Eukaryotic Gene Expression: Basics & Benefits

P N RANGARAJAN

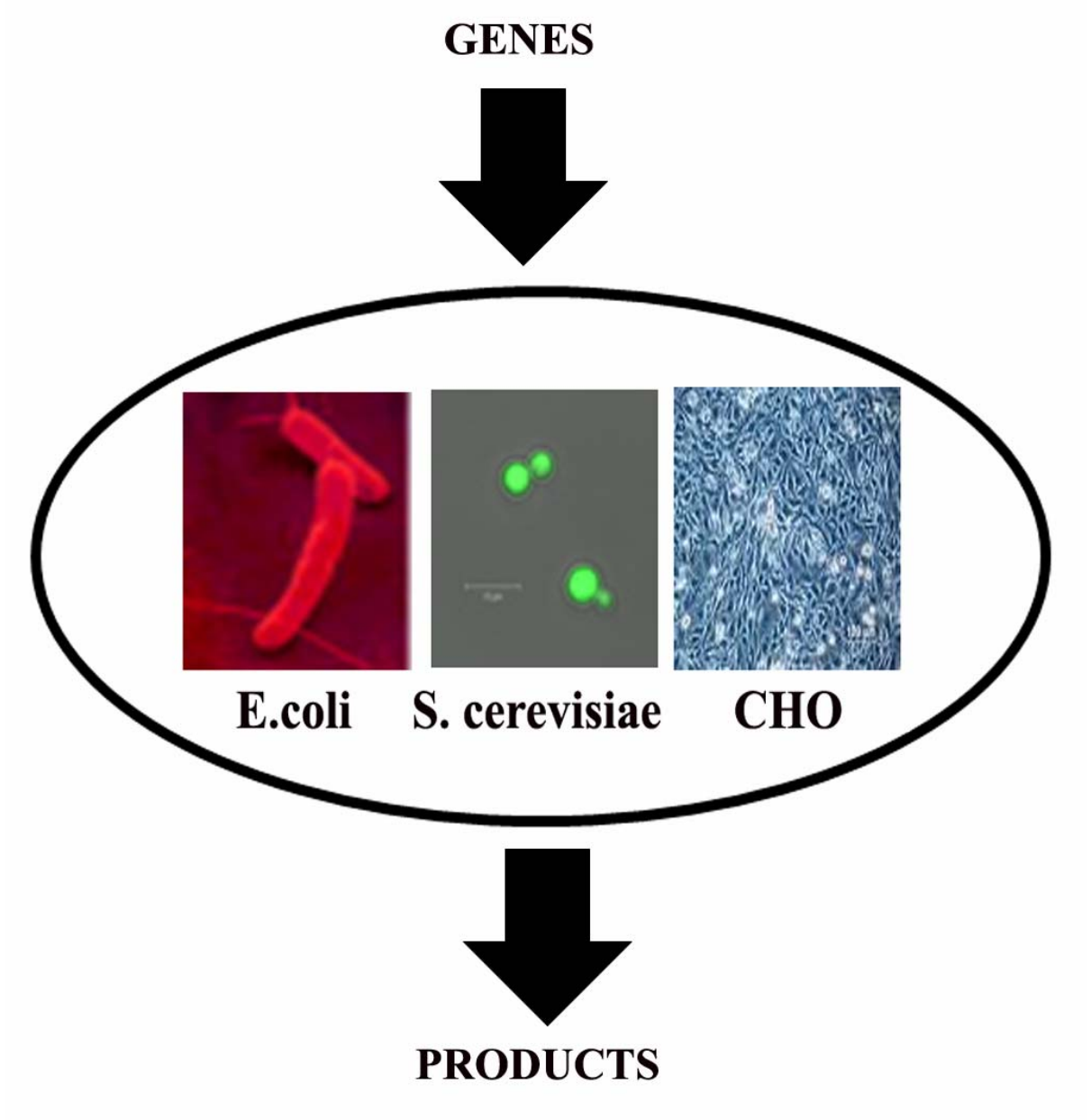
Lecture 40

Metabolic Engineering
&
Synthetic Biology

METABOLIC ENGINEERING

Engineering biosynthetic pathways for low-cost production of useful products

Metabolic engineering overcomes the cellular regulation to produce product of our interest or creates a new product that the host cells normally don’t need to produce.


GENES
E.coli
S. cerevisiae
CHO
PRODUCTS

Pathway and metabolic engineering for biochemical production is a major research area and is distinct from the over-production of therapeutic proteins. In the latter case, a protein is the final product, and machineries already exist in cells to produce proteins from genes.

In metabolic or pathway engineering however, cells do not typically have the machineries for its production, and multiple genes need to be heterologously expressed to synthesize enzymes of a complete pathway.

The unique challenges in such engineering efforts include balancing of promoter strengths, enzyme expression and coordination of expression, and interface with native metabolism.

Several biomolecules of therapeutic value are produced by complex metabolic pathways in higher eukaryotes especially plants.

Isolation of these compounds from these natural sources is often cumbersome, inadequate and uneconomical

Can such molecules be produced in prokaryotic or eukaryotic microorganisms by metabolic engineering?

Alteration of primary metabolism of the host

Heterologous expression of the genes encoding the natural product biosynthetic pathway

Efficient supply of the required biosynthetic intermediates to support the engineered natural product biosynthesis.

