Synthetic Metabolism: Lessons from Metabolic Engineering

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Limitations by network structure

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Structural limitations

Problem

Any introduced pathway must obtain carbon skeletons and coenzymes (NADPH, ATP, etc) from the host network. This introduces couplings, and hence constraints, to the host metabolic network.

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Solution

Apply structural analysis techniques (e.g. elementary modes analysis, linear programming (FBA)) to the combined network to determine overall stoichiometries and optimal conversion efficiencies of potential routes.

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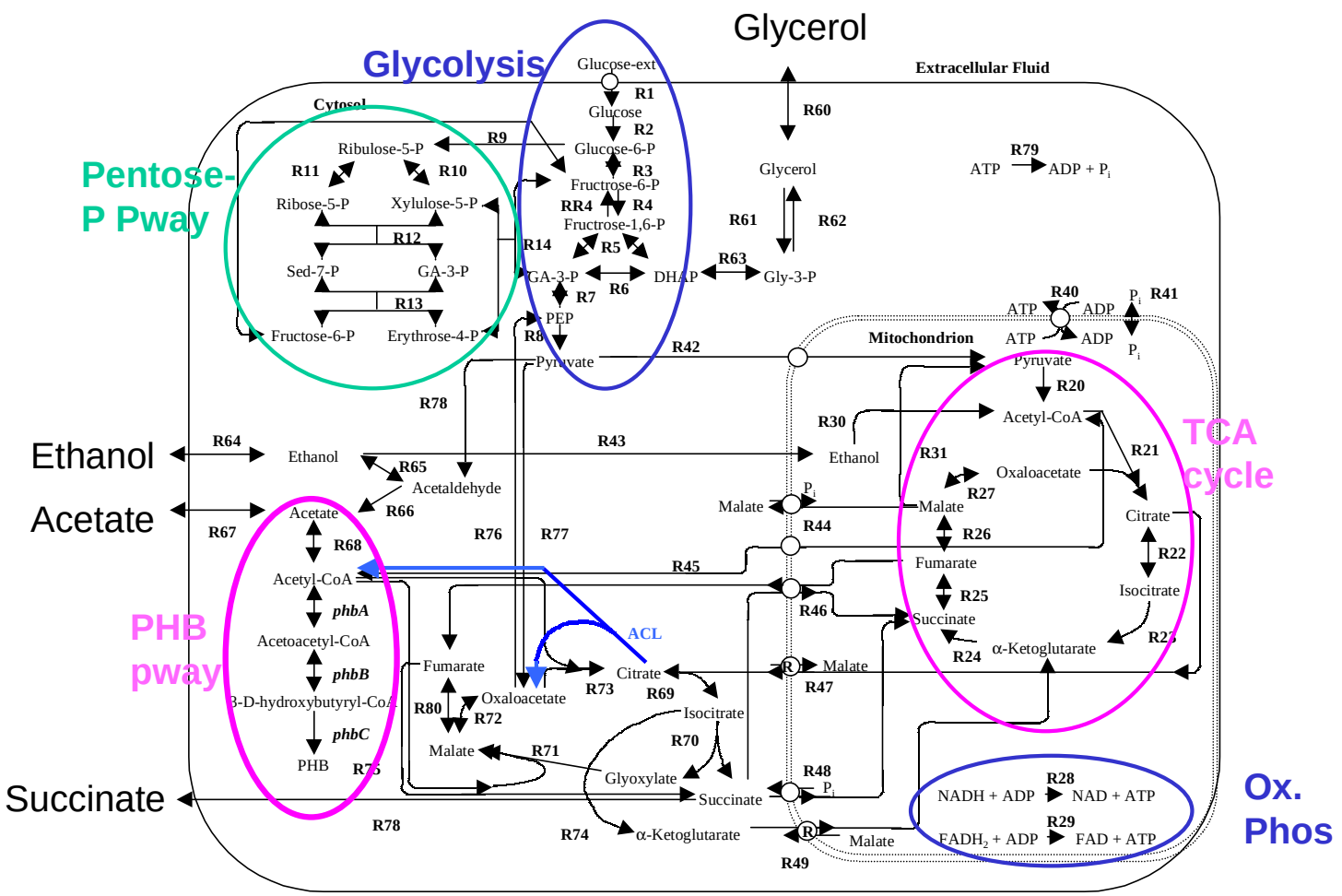
Polyhydroxybutyrate synthesis in yeast



