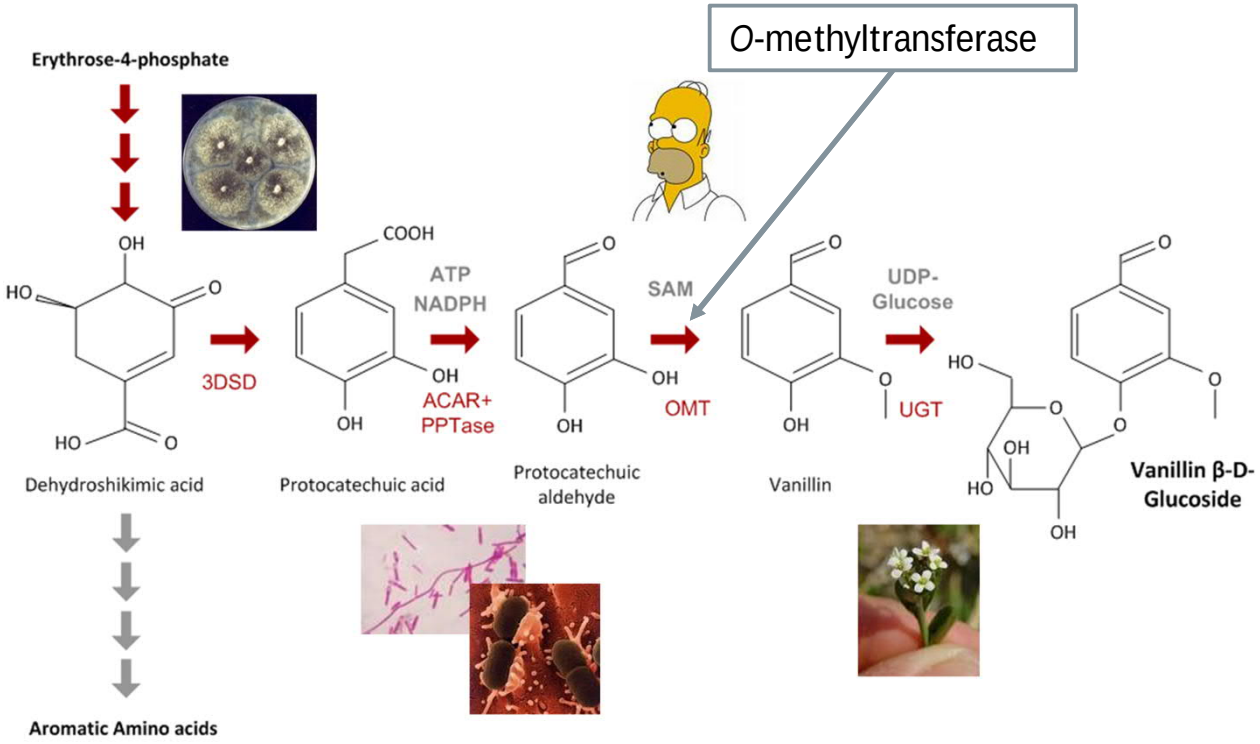

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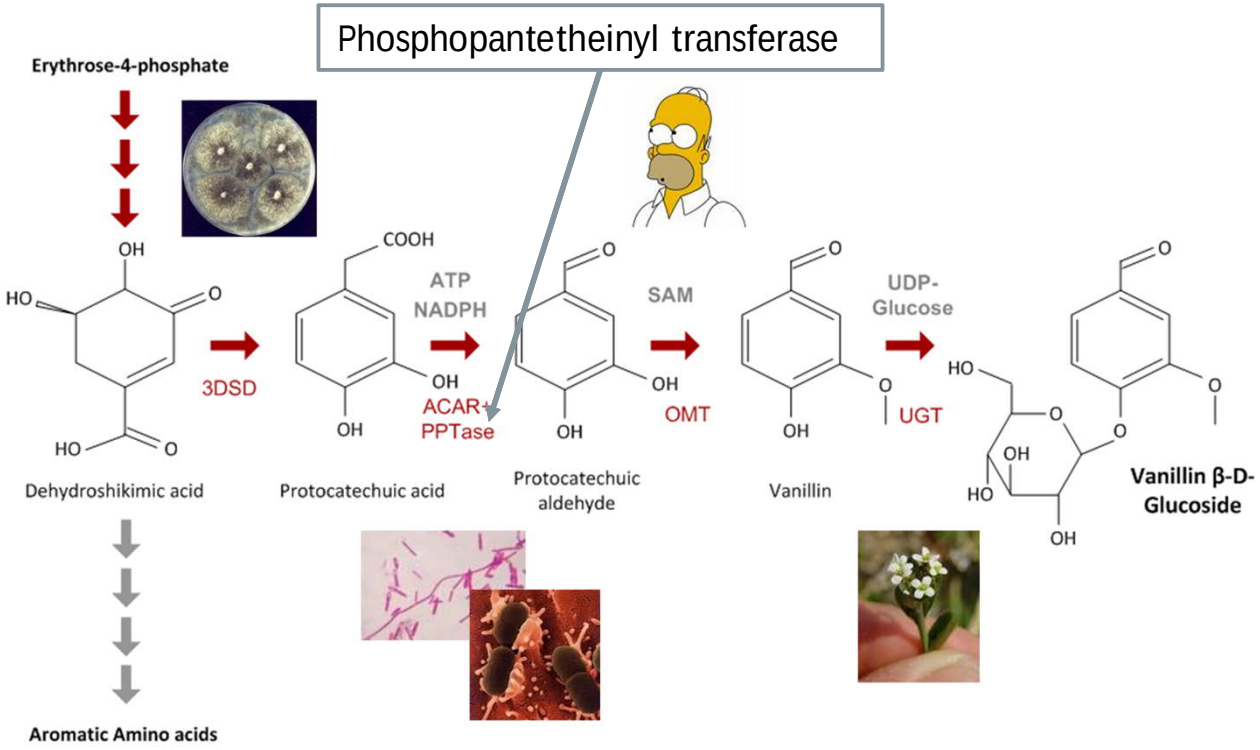

Hansen *et al.*, AEM, 2009
Brochado *et al.*, 2010

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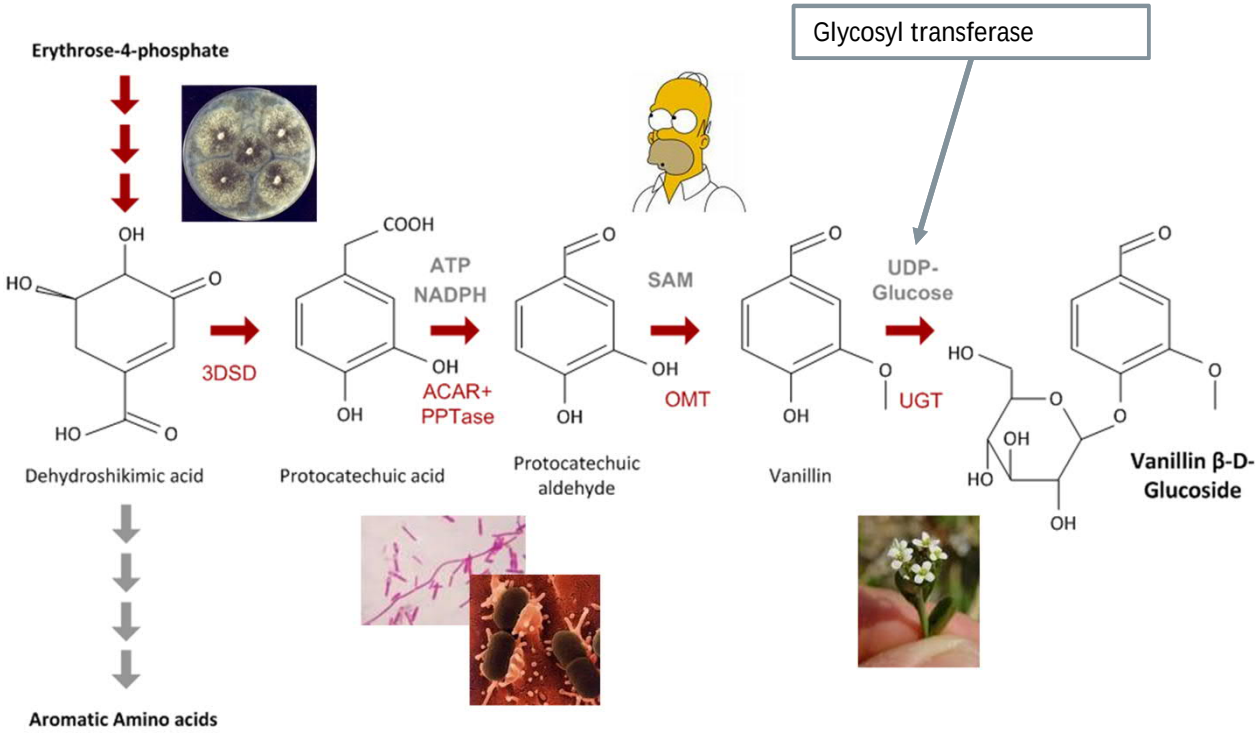
Synthetic pathway for vanillin production in yeast