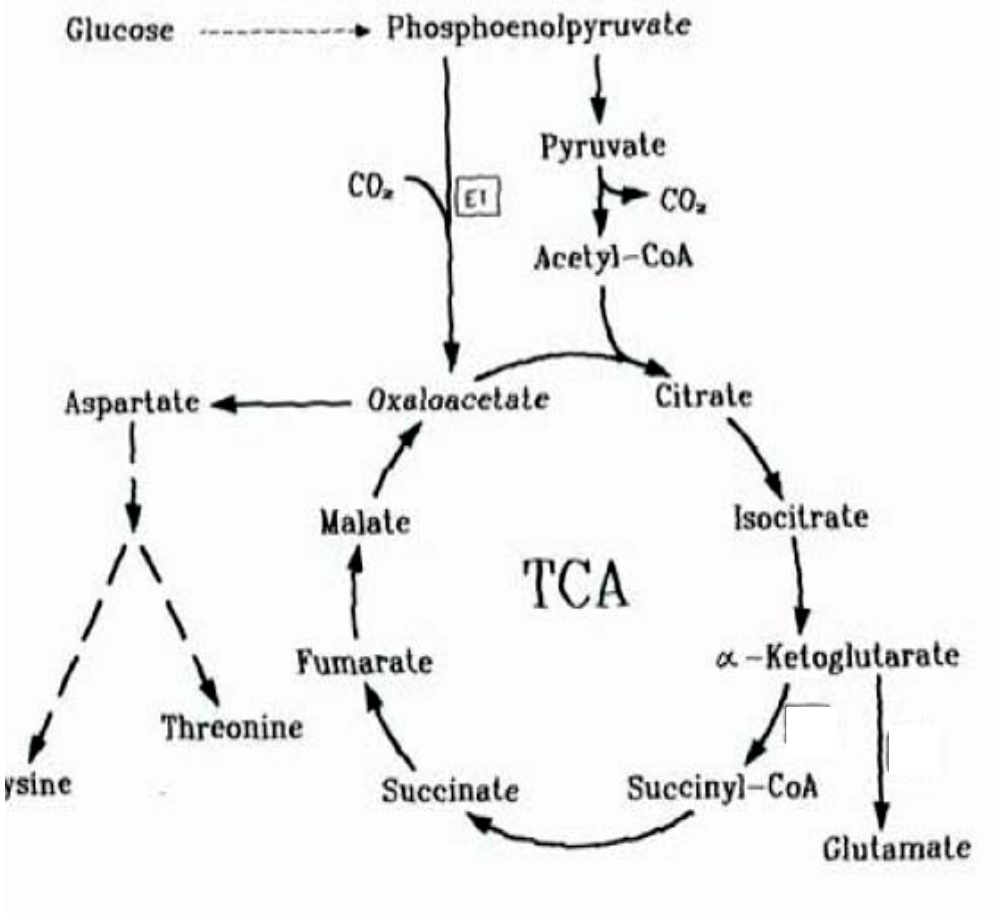
Amino acids

Annual worldwide production of over 400,000 tons

Used as food additives, (Glutamate as MSG in foods) medicines, starting material in chemical synthesis.

Ex. glutamic acid, lysine

Corynebacterium glutamicum for efficient overproduction of glutamate (1957)



Metabolic Engineering of Glutamate Production

Eiichiro Kimura

Microbial Production of l -Amino Acids
Advances in Biochemical Engineering/Biotechnology,
2003, Volume 79/2003, 37-57

Citric acid

- over 130,000 tons produced worldwide each year
- used in foods and beverages
- iron citrate as a source of iron
- preservative for stored blood, tablets, ointments,…
- in detergents as a replacement for polyphosphates
- a microbial fermentation for production of citric acid developed in 1923
- >99% of the world's output produced microbially *Aspergillus niger*

Antibiotics

- Antibiotics are small molecular weight compounds that inhibit or kill microorganisms at low concentrations
- often products of secondary metabolism
- antibiotics produced by various bacteria, actinomycetes & fungi

Bacillus
Streptomyces
Penicillium

S. venezuelae	Chloramphenicol
S. aureofaciens	Tetracycline
S. aureofaciens	Chlortetracycline
S. noursei	Nystatin
S. erythreus	Erythromcin
S. lincolnensis	Clindamycin
S. griseus	Streptomycin
S. fradiae	Neomycin