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Optimal yields of PHB synthesis

Wild-type yeast + PHB pathway

1. 2 Acetate + EtOH $\longrightarrow$ PHB + 2 CO_2 0.67
2. 65 Ac. + 31 EtOH $\longrightarrow$ 30 PHB + 72 CO_2 0.63

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Wild-type yeast + ATP–citrate lyase + PHB pathway

3. 12 EtOH $\longrightarrow$ 5 PHB + 4 CO_2 0.83
4. 77 EtOH + 31 Glycerol $\longrightarrow$
   48 PHB + 4 Ac. + 47 CO_2 0.78

(Number following each mode is the fractional carbon conversion.)

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Metabolic flux is not proportional to enzyme amount

Non–linearity of Flux–Enzyme Relationship

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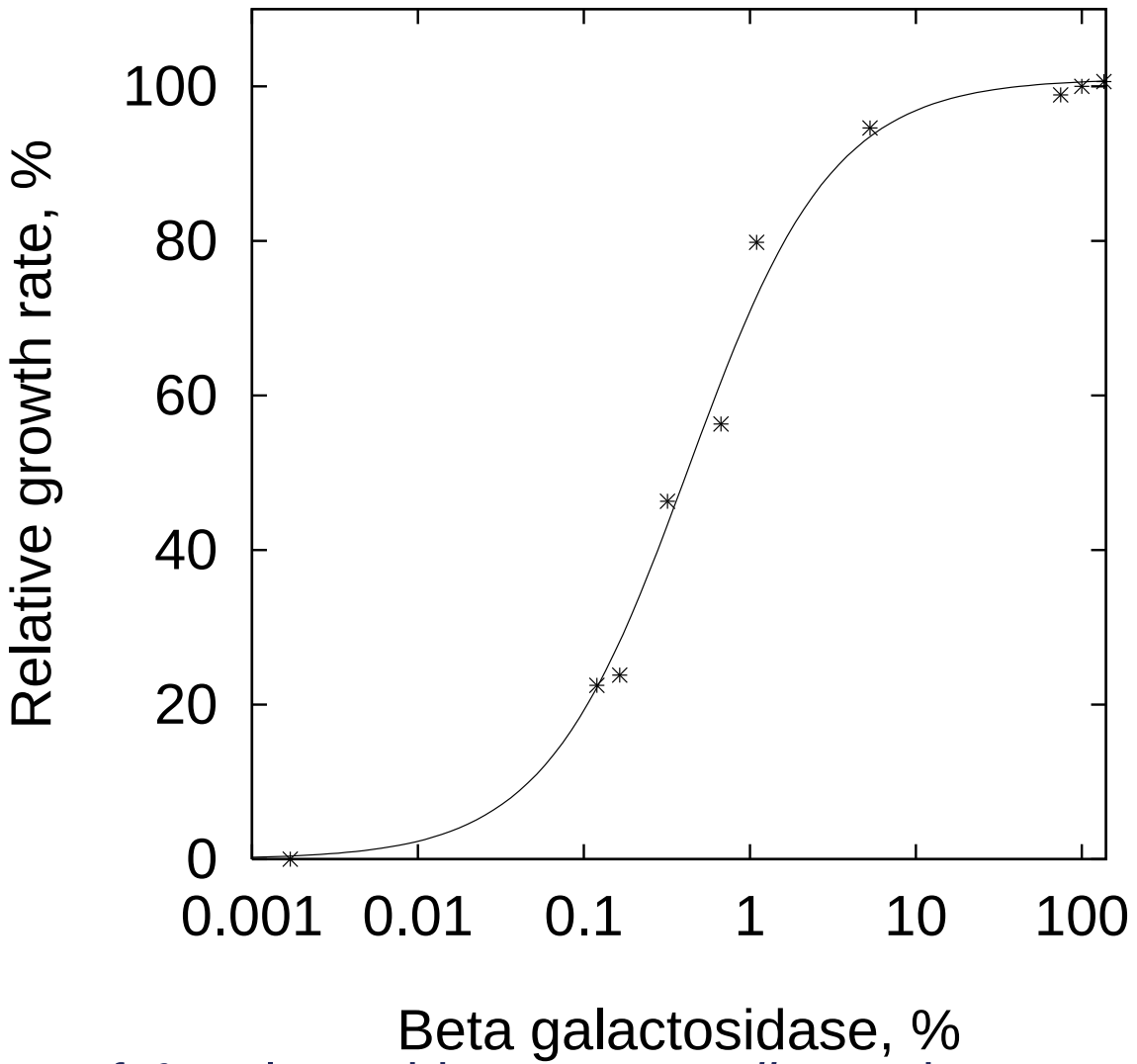
Solutions