Hansen *et al.*, AEM, 2009
Brochado *et al.*, 2010

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EMBL

Synthetic pathway design

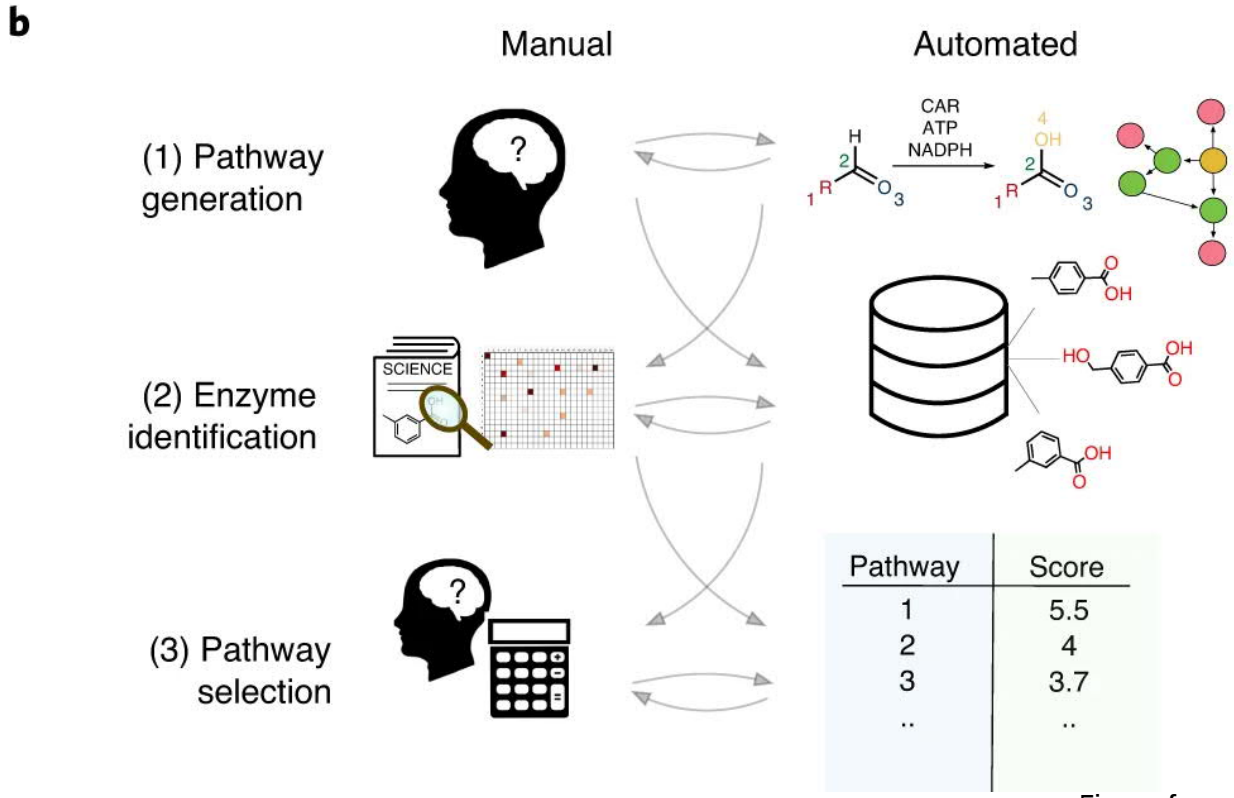


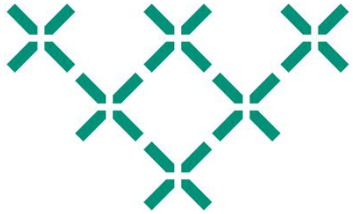
Figure from Finnigan et al. *Nature Catalysis* volume 4, pages98–104(2021)

Metabolic reaction databases

https://www.genome.jp/kegg/

https://www.rhea-db.org/

https://metacyc.org/



https://www.metanetx.org/

Task: Which reactions are needed to convert 3,4-dihydroxybenzoate to cis,cis-muconate? What are the proteins (uniprot entries) that could be used?

https://www.genome.jp/kegg/ https://www.rhea-db.org/

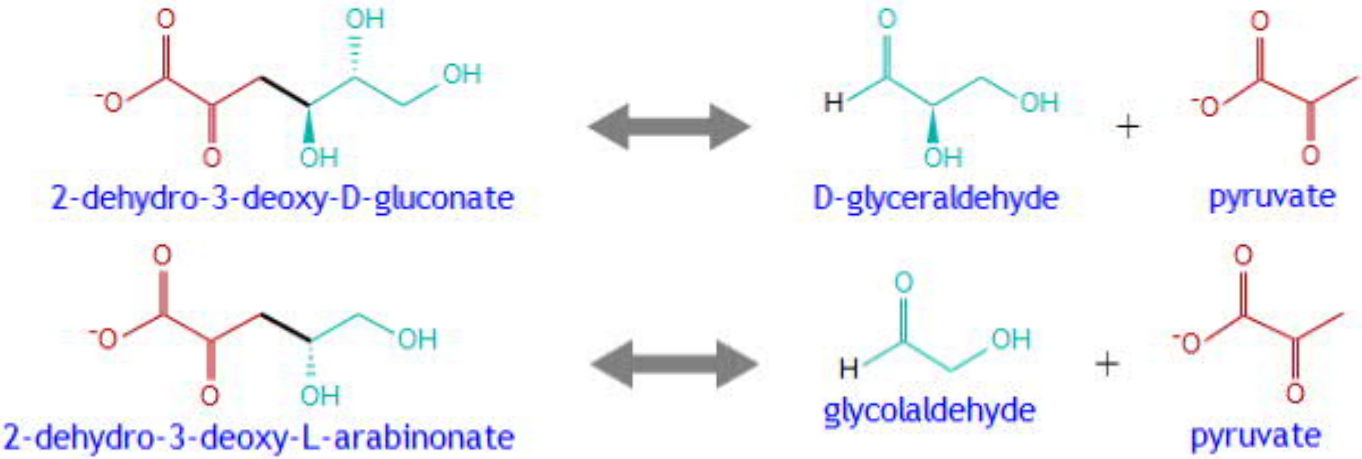
https://metacyc.org/ https://www.metanetx.org/

Protein database: https://www.uniprot.org/

Enzymes may be promiscuous

- Enzymes may catalyze alternative reactions
  - catalytic promiscuity = "ability of an enzyme to catalyze a secondary reaction at the same active site where its primary activity occurs, and the secondary activity has a different mechanism"
  - substrate promiscuity = substrate ambiguity

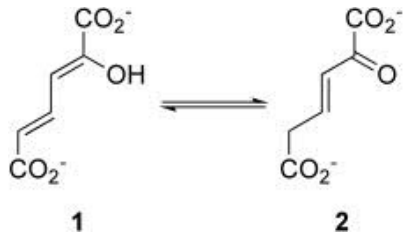
Reactions catalyzed by KDG aldolase from *Sulfolobus solfataricus*



http://www.jbc.org/content/279/42/43886.full.html
http://www.jbc.org/content/285/44/33701.long