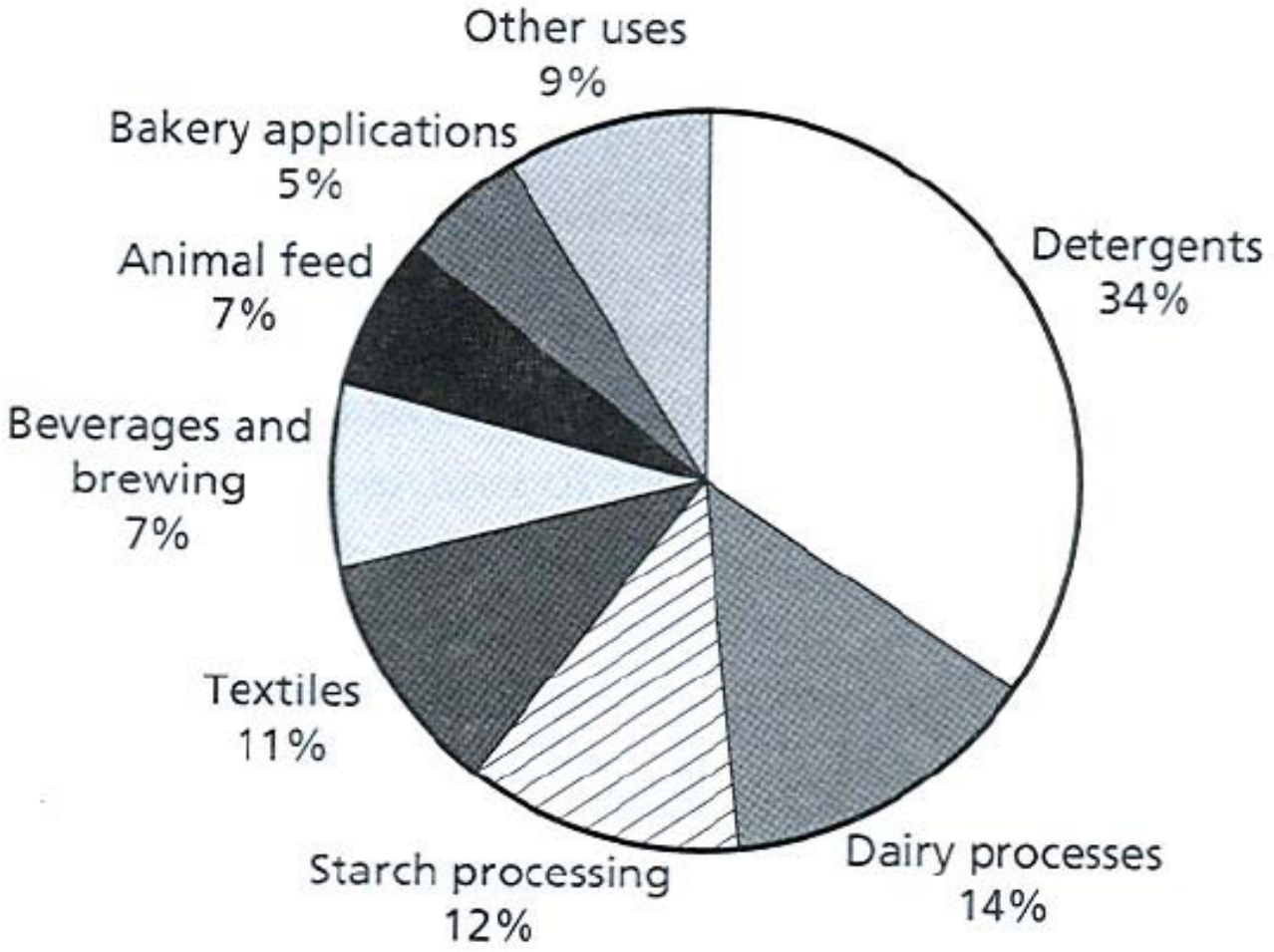
Enzymes

Commercially produced enzymes:

• enzymes used in *industry*, such as amylases, proteases, catalases, isomerases

• enzymes used for *analytical* purposes, such as glucose oxidase, alcohol dehydrogenase, hexokinase, cholesterol oxidase

• enzymes used in *medicine*, such as asparaginase, proteases, lipases



Uses of microbial enzymes

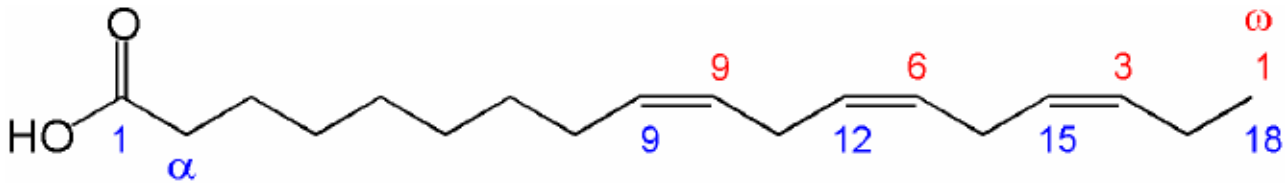
Bacterial proteases	*Bacillus, Streptomyces*	Detergents
Asparaginase	*Escherichia, Serratia*	Antitumor agent
Glucoamylase	*Aspergillus*	Fructose syrup production
Bacterial amylases	*Bacillus*	Starch liquefaction, brewing, baking, feed, detergents
Glucose isomerase	*Bacillus, Streptomyces*	Sweeteners
Rennin	*Alcaligenes, Aspergillus, Candida*	Cheese manufacture
Pectinase	*Aspergillus*	Clarify fruit juice
Lipases	*Micrococcus*	Cheese production
Penicillin acylase	*Escherichia*	Semisynthetic penicillins
Taq polymerase	*Thermus aquaticus*	Polymerase chain reaction

Metabolic engineering: prokaryotes *vs* eukaryotes

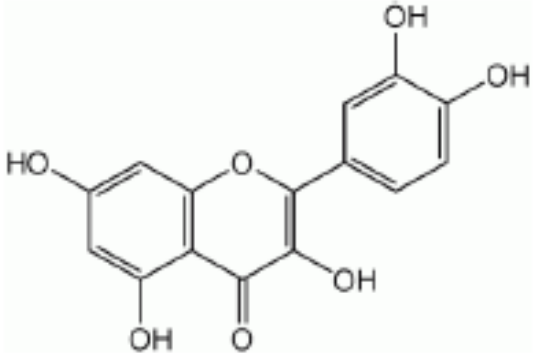
Prokaryotes have evolved operons for the economical coexpression of several genes required to achieve a desired metabolic state. These operons typically consist of a promoter which drives expression of a single multicistronic message.