1. Find, and then over–express the limiting enzymes; repeat; repeat; repeat; repeat . . .
2. Bring about balanced changes in all the enzymes along the route.

Dependence of *E. coli* growth rate on β-galase



The effect of β–galactosidase on *E. coli* growth rate on lactose.
Dykhuizen et al, Genetics, 115, 25-31 (1987).

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Engineering Aromatic Biosynthesis

Synthesis of DAHP in *E. coli* studied in an *aroB* mutant that excretes DAHP.

- Synthesis increased by overproduction of a feedback resistant DAHP synthetase (AroG).
- Further increase obtained by overproduction of transketolase (yields E4P).
- Still further increase obtained overexpressing pyruvate, water dikinase to increase formation of PEP.

Pyruvate, water dikinase only has an effect when the other two enzymes are first overexpressed.

R. Patnaik et al, *Biotech. Bioeng. 46*, 361–370 (1995).

The Example of Yeast Trp Biosynthesis

Genes overexpressed					Mean	Relative
2	4	1	3	5	change	trp flux
-	-	-	-	-	1	1.0
-	-	+	+	-	58	2.0
+	+	-	+	-	35	2.4
+*	-	+	+	-	34	1.2
+*	+	+	+	-	30	2.1
+	+	-	+	+	19	8.2
+*	+	+	+	+	23	8.8

'+' indicates the enzyme was overexpressed; '-' indicates wild–type level. The mean change column gives the average fold over–expression.