Example of catalytic promiscuity: 4-Oxalocrotonate Tautomerase

Primary reaction

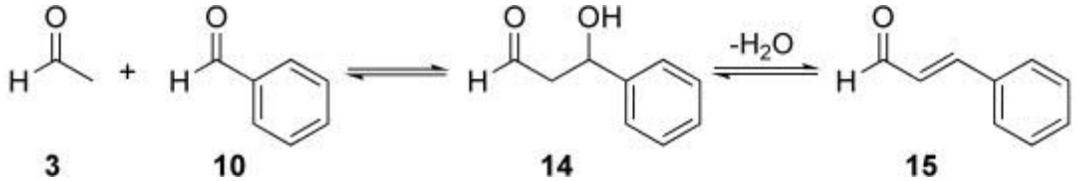


1 2

2-hydroxy-2,4-hexadienedioate 2-oxo-3-hexenedioate

https://chemistry-europe.onlinelibrary.wiley.com/doi/full/10.1002/cbic.201000633

Secondary reaction: aldol condensation



3 10 14 15

acetaldehyde benzaldehyde cinnamaldehyde

Reaction rules model possible enzyme catalyzed reactions

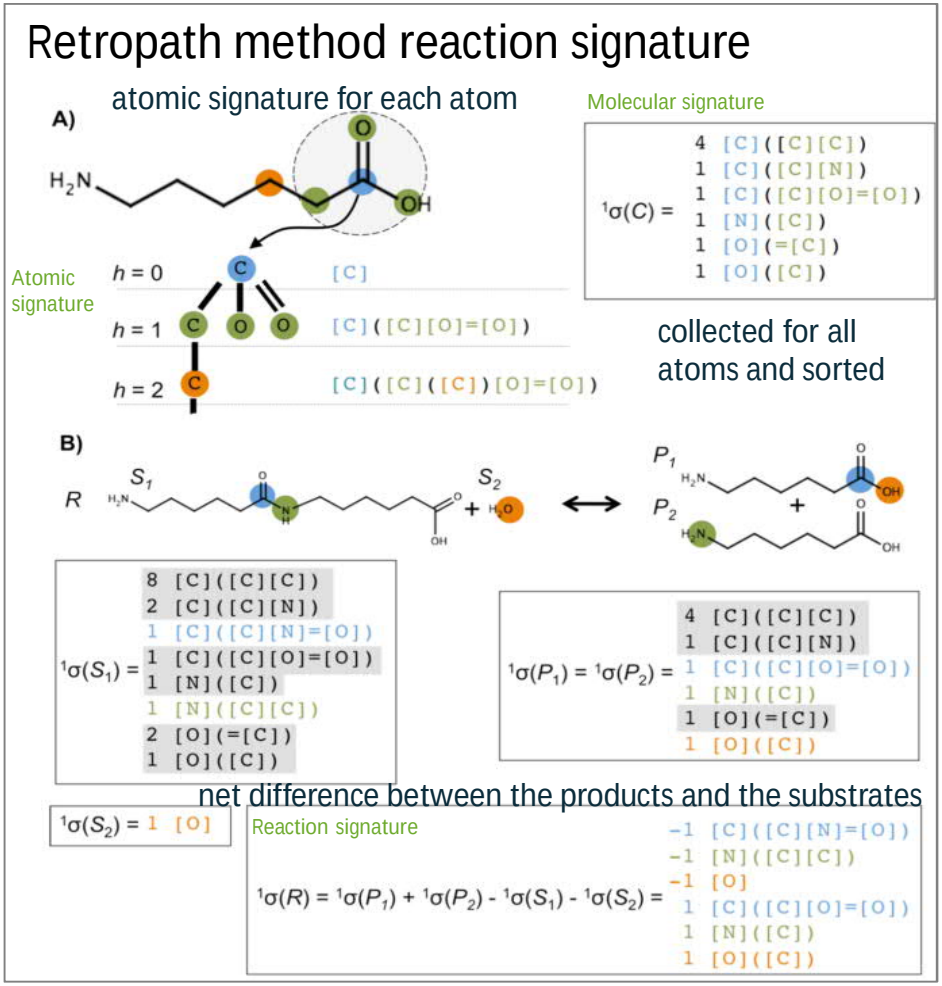
- Rules model similarities to known reactions (i.e. similarities of reactants)
- Assume that if the core of the reaction (where the bonds break) remains the same then an enzyme could be found/built for the novel reaction
- Define different dimensions of the core

- Reaction rules create extended metabolic space

Table 1 Reactions in the EMRS

height *h*	reactions	% increase from canonical
2	9083	17.72%
3	7882	2.15%
4	7800	1.09%
5	7752	0.47%
6	7725	0.12%
canonical	7716	0%

Number of novel generated putative reactions in the EMRS for different heights *h*.



Carbonell, P., Planson, A.-G., Fichera, D., & Faulon, J.-L. (2011). A retrosynthetic biology approach to metabolic pathway design for therapeutic production. BMC Systems Biology, 5(1), 122.

Task:

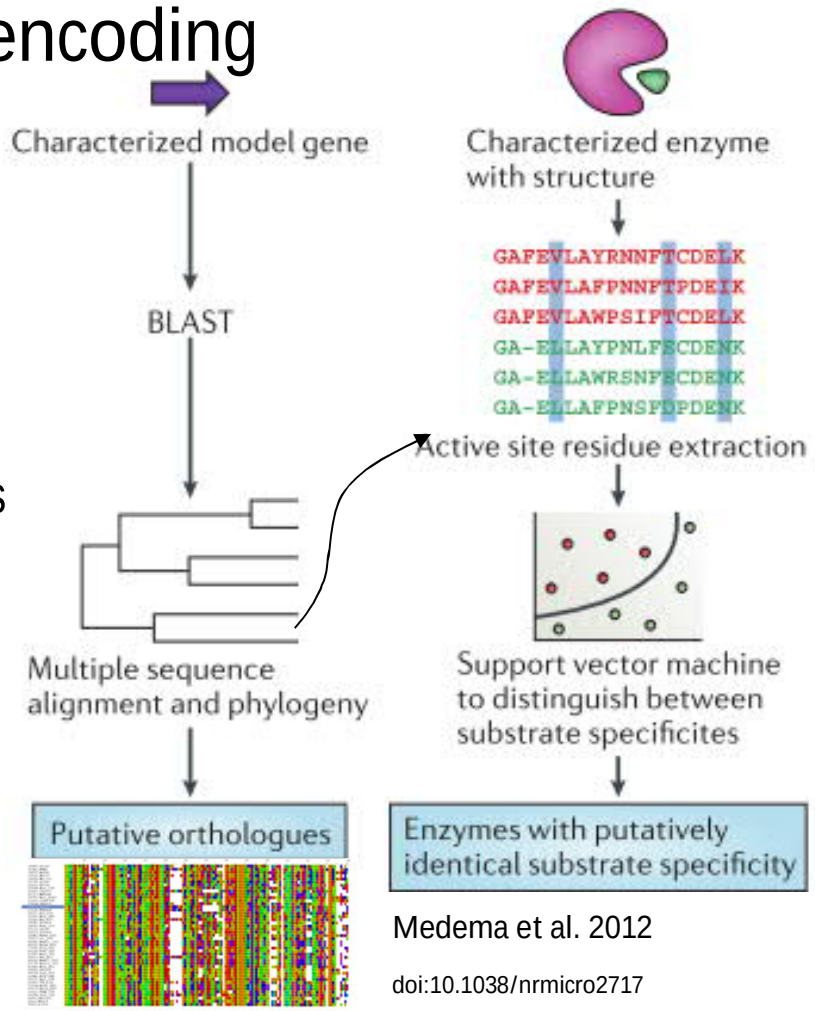
If the reaction rules are used to create extended metabolic space it becomes larger when..?

a) dimension parameter is smaller

b) dimension parameter is bigger

Selecting candidate enzyme encoding sequences

- Gene/protein databases include references to enzyme mechanisms (e.g. EC numbers)
- Further candidates (orthologs) by genome mining of sequence databases
- Screening candidates' performances



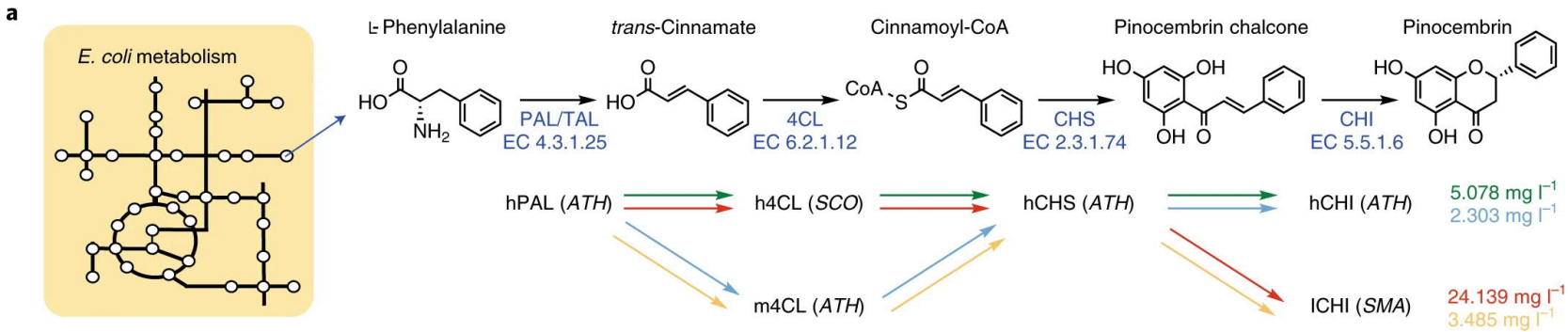
Medema et al. 2012

doi:10.1038/nrmicro2717

Orthologs: genes in different species evolved from a common ancestral gene.
Paralogs: gene copies created by a duplication event within the same genome.

Slide adopted from Merja Oja

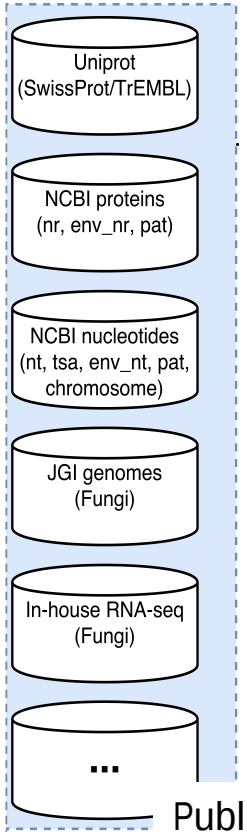
Synthetic pathway to pinocembrin to *E.coli*



Alternative enzyme options result in different pinocembrin titers
Pathway optimization could involve optimizing the enzyme levels or the actual enzymes

Figure from Lee et al. Nature Catalysis 2,18–33(2019) but data from Feher, T. et al. Biotechnol. J. 9, 1446–1457 (2014).