In eukaryotes, majority of the genes are organized in monocistronic regulons scattered on the mammalian chromosome. One of the strategies for expressing multiple genes of a pathway in mammalian cells is to assemble multicistronic eukaryotic operons by cloning several *IRES*-containing translation units in consecutive orientation under control of a single eukaryotic promoter. The terminal cistron contains a single polyadenylation site for proper processing of the multicistronic message.

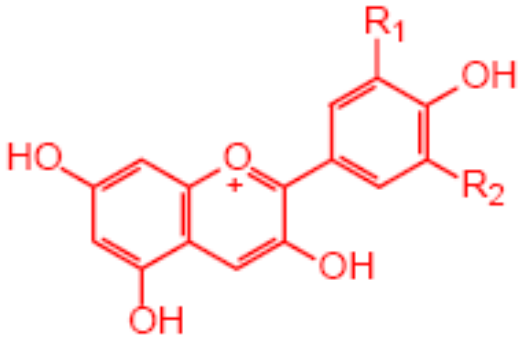
Plant primary metabolites	***Plant Secondary metabolites (Plant natural products)***
1. Organic compounds produced in the plant kingdom 2. Have metabolic functions essential for plant growth and development 3. Produced in every plant 4. Include carbohydrates, amino acids, nucleotides, fatty acids, steroids and lipids	1. Organic compounds produced in plant kingdom 2. Don't have apparent functions involved in plant growth and development 3. Produced in different plant families, in specific groups of plant families or in specific tissues, cells or developmental stages throughout plant development. 4. Include terpenoids, special nitrogen metabolite (including, non-protein amino acids, amines, cyanogenic glycosides, glucosinolates, and alkaloids), and phenolics.



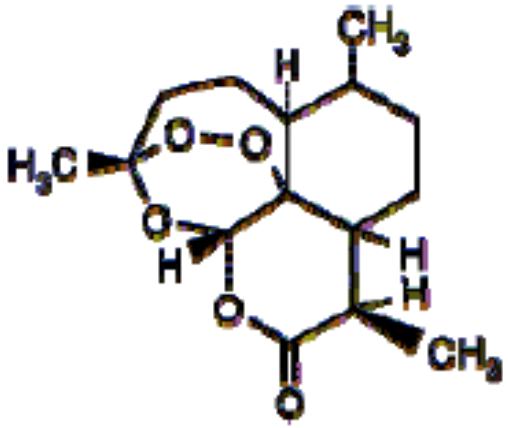
α-Linolenic acid (polyunsaturated omega-3 fatty acid, $C_{18}H_{30}O_2$), in walnut and flaxseeds



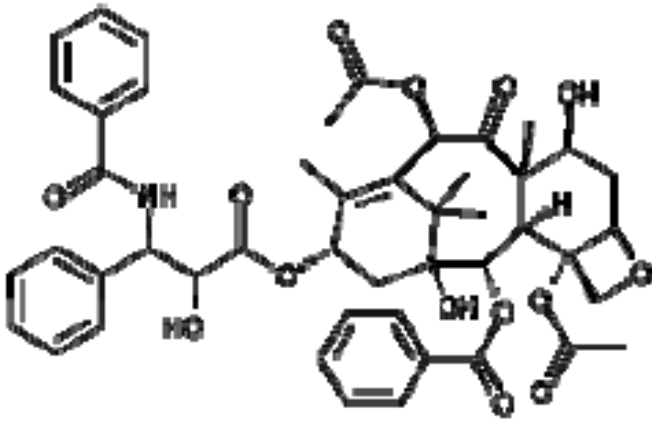
Quercetin (flavonoids)



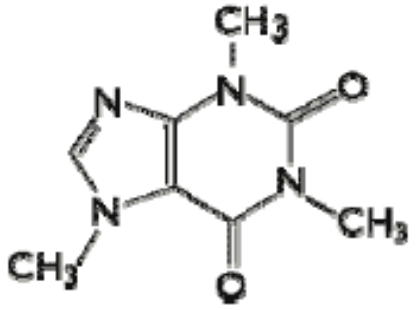
Anthocyanidins (flavonoids)



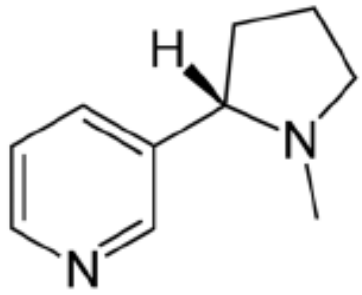
Artemisinin (terpenes)



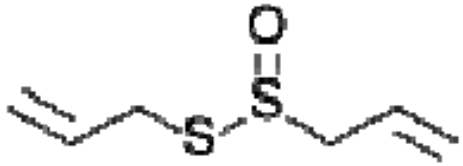
Paclitaxel (terpenes)



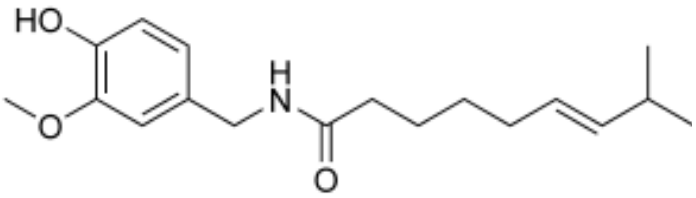
Caffeine (alkaloids)