P. Niederberger et al, *Biochem. J. 287*, 473–479 (1992)

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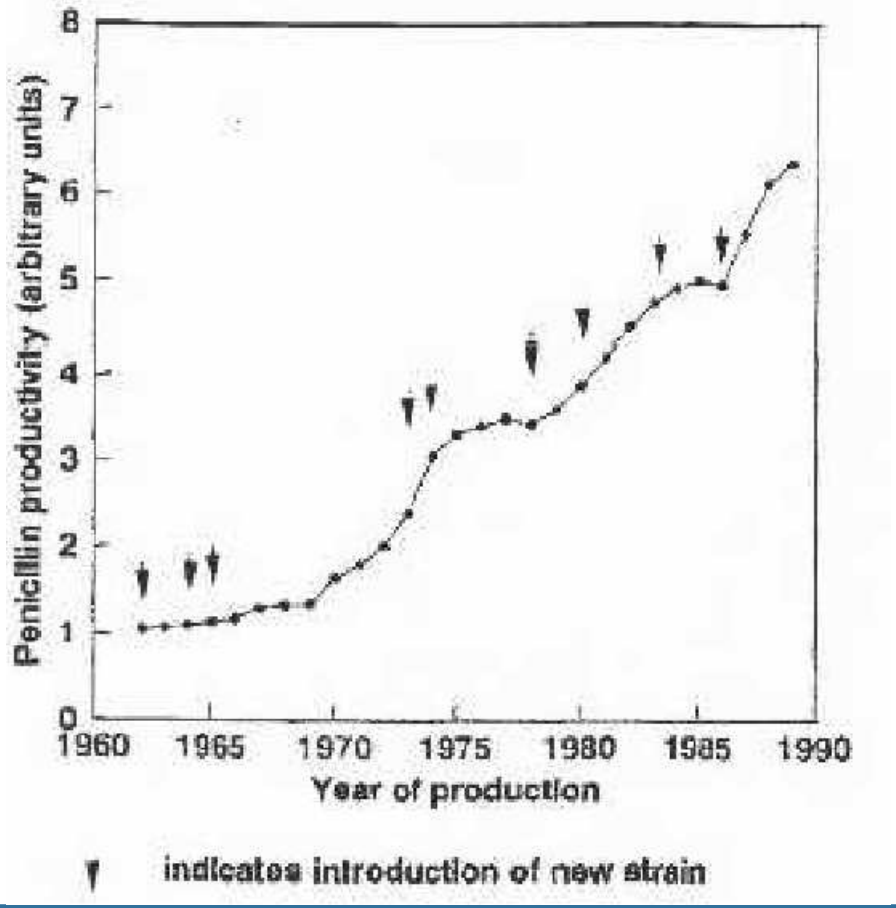
Increasing Productivity Gets Harder

Productivity was increased by mutation and selection rather than rationally, but changes occurred in many aspects of precursor uptake and metabolism.



Penicillin Synthesis — 1

There are 3 enzymes on the main route from amino acids to penicillin V.

aminoadipate + cys + val

↓ ACVS

LLD–ACV

↓ IPNS

isopenicillin N

↓ IAT

penicillin V

Altering enzymes affects metabolites more than fluxes

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Enzyme Effects on Metabolites

Problem

The concentrations of metabolites generally respond more sensitively to changes in a single enzyme activity than do fluxes. Adverse effects can follow through osmotic effects or metabolite toxicity.

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Solution

Proportionate changes in all enzymes along the path (Kacser & Acerenza Universal Method), or activating whole pathways by manipulating a global regulator.