Novel machine learning approaches reach beyond homology based enzyme finding



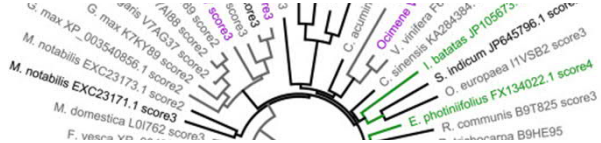
Public and proprietary sequence resources in use

Challenge

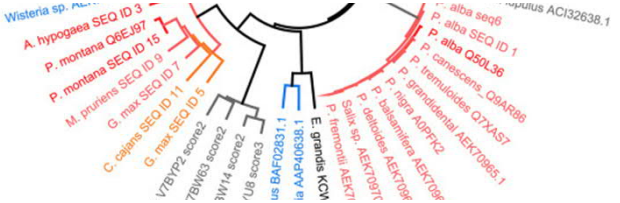
- Interesting enzymes may lack homology to known genes
- How should novel enzyme sequences be?

Our strategy

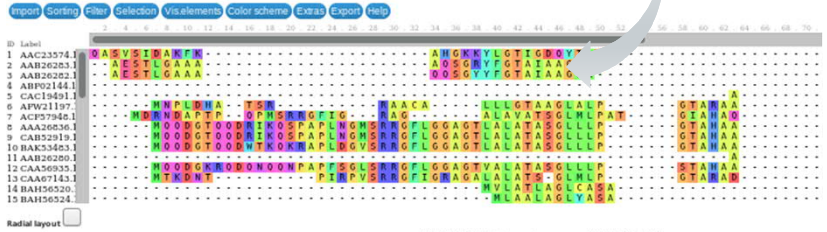
- Complementing conventional sequence mining with machine learning

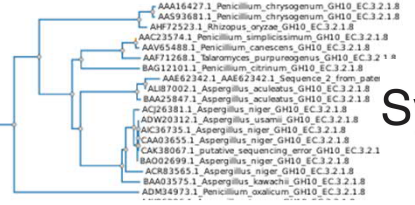


Homology based search of isoprene synthases



Autoencoder modelled proteins

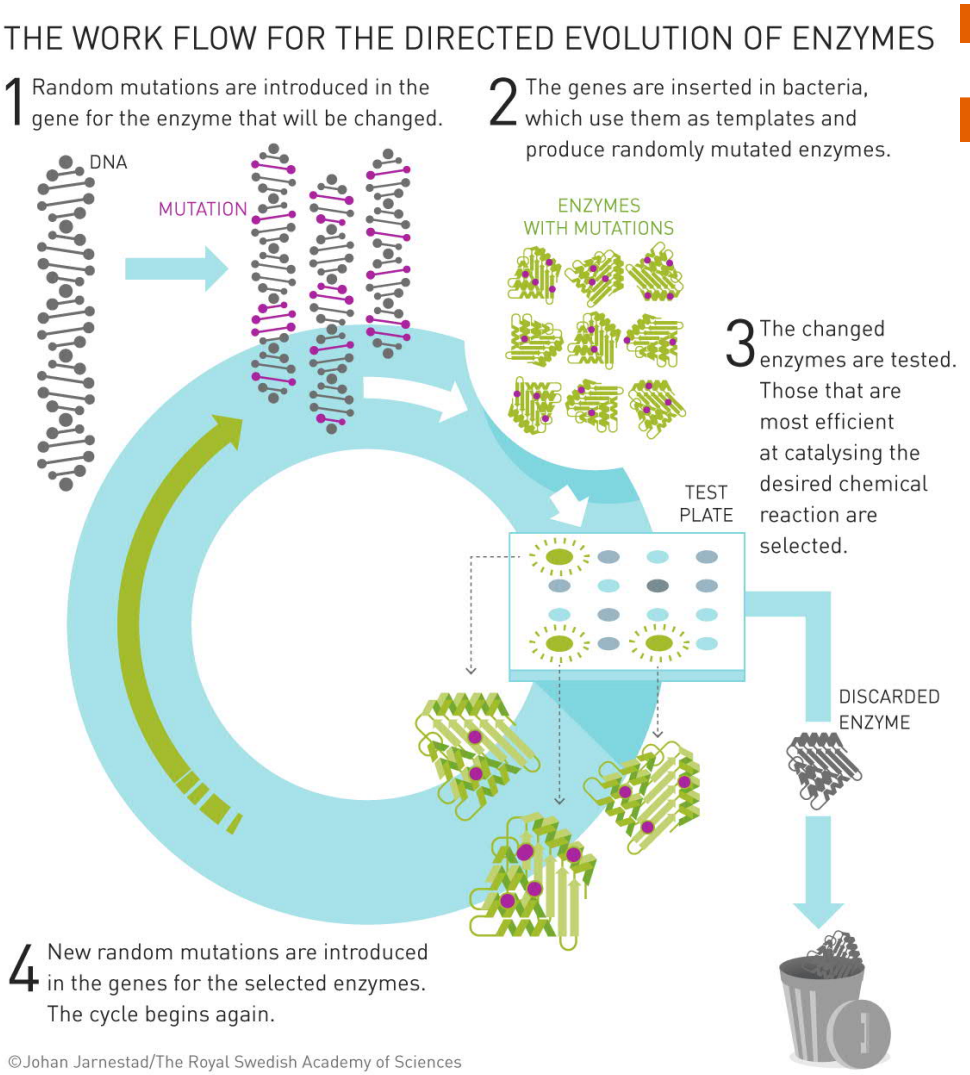




M. Ilmén *et al.*, *Metab. Eng.*, 2015; M. Oja *et al.*, *ISMB Comm J*, 2017

Frances H. Arnold received the Noble prize for directed evolution of proteins in 2018

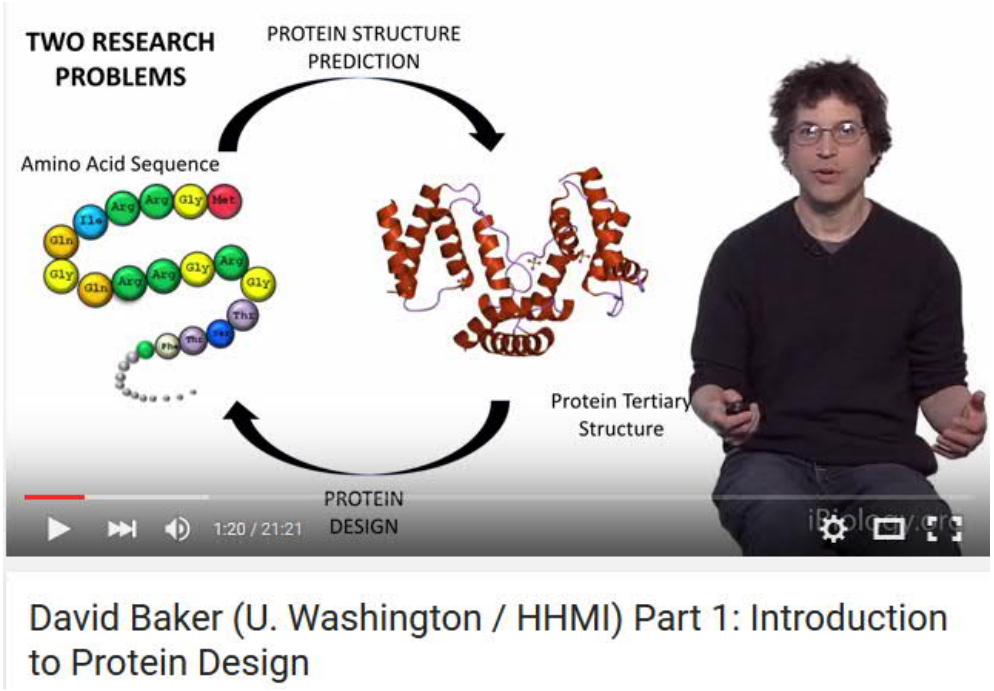
https://www.quantamagazine.org/frances-arnold-george-smith-and-gregory-winter-win-chemistry-nobel-for-directing-evolution-20181003/



Design of new-to-nature proteins

Protein structure prediction

https://www.rosettacommons.org/



David Baker (U. Washington / HHMI) Part 1: Introduction to Protein Design

https://www.youtube.com/watch?v=0LetJMbu7uY

David Baker, PhD, Director of the Institute for Protein Design
"His research group is a world leader in computational protein design and protein structure prediction."
Rosetta computational prediction and design method

David Baker (U. Washington / HHMI) Part 2: Design of New Protein Functions
https://www.youtube.com/watch?v=ZrAwWx7meTk

Task: Find out who are DeepMind and what was the breakthrough they demonstrated in 2020 – Let's discuss this tomorrow

Criteria for choosing pathways for experimental implementation?