Nicotine (alkaloids)



Allicin (non-protein amino acids)



Capsaicin (amines)

Fungal secondary metabolites

Non-ribosomal peptides (NRPS)

b-lactam antibiotics
Cyclosporin – immunosuppressant
Echinocandin – antifungal drug

Polyketides (PKS)

Lovastatin – cholesterol lowering agent
Aflatoxin – carcinogen

Indole alkaloids

Ergotamine – migraine treatment
– control of post partum bleeding

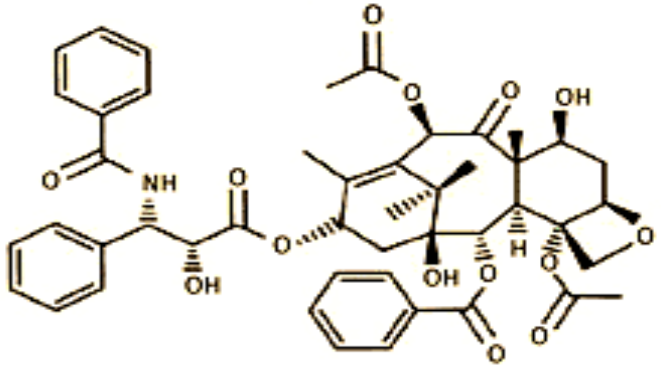
Metabolic engineering requires multigene expression

Taxol (paclitaxel) is a widely used cancer drug that is isolated from the bark of the Pacific yew tree, *Taxus brevifolia.*

One has to sacrifice two to four fully grown trees to get sufficient taxol for treatment of one patient.

During early stages of its clinical use, 130 kg of Taxol were extracted from 1000 tons of bark, which required cutting down more than 500,000 mature Pacific
yew trees.

At present, taxol is produced either through chemical conversion of a related molecule derived from needles of the more prevalent European yew, *T. baccata*, or from plat tissue culture.



Taxol

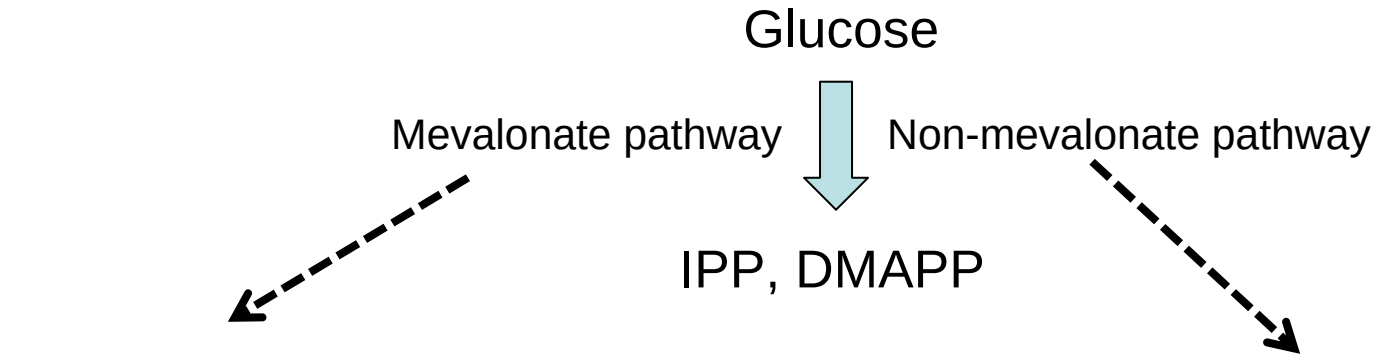
Isoprenoid Pathway Optimization for Taxol Precursor Overproduction in Escherichia coli
Ajikumar P et al., (1 October 2010) Science 330 (6000), 70

Taxol belongs to a large family of compounds known as terpenes, which includes artemisinin (a malaria drug), carvone (a flavoring agent), and pinene (the main component of turpentine).

The carbon skeletons of all terpene molecules are made from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are present in all cells that metabolize glucose.

Each terpene skeleton is then modified by a series of enzymatic reactions ultimately leading to synthesis of the final natural product.

The conversion of IPP and DMAPP into Taxol is a complex process that is present only in cells from the Pacific yew bark.