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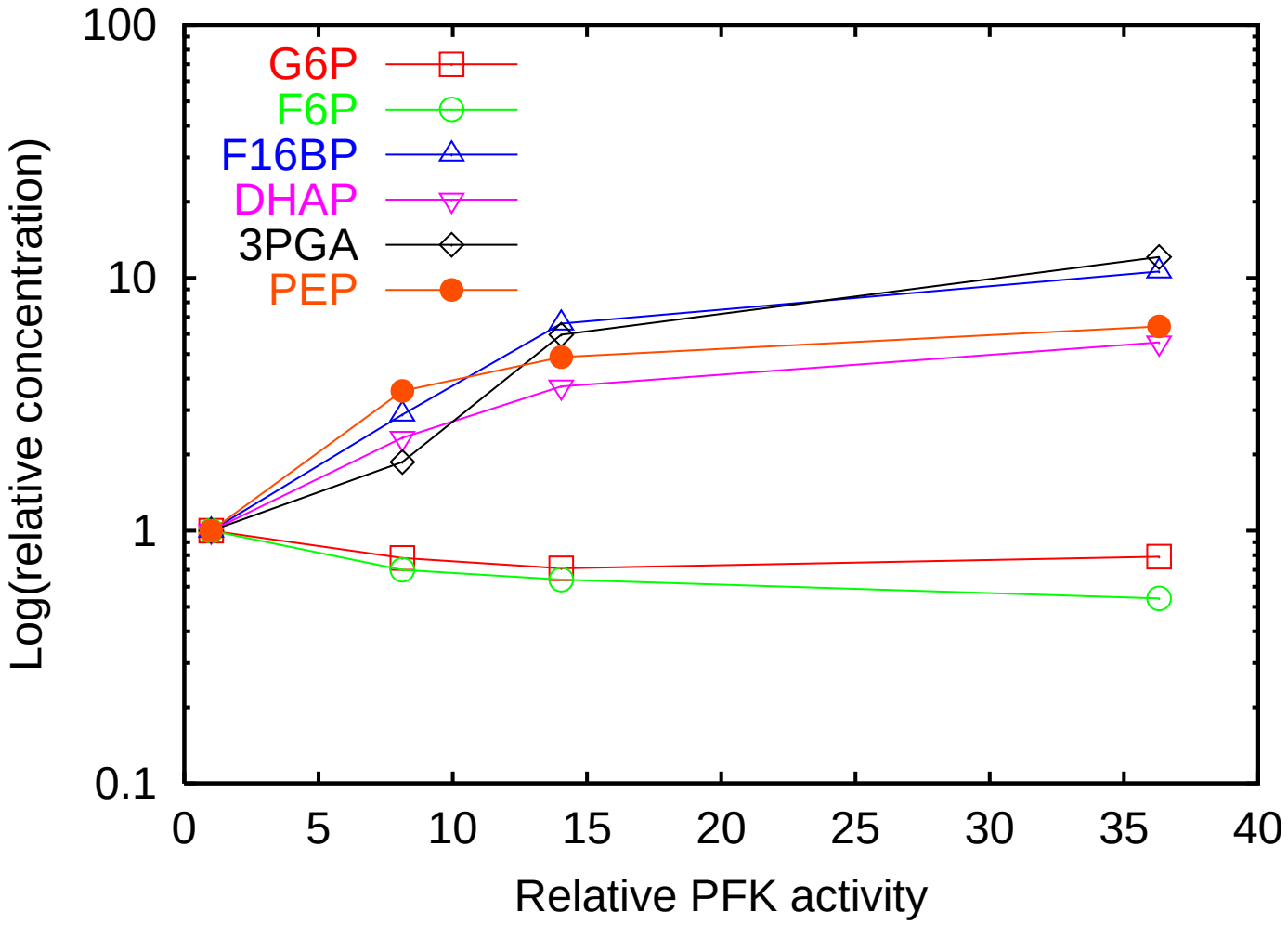
Over–Expression of Potato PFK



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Engineering Tryptophan Production

Increased trp production in *Corynebacterium glutamicum* by:

- Starting with a phe, tyr auxotroph derepressed to overexpress all trp enzymes a few fold.
- Overexpressing DAHP synthase (8x) and all the trp genes (11x) after chorismate.
- Abolishing feedback on PRT to stop accumulation of toxic anthranilate caused by imbalance in ANS-PRT activities.

but

- Continuous selection pressure needed to retain plasmid.

R. Katsumata & M. Ikeda, *Biotechnology 11*, 921–925, (1993).

Abolishing feedback inhibition is a bad idea

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Abolishing feedback inhibition

Problem

Feedback inhibition has long and widely been stated to be a control mechanism on metabolic fluxes. Hence abolishing these effects has often been tried as a first step.

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Solution

Take note that:

1. Feedback inhibition is an antidote to the tendency of reactions at the start of a pathway to have the greater control of flux.
2. Feedback inhibition improves homoeostasis of the concentration of the feedback metabolite; increased cooperativity of the inhibition specifically enhances this effect.
3. Feedback inhibition improves the stability of pathways in that it speeds up the return to a steady state after random perturbation,

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Yeast Glycolysis

The allosteric PFK of yeast glycolysis has been replaced with a non–regulated version by complementation of *pfk*- mutants with a non–allosteric enzyme from *D. discoideum*.

Results:

1. The glycolytic rate and growth rate were unchanged from the wild–type with a regulated PFK.
2. Even if just the F26BP regulation was abolished, The concentrations of glycolytic intermediates were substantially higher.
3. In the latter case, yeast showed slower aerobic-anaerobic transitions because of the raised metabolite levels.