- Yield
- Thermodynamics
- Pathway length
- Number of new-to-nature reactions
- Possible host
- Toxicity

Tasks:

How do get the theoretical yield of the target compound for a candidate pathway?

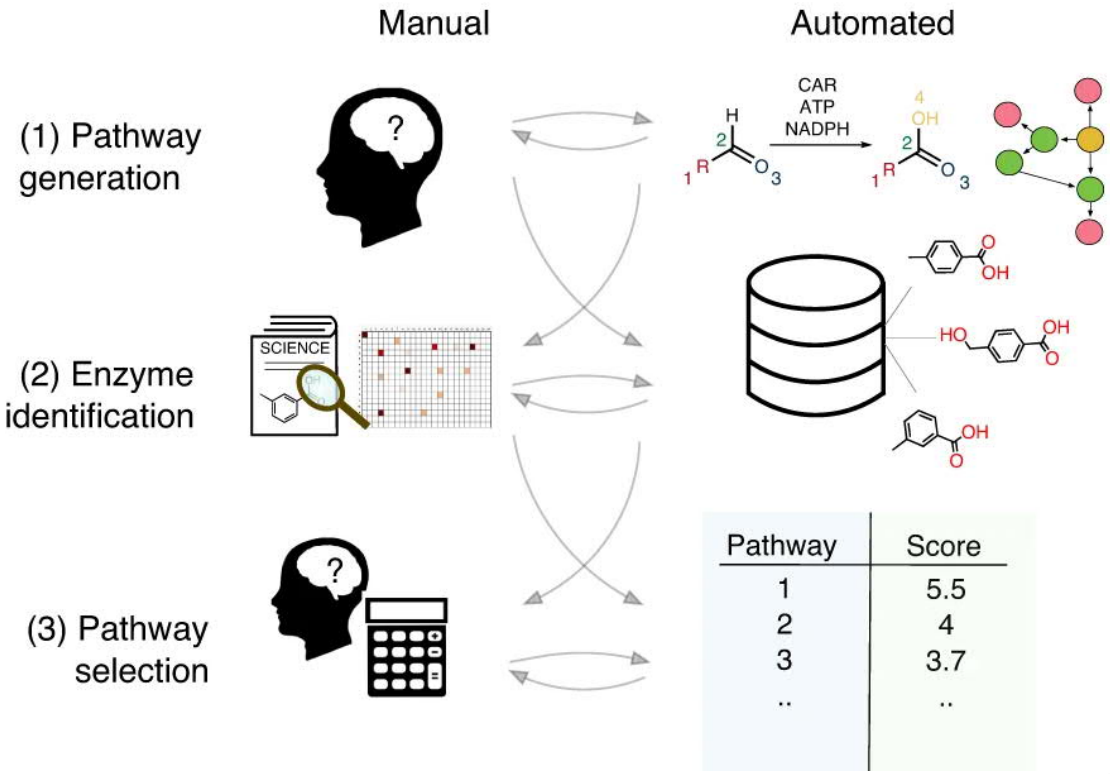
Could this pathway be used to produce 3-hydroxypropanoate in a yeast host?

acetyl-CoA + CO2 + NADH + H+ <=> 3-oxopropanoate + CoA + NAD+

3-hydroxypropanoate + NAD+ <=> 3-oxopropanoate + NADH + H+

(https://equilibrator.weizmann.ac.il/)

Synthetic pathway design



Pathway search in
- Known (bio)chemical reactions
- Extended metabolic space

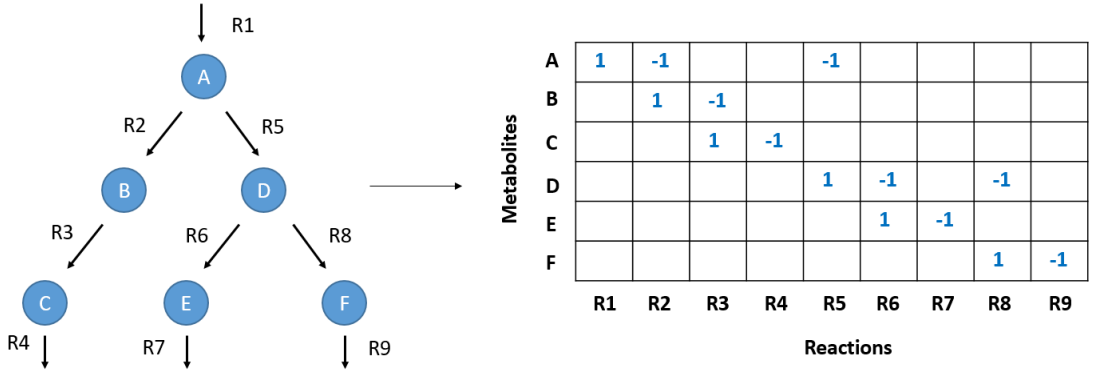
-> in graph or in genome-scale metabolic model

Candidate enzyme sequences from
- Homology based search in sequence resources
- Machine learning beyond sequence features
- Directed evolution
- Design of new-to-nature (Near Future?)

Pathway ranking

Figure from Finnigan et al. *Nature Catalysis* volume 4, pages98–104(2021)

Conversion to a mathematical representation



Stoichiometric matrix

Metabolites	R1	R2	R3	R4	R5	R6	R7	R8	R9
A	1	-1			-1				
B		1	-1						
C			1	-1					
D					1	-1		-1	
E						1	-1		
F								1	-1

Reactions

Figure modified by Tuula Tenkanen from O'Brien et al. 2015

Obeying the law of conservation of mass,
metabolite mass balances constrain metabolic phenotypes

$$\frac{d\boldsymbol{X}}{dt} = \boldsymbol{S} \cdot \boldsymbol{v} = \boldsymbol{S} \cdot \boldsymbol{f}(\boldsymbol{e}(\boldsymbol{t}), \boldsymbol{s}(\boldsymbol{t}), \boldsymbol{p}) \qquad \text{(Equation 1)}$$

Steady state assumption linearizes the mass balances

$$\frac{dX}{dt} = \boldsymbol{S} \cdot \boldsymbol{v} = \boldsymbol{S} \cdot \boldsymbol{f}(\boldsymbol{e}(\boldsymbol{t}), \boldsymbol{s}(\boldsymbol{t}), \boldsymbol{p}) = 0 \qquad \text{(Equation 2)}$$

Constraints:
1) $\boldsymbol{Sv} = 0$
2) $\boldsymbol{v,lb} < \boldsymbol{v} < \boldsymbol{v,ub}$

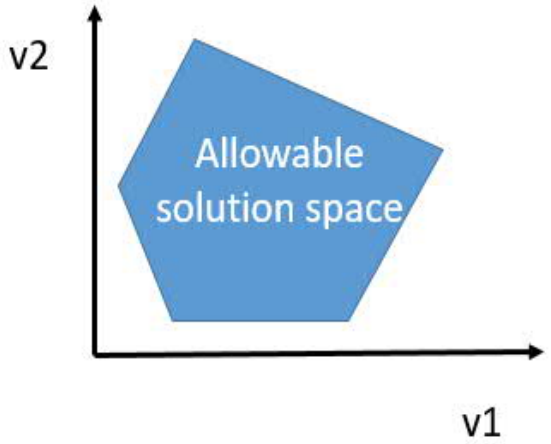


Figure modified by Tuula Tenkanen from O'Brien et al. 2015

The linear system is lighter to solve and free of kinetic equations and parameters
Additional constraints introduced to obey the second law of thermodynamics

Linear optimization can be used to identify optimal metabolic states

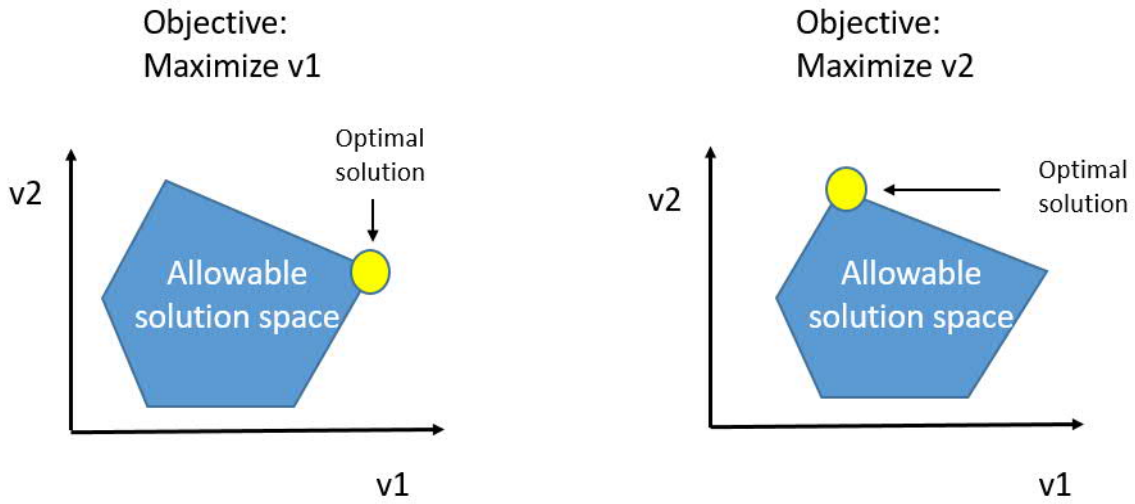


Figure modified by Tuula Tenkanen from O'Brien et al. 2015

Flux Balance Analysis (FBA)

Varma and Palsson, 1993; Varma and Palsson, 1994

maximize (or minimize) $\boldsymbol{c}' \cdot \boldsymbol{v}$

subject to $\boldsymbol{S} \cdot \boldsymbol{v} = 0$ (Equation 3)

$v,lb < v < v,ub$

Linear optimization can be used to identify optimal metabolic states

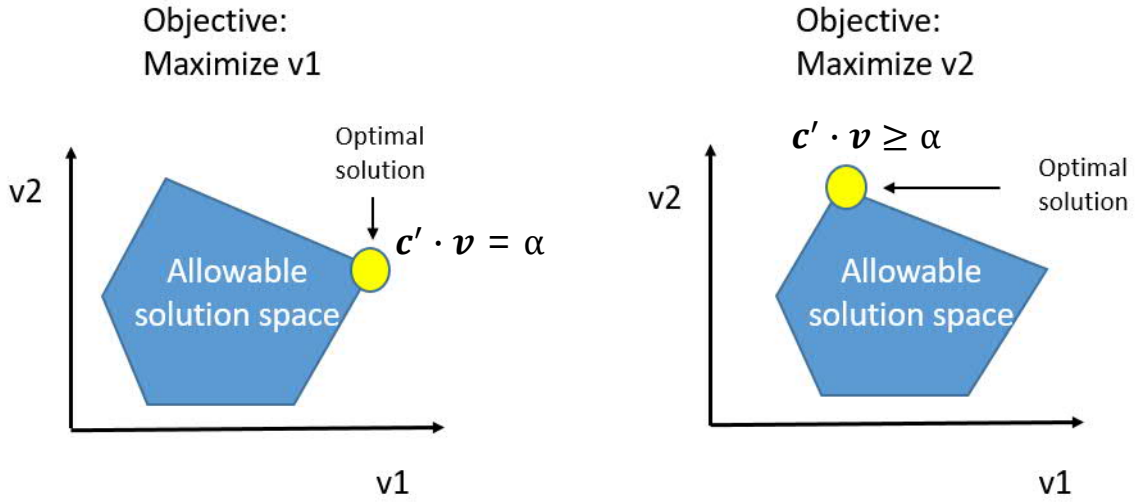


Figure modified by Tuula Tenkanen from O'Brien et al. 2015

Flux Variability Analysis (FVA)

Mahadevan et al. 2003

maximize and minimize v_i

subject to $S \cdot v = 0$

$c' \cdot v \geq \alpha$

$v,lb < v < v,ub$

(Equation 3)

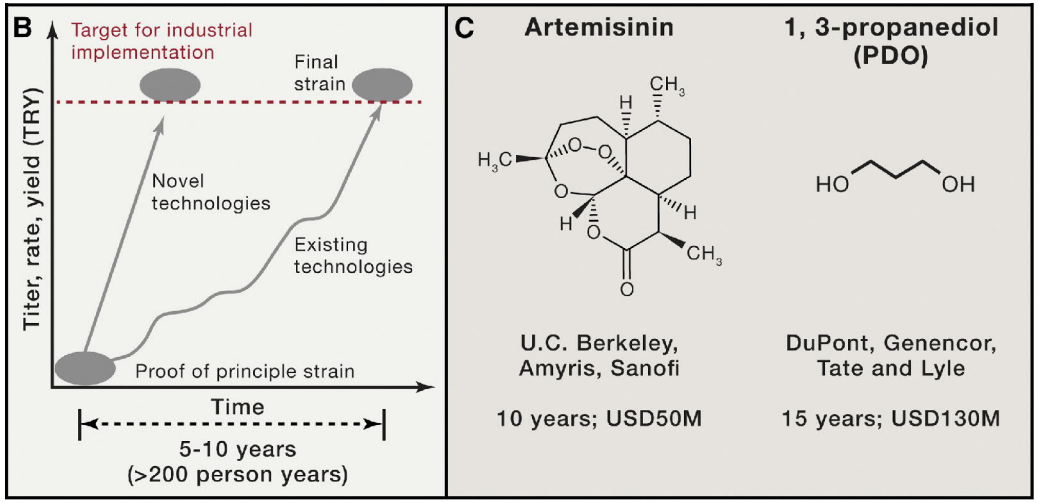
α is the optimal value of the inital objective

⇒ While the objective has the optimal value other fluxes may vary

⇒ The ones that are non-zero are essential for the optimal value of the objective

1. Design of engineering strategies for optimizing production

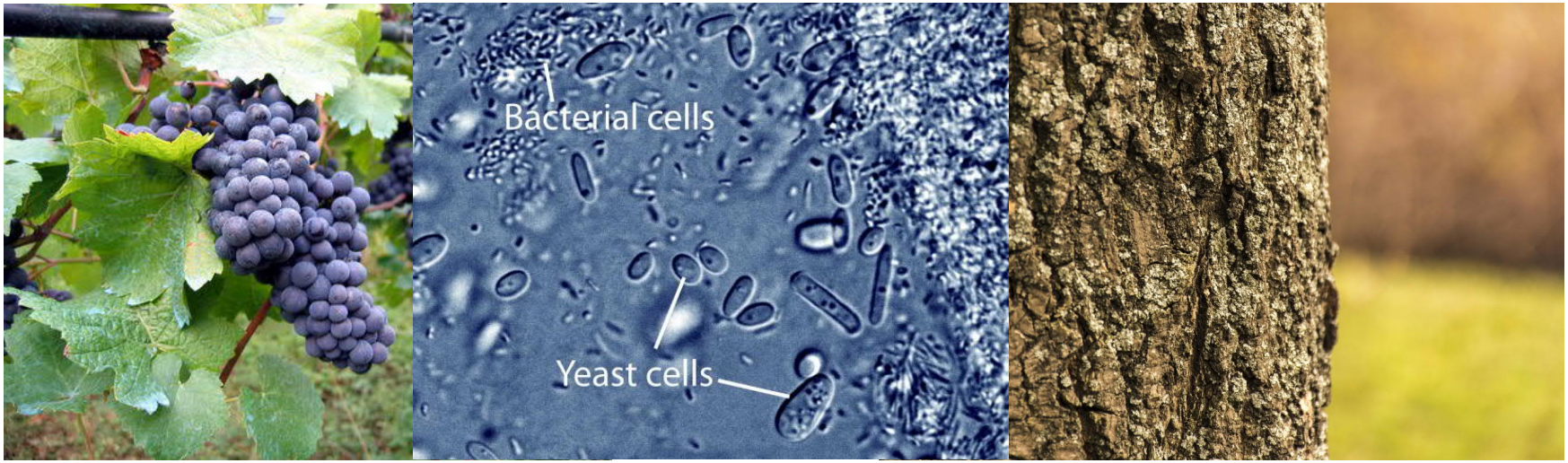
From laboratory demonstration to industrial feasibility



Nielsen J and Keasling JD. *Cell* 2016 164, 1185-1197DOI: (10.1016/j.cell.2016.02.004)

Review on possibilities: Lee SY et al. (2019) https://www.nature.com/articles/s41929-018-0212-4

Microbial metabolism is optimized in evolution for survival and growth



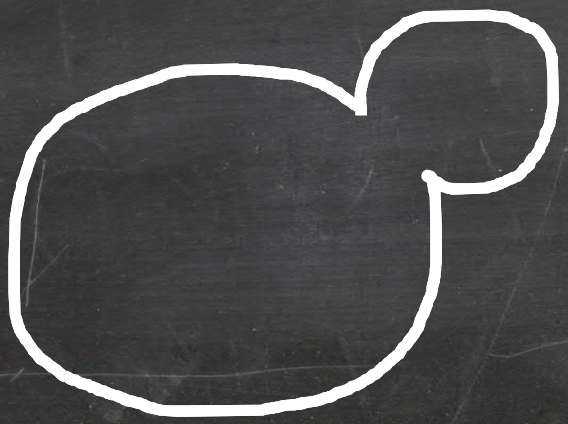
Determinants of industrially feasible production:

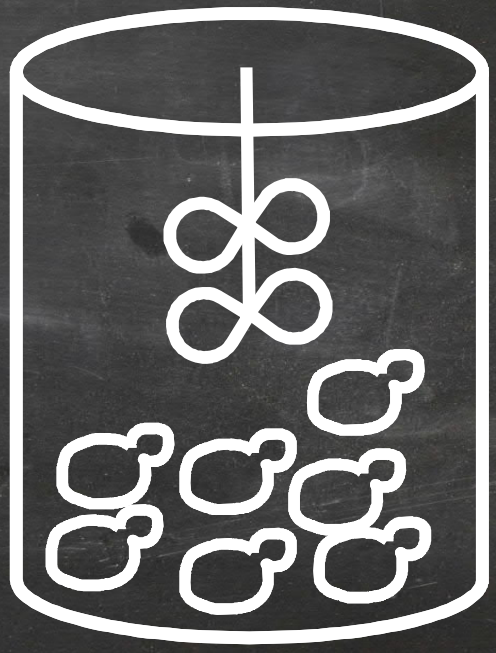
1. *YIELD*
2. *TITER*
3. *VOLUMETRIC PRODUCTIVITY*
4. *SPECIFIC PRODUCTIVITY*

Task: what are the units of these?

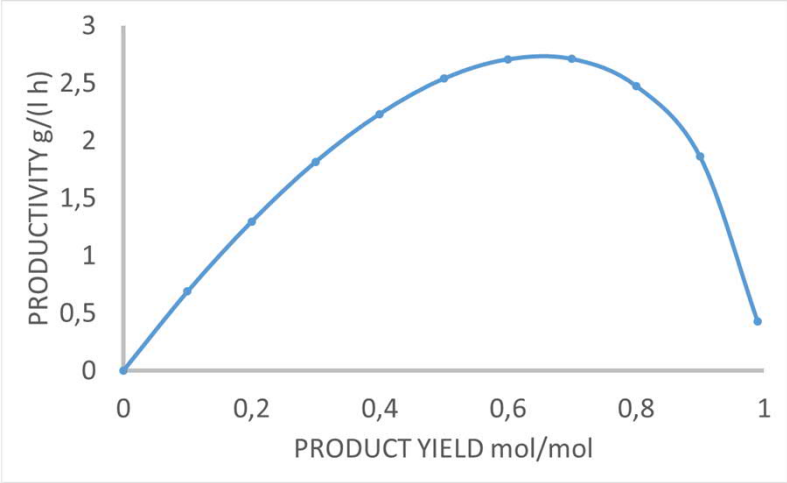
1. YIELD:
   g product/g substrate
2. TITER:
   g product/l
3. VOLUMETRIC PRODUCTIVITY:
   g product/ (l h)
4. SPECIFIC PRODUCTIVITY:
   g product/ (g biomass h)

```
g product/ (l h) =
g product/ (g biomass h) * g biomass/ l
```





If production draws substantial resources from growth, PRODUCTIVITIES remain low and industrially infeasible



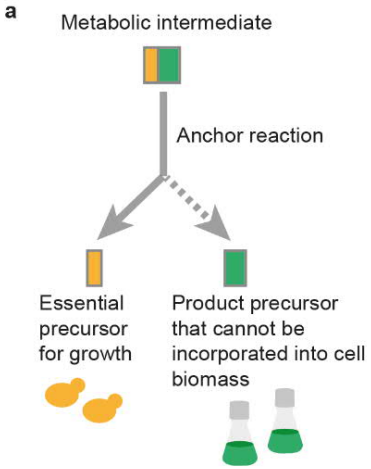
Batch process Monod model simulation for an example of a small molecule heterologous product in *S. cerevisiae*

In silico design of engineering strategies using genome-scale metabolic models

- Growth-product coupling: the cells can only grow if they produce
- Push-pull strategies: expression levels are modified to push and pull more resources to production

Growth-product coupling

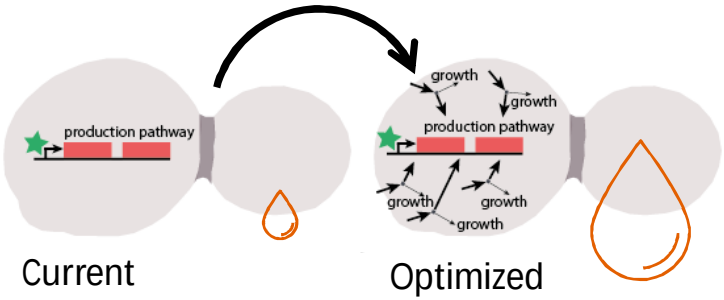
Algorithms use genome-scale metabolic models for identifying knock-out targets



Jouhten P. et al. Metab Eng. (2017)

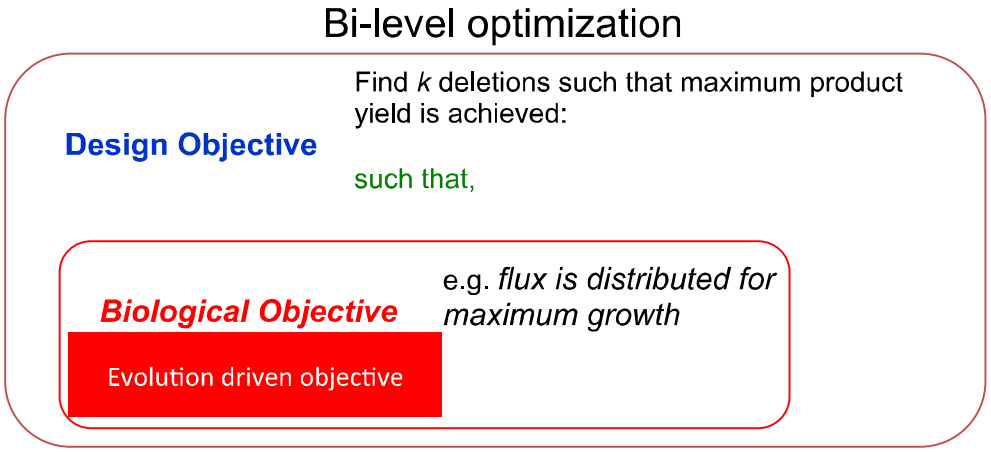
Push-pull strategies

Algorithms use genome-scale metabolic models for identifying deletion and re-regulation targets



Jouhten P. et al. *unpublished work* with Kiran Patil, EMBL Heidelberg

Growth-product coupling elegantly aligns biological and engineering objectives through network reduction



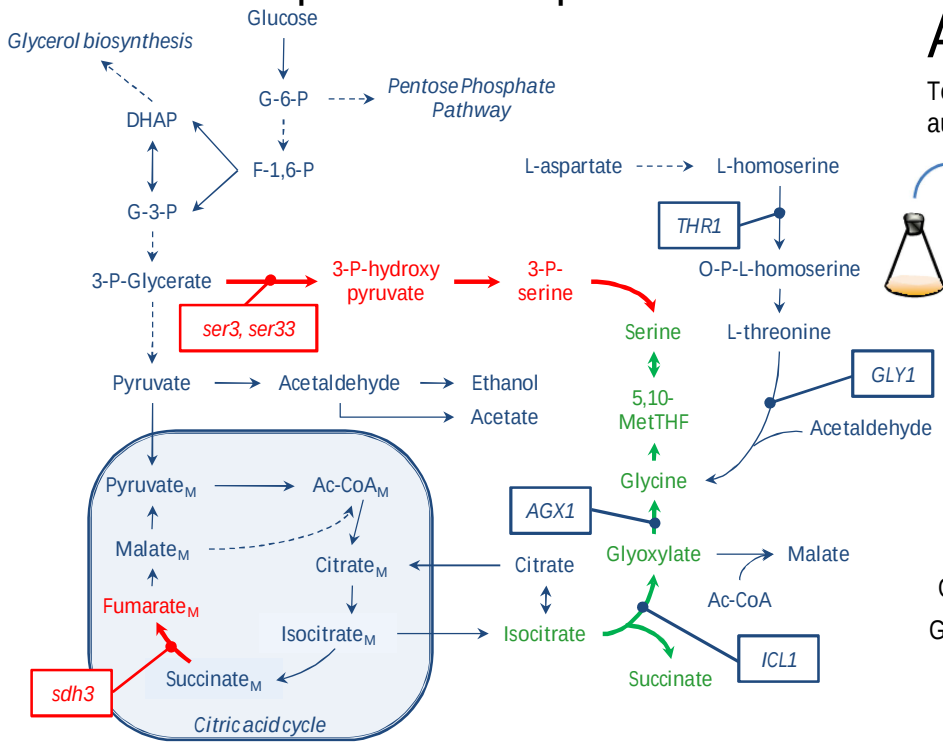
OptKnock: Burgard et al. (2003)
OptGene: Patil et al. (2005)

Slide modified from
Kiran Patil

EMBL

Growth-product coupling allows using adaptive laboratory evolution for improving production

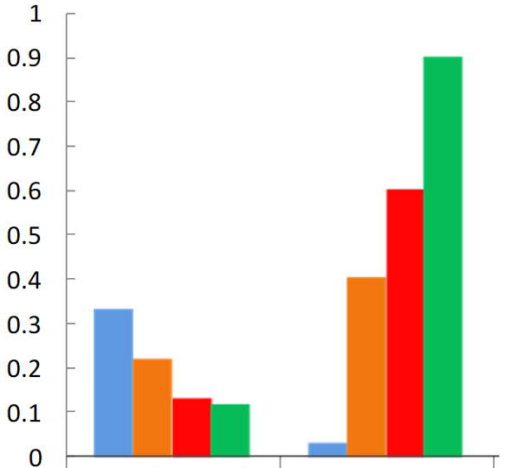
Proof of concept: succinate production in *S. cerevisiae*



Otero *et al.* PLoS One. (2013) 8:e54144.

ALE

To recover from Gly auxotrophy



	Specific Growth Rate (1/h)	Maximum Titer (g/L)
Gly auxotrophic / Gly prototrophic		
REF	0.33	0.03
8D	0.22	0.40
8D Evolved	0.13	0.60
8D Evolved + pICL1	0.12	0.90

Slide from Kiran Patil

Tasks:

Which determinants of industrial feasibility can be improved in growth-product coupled strains using adaptive laboratory evolution?

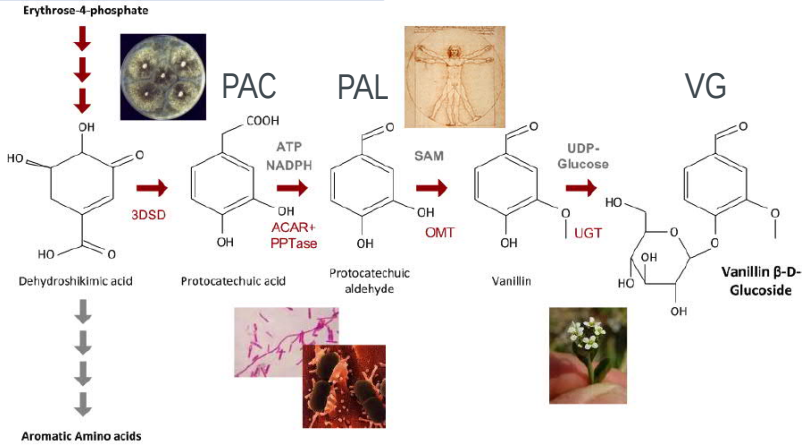
a) Product yield

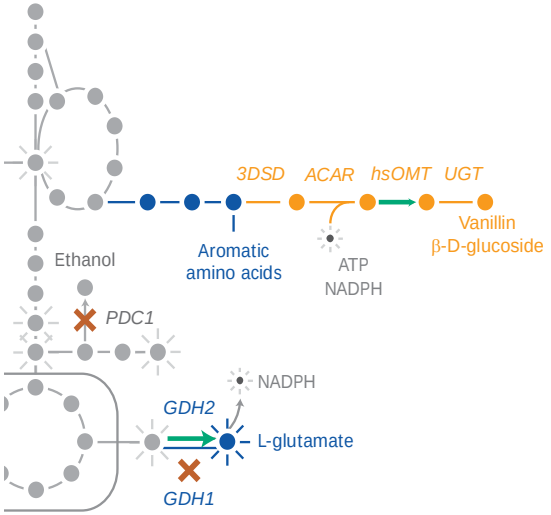
b) Productivity

c) Fitness of the strain

Pathway optimization improved vanillin production only after designed optimization of network

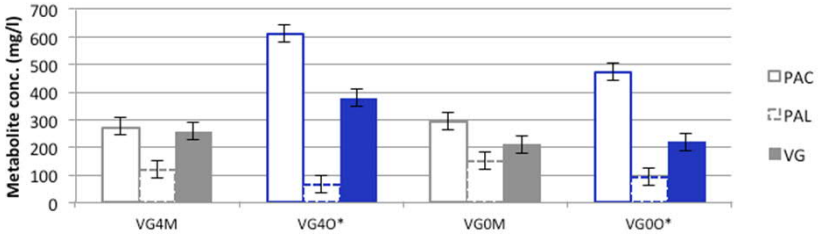
Synthetic vanillin pathway





Experimental validation

- Overall 5-fold productivity improvement
- Project continuation @ Evolva A/S: commercial production



Slide from Kiran Patil

EMBL

Brochado et al. (2011, 2013). Dr. Kiran Patil in collaboration with Evolva A/S (Denmark)

Platforms for genome-scale metabolic model manipulations and simulations

	Platform	Description	Link
For developers	COBRApy	Python package	https://opencobra.github.io/cobrapy/
	OpenCOBRA	Matlab functions	https://opencobra.github.io/cobratoolbox/stable/
	COBRA.jl	Julia package	https://opencobra.github.io/COBRA.jl/stable/
	Sybil	R-package	https://rdrr.io/cran/sybil/man/sybil-package.html
For end users	CAMEO	COBRApy compatible platform with *in silico* metabolic engineering tools	https://cameo.bio/
	BIOMET Toolbox	Web based platform with tools for reconstruction and analysis of models	http://biomet-toolbox.chalmers.se/
	MetaFlux	GUI or lisp API for model reconstruction and FBA	http://bioinformatics.ai.sri.com/ptools/metaflux.shtml
	OptFlux	Java based tool for *in silico* metabolic engineering	http://www.optflux.org/
	CellNetAnalyzer	GUI for model analysis using elementary flux modes approach, Matlab based	http://www2.mpi-magdeburg.mpg.de/projects/cna/cna.html

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Merja Penttilä, VTT Ltd and Aalto

Kiran Patil's group at EMBL Heidelberg & Cambridge University

Production host engineering team at VTT Ltd

Merja Oja, Roal Oy

Juho Rousu, Aalto

ACADEMY OF FINLAND

Federal Ministry of Education and Research

EMBL European Molecular Biology Laboratory

FBA derived tools demonstrated in strain design

Methods for designing genetic engineering strategies (e.g. growth-product coupling) for wetlab metabolic engineering

- gene deletion(s)
  - OptKnock [Burgard, et al. 2003]
  - OptGene [Patil et al. 2005]
- gene additions / deletions
  - OptStrain [Pharkya, et al. 2004]
- gene overexpressions / known down
  - e.g. OptForce [Ranganathan, 2010]
- FSEOF for overexpression by scanning towards increasing production, [Choi et al. 2010]
- K-OptForce includes kinetics, [Chowdhury et al. 2014]
- tSOT, considers gene expression data, [Kim et al. 2016]

Table 5.2 Examples of reported overproducer yeast strains whose development has been involved using genome-scale metabolic model simulation tools

Product	Species	Tools	Year	Ref.
Ethanol	*S. cerevisiae*	in house script (FBA)	2006	Bro et al. (2006)
Sesquiterpene	*S. cerevisiae*	MOMA, OptGene	2009	Asadollahi et al. (2009)
Vanillin	*S. cerevisiae*	MOMA, OptGene, OptKnock	2010	Brochado et al. (2010)
2,3-butanediol	*S. cerevisiae*	OptKnock	2012	Ng et al. (2012)
Fummaric acid	*S. cerevisiae*	FBA	2012	Xu et al. (2012)
Succinic acid	*S. cerevisiae*	OptGene	2013	Otero et al. (2013)
Tyrosine	*S. cerevisiae*	OptKnock	2013	Cautha et al. (2013)
Dihydroartemisinic acid	*S. cerevisiae*	MOMA, OptStrain, OptForce, OptKnock	2013	Misra et al. (2013)
Muconic acid	*S. cerevisiae*	FBA	2013	Curran et al. (2013)
Malate	*C. glabrata*	FBA	2013	Chen et al. (2013)
Triacetic acid lactone	*S. cerevisiae*	OptKnock	2014	Cardenas and Da Silva (2014)
Human recombinant protein	*P. pastoris*	FSEOF, MOMA	2014	Nocon et al. (2014)
Ethanol	*S. cerevisiae*	FBA, EMA	2014	Toro et al. (2014)
Acetoin	*C. glabrata*	FBA	2014	Li et al. (2014)
Amorphadiene	*S. cerevisiae*	MOMA, FBA	2014	Sun et al. (2014)
Succinate	*S. cerevisiae*	FBA	2014	Rosdi and Abdullah (2014)
3-hydroxypropionic acid	*S. cerevisiae*	FBA	2015	Borodina et al. (2015)
Patchoulol	*S. cerevisiae*	EMA	2015	Gruchattka and Kayser (2015)
Lipid	*Y. lipopytica*	FBA	2015	Kavscek et al. (2015)
Tyrosine	*S. cerevisiae*	OptKnock	2015	Gold et al. (2015)
β-Farnesene	*S. cerevisiae*	pFBA	2016	Meadows et al. (2016)
3-hydroxypropionic acid	*S. cerevisiae*	pFBA	2016	Kildegaard et al. (2016)
Muconic acid	*S. cerevisiae*	FBA	2016	Suastegui et al. (2016)
Biomass	*S. stipitis*	FBA	2016	Unrean et al. (2016)
Growth on Methanol or glycerol	*P. pastoris*	FBA	2017	Tomas-Gamisans et al. (2018)
Polymalic acid	*A. pullulans*	FBA	2017	Feng et al. (2017)
Ethanol	*S. stipitis*	FBA	2017	Acevedo et al. (2017)
Triacylglycerol	*Y. lipopytica*	FBA	2018	Koivuranta et al. (2018)
Lipid	*R. toruloides*	FBA	2018	Castañeda et al. (2018)

UNCORRECTED PROOF