J. Kirby, J. D. Keasling,
Annu. Rev. Plant Biol. 60, 335 (2009).

P. K. Ajikumar *et al*.,
Science **330**, 70 (2010)

Taxol precursor

P. K. Ajikumar *et al*.,
Science **330**, 70 (2010)

Taxadiene (taxol precursor) levels in E. coli: 8 mg/liter per hour

Previous attempts increased taxadiene levels in E. coli upto 1.3 mg/liter

P. K. Ajikumar *et al*: taxadiene levels upto 60 mg/litre

Taxol precursor

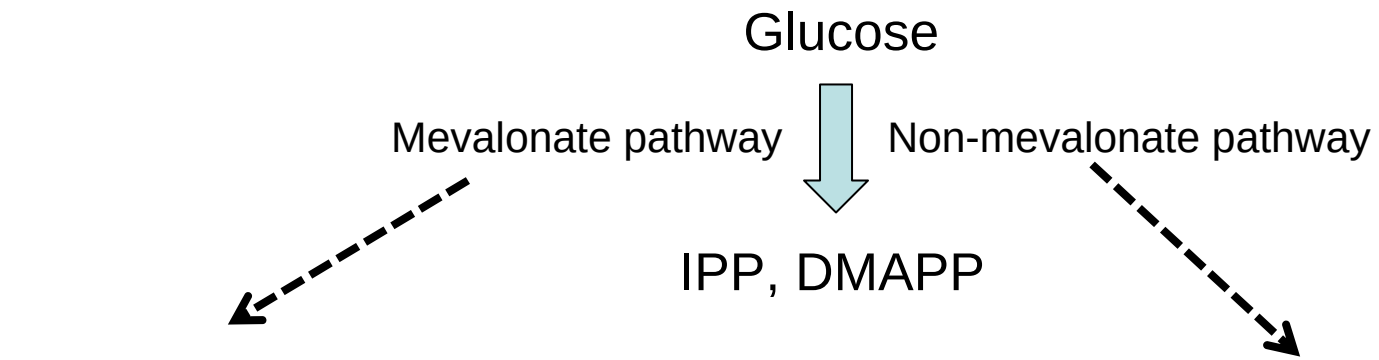
P. K. Ajikumar *et al*.,
Science **330**, 70 (2010)

- Improvement in the rate of IPP and DMAPP synthesis by careful optimization of the absolute and relative levels of four key enzymes in E. coli

- Intracellular activities of two plant enzymes (GGPP synthase and taxadiene synthase) were enhanced substantially through codon optimization and deletion of an N-terminal plastid transit peptide

- The upstream segments to IPP/DMAPP and downstream segments to taxadiene were balanced by varying promoter strength, plasmid copy number and genotype

- Engineering a chimeric cytochrome P450 reductase enzyme harboring both oxygenase and reductase activities without an membrane anchor



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Semi-biosynthetic Route for Artemisinin Production

Semi-biosynthetic Route for Artemisinin Production

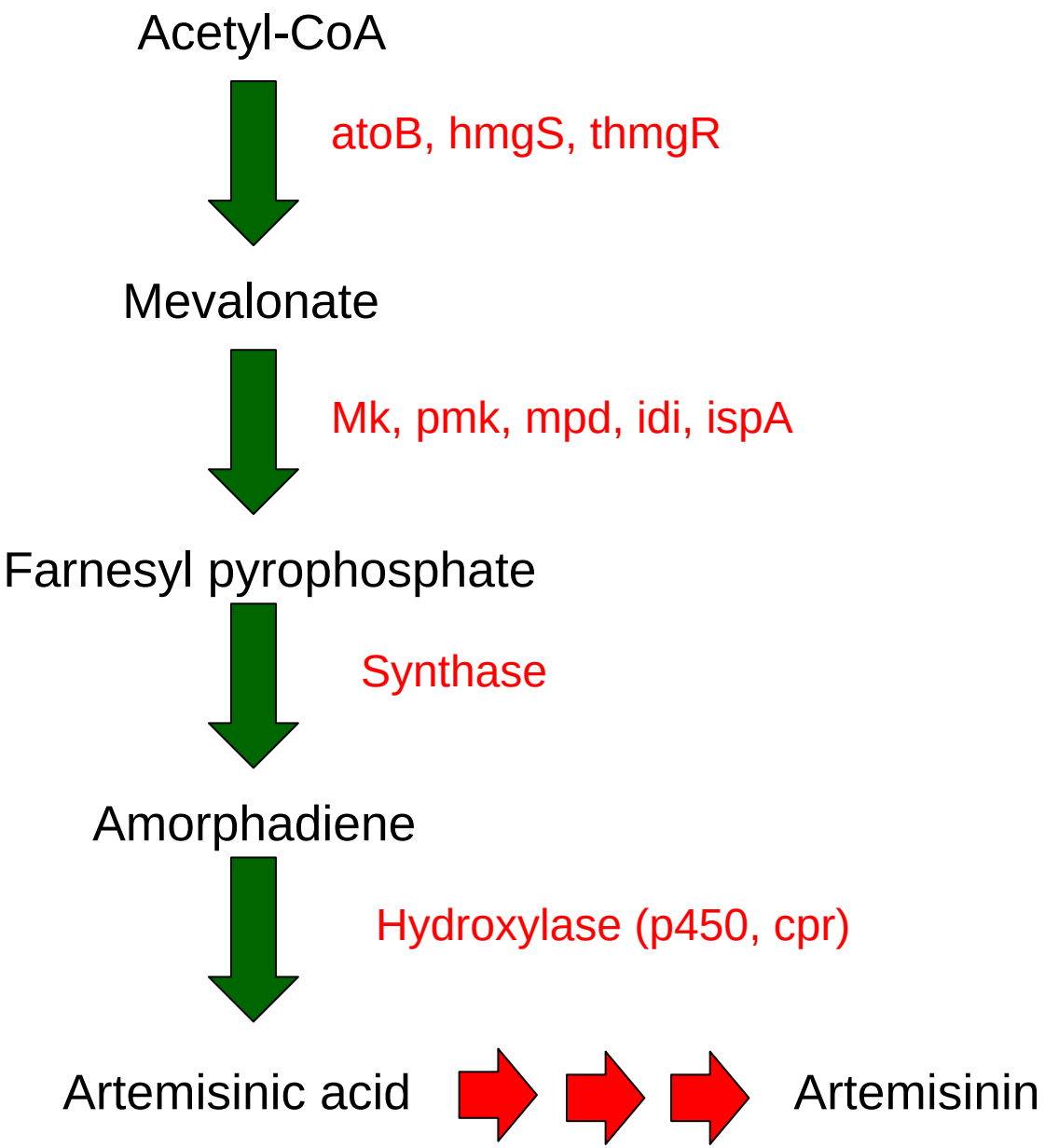
Towie N., Nature. 2006 Apr 13;440(7086):852-3.
Malaria breakthrough raises spectre of drug resistance.

Ro DK et al., Nature. 2006 Apr 13;440(7086):940-3.
Production of the antimalarial drug precursor artemisinic acid in engineered yeast.

Engineering mevalonate pathway in *S. cerevisiae* to produce upto 100mg/litre of artemisinic-acid

(1)Engineering the farnesyl pyrophosphate (FPP) biosynthetic pathway to increase FPP production
(2)Introduction of amorphadiene synthase gene (*ADS*) from *A. annua* into the high FPP producer to convert FPP to amorphadiene, and
(3)Cloning a novel cytochrome P450 that performs a three-step oxidation of amorphadiene to artemisinic acid from *A. annua* and expressing it in the amorphadiene producer

Semi-biosynthetic Route for Artemisinin Production



Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid

Dae-Kyun Ro BMC Biotechnol. 2008; 8: 83.

Three plant genes coding for amorphadiene synthase, amorphadiene oxidase, and cytochrome P450 reductase, which in concert divert carbon flux from farnesyl diphosphate to artemisinic acid, were expressed from a single plasmid.

The artemisinic acid production in the engineered yeast reached 1gram /litre in bio-reactors.

Metabolic Engineering

Isolate genes from higher eukaryotes (plants) and express them in E. coli/yeast

Plant genes are not expressed efficiently in E. coli

Synthetic genes with codons optimized for high level expression in E. coli

Assemble these genes into synthetic operons (in case of E. coli)

Generate strains in which specific genes of the endogenous pathway are knocked out

Prevent accumulation of intermediates which often inhibit growth

Failure to balance the flux in the synthetic pathway will result in a bottleneck and the accumulation of intermediates.

One way to balance the flux is through transcription optimization of the various genes in the pathway.

By fusing a library of promoters to the various enzymes in the isoprenoid production pathway, it is possible to engineer a flux-balanced pathway with improved yield and reduced metabolic burden.

Well-characterized families of transcription regulators have emerged as powerful tools in metabolic engineering as they allow rational coordination and control of multigene expression.

Balancing a heterologous mevalonate pathway for improved isoprenoid production in Escherichia coli.
Pitera DJ, Paddon CJ, Newman JD, Keasling JD.
Metab Eng. 2007 Mar;9(2):193-207

Ethanol production in Yeast

Yeast is a natural producer of ethanol but is unable to break down cellulose and hemicellulose, major components of biomass, and it is unable to utilize pentoses such as d-xylose and l-arabinose, which come from the breakdown of hemicellulose.

Thus, engineering yeast to break down cellulose and hemicellulose and utilize all the sugars from their breakdown is currently a major research effort in the area of bioenergy.

In order to endow yeast with the above functions, multiple enzymes and pathways need to be inserted and optimized.

To break down cellulose to glucose, at least three classes of enzymes (cellobiohydrolase, endoglucanase, and beta-glucosidase) need to be expressed.

To break down hemicellulose to d-xylose and l-arabinose, at least four classes of enzymes (xylanase, xylosidase, glucuronidase, and arabinosidase) need to be expressed.

All these enzymes need to be expressed in the right proportion for the best synergy.

Further, to increase the utilization efficiency of the pentose sugars, pentose transporters need to be introduced.

Ethanol production from xylose in engineered Saccharomyces cerevisiae strains: current state and perspectives.
Matsushika A, Inoue H, Kodaki T, Sawayama S.
Appl Microbiol Biotechnol. 2009 Aug;84(1):37-53.

ENGINEERING YEAST GLYCOSYLATION PATHWAYS FOR THE PRODUCTION OF “HUMANIZED” GLYCOPROTEINS

DEVELOPMENT OF GLYCO-ENGINEERED YEAST STRAINS

Glycoproteins comprise about 70% of all approved therapeutic proteins.

It is estimated that ~30% of new drugs that are likely to be licensed during the next decade will be based on antibody products.

Glycosylation plays a crucial role in determining the biological activity, half life, tissue distribution etc., of proteins.

For example, human IgG is a glycoprotein and serum IgG consists of multiple glycoforms owing to the differential glycosyltion of the Fc region. While glycosylation does not affect antigen binding, it has a profound effect on the biological mechanisms that are activated by the immune complexes formed as well as the solubility and stability of antibodies.

Mammalian cell culture, especially, Chinese hamster ovary (CHO) cell lines, emerged as the expression host of choice for the production of therapeutic glycoproteins.

To date mammalian cell culture has been the only process to yield human-like glycoproteins but in some cases human proteins expressed in mammalian cell lines does pose a problem.

For example, glycoproteins from human cells usually contain a mixture of both (2,6)- and (2,3)-sialic acid attachments, the sialic acid linkages in CHO cells are exclusively of the (2,3)-type.

To overcome this problem, CHO cells to express the gene, (2,6)-sialyltransferase was expressed in CHO cells and the recombinant tissue plasminogen activator (tPA) produced from these genetically altered CHO cells contained oligosaccharides with (2,6)-sialic acid linkages.

Expressing glycosyl transfereases emerged as one of the most challenging areas of Glycoengineering in recent years.

Producing recombinant proteins in mammalian cells is expensive.

Can we produce large volume therapeutic proteins with humanized glycosylation using yeast ?

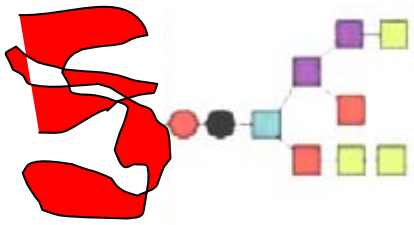
Protein glycosylation in yeast is often different from that in mammalian cells.

When mammalian glycoproteins are produced in yeast, high-mannose type N-glycans attached to recombinant glycoproteins which can be cleared rapidly from the human bloodstream due to interaction with human mannose receptors and they can cause immunogenic reactions in humans.

To overcome these problems, it is necessary to reengineer the N-glycosylation pathway in yeast species most frequently used for the production of heterologous proteins (*S. cerevisiae, Pichia pastoris, Yarrowia lipolytica, Hansenula polymorpha etc.)*.

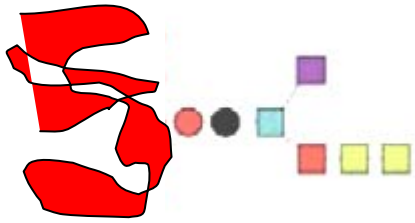
One approach relies on deletion of certain yeast-specific genes involved in hypermannosylation (mannosyltransferases) coupled with overexpression of several glycosyltransferases and glycosidases derived from various species to produce humanized oligosaccharides on the produced glycoproteins.

Endoplasmic Reticulum
(human or *P. pastoris*)

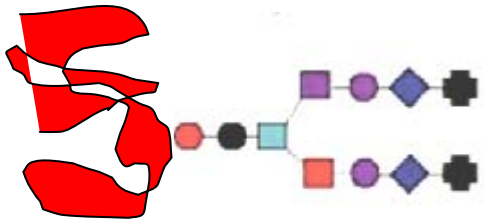


Endoplasmic Reticulum
(*P. pastoris*, *Δalg3, Δoch1*)

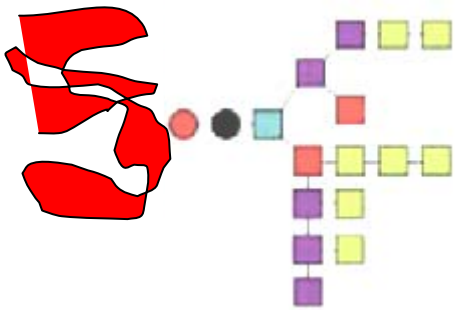
ALG3: mannosidase II
OCH1: α-1,6 mannosyltransferase



Golgi (human)



Golgi (*P. pastoris*)



Golgi (Humanized *P. pastoris*)

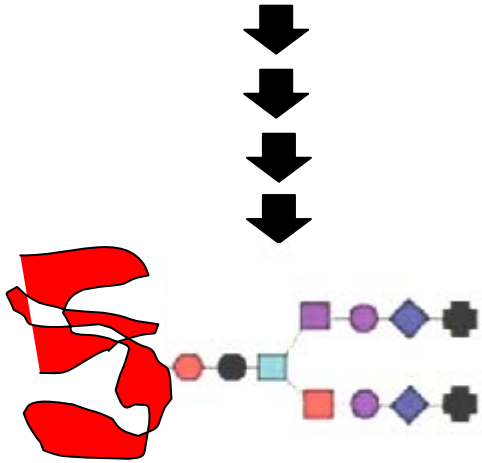


Figure adapted & modified under License number 2545290275749 from:
Glycosylation engineering in yeast: the advent of fully humanized yeast.
S R Hamilton and T U Gerngross. Current Opinion in Biotechnology 2007, 18:387–392

http://www.glycofi.com/

Li H et al., (2006) Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nat Biotechnol 24:210–215

P. pastoris strains were glycoengineered to produce six N-glycoforms of anti-CD20 monoclonal IgG antibodies which bind to CD20 present on the surface of B cells and destroy B cells.

They are used in the treatment of many lymphomas, leukemias, transplant rejection and some autoimmune disorders.

Choi BK et al., (2008)

Recombinant human lactoferrin expressed in glycoengineered Pichia pastoris: effect of terminal N-acetylneuraminic acid on in vitro.

Synthetic Biology

The goal of synthetic biology is to develop designer organisms
for the sole purpose of serving people.

To accomplish this higher goal, scientists first need to understand what is life.

To address the problem, scientists have taken two main approaches:

In one approach, an organism is stripped to its bare minimum genome in an effort to understand what is required to create a self-replicating organism.

In another approach, genes are synthesized and inserted into a ‘shell’ to create a living organism.

The day is not very far when design new biological systems will be designed the same way engineers design electronic or mechanical systems.

Minimal genome project

M. genitalium, is an obligate intracellular parasite whose genome consists of 482 genes comprising 582,970 base pairs, arranged on one circular chromosome (the smallest genome of any known natural organism that can be grown in free culture).

By systematically removing genes, a minimal set of 382 genes were identified that can sustain life.

This effort was also known as the Minimal Genome Project.

The challenge is to synthesize chromosome DNA sequences consisting of these 382 genes and transplant it into a *M. genitalium* cell to create *M. laboratorium*.

The resulting