A. M. Estévez et al, FEBS Lett, 374, 100–104, (1995). E. Boles et al, Mol. Microbiol. 20, 65–76 (1996).

Pull, don’t push.

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Problem

Metabolism can be seen to be resistant to stimulating biosynthesis by expressing or over–expressing pathway enzymes. Control of the flux soon passes elsewhere, and metabolite concentrations rise. But what’s the alternative?

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Solution

Exploit the natural homeostatic mechanisms of the cell: pull the products out, rather than push precursors towards them. The metabolic network will tend to replace material that’s removed. Feedback loops in metabolism transfer control from the ‘supply’ steps near the beginning to the ’demand’ reactions after the feedback loop.

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Lysine biosynthesis

Figure of lysine biosynthesis pathway from aspartate showing feedback inhibition.

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Lysine — Partial Success

In *Corynebacterium glutamicum*,

- Of the 6 enzymes tested between asp and lys, only overexpression of feedback-resistant aspartate kinase and dihydrodipicolinate synthase leads to excretion of lysine.
- Overexpression of both these together gives higher yields than either separately.
- In aspartate kinase overexpressers, overexpression of phosphoenolpyruvate carboxylase increases lysine synthesis, though it has no effect alone.
- However, the plasmid coding the feedback resistant aspartate kinase is very unstable.

J. Cremer et al. *Appl. Env. Microbiol. 57*, 1746–1752 (1991)

C glutamicum and Lysine Export

- Strains with different lysine productivities have different lysine export activities.[1]
- A lysine–inducible, energy–dependent lysine exporter (lysE) has been isolated.[2]
- Over–expression of lysE in wild–type C glutamicum causes a 5–fold increase in lysine excretion and is more effective than making aspartate kinase resistant to feedback inhibition by lysine.[2]

[1] Broer et al, *Appl. Env. Microbiol. 59,* 316–321, 1993. [2] Vrljic et al, *Mol. Microbiol. 22*, 815–826, 1996.

Coenzyme Imbalance

Cofactor Imbalance

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It can be difficult to reconcile the poise of coenzyme couples to be suitable for both general cellular metabolism and the desired pathway.

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Solution

? This is really difficult. Changing the source/ kinetics of the enzymes involved may help.

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Xylose metabolism

- Pentilla and colleagues are engineering *S*. cerevisiae to metabolise the xylose component of hemicellulose to ethanol.
- The pathway involves reduction by an NADPH-linked xlose reductase to xylitol. This is then oxidised by an NAD–linked xylitol dehydrogenase to give xylulose. After phosphorylation, this enters the pentose phosphate pathway as xylulose 5–P.
- Conditions are suitable for the first reaction with NADPH generated adequately. The second reaction is limited by NAD supply.
- This is difficult to fix without disrupting the cell's metabolism.

Success story

Artemisinin by Synthetic Biology

Figure or artemisinin pathway

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Artemisinin Production

Jay Keasling's group achieved good productivity of the malaria drug artemisinin in *S. cerevisiae* by:

- Engineering the farnesylPP pathway to increase productivity and reduce diversion to sterols;
- Introduced the amorphadiene synthase gene (ADS) from *Artemisia annua*;
- Introducing a P450 enzyme to carry out the three–step oxidation of amorphadiene to artemisinin.

Ro et al, Nature 440, 940–943, 2006

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Multiple Metabolic Manipulations

Figure showing productivity of strains as successive changes are introduced form: Ro et al, Nature 440, 940–943, 2006

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Summary

- Molecular biologists suffered many disappointments through naïve attempts to modify metabolism to increase productivity of desired products.
- Metabolic engineering started to achieve better results when it absorbed concepts from modern theory of the control of metabolism.
- The artemisinin project confirms that synthetic biology projects need to build on the lessons from metabolic engineering.

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Read the Theory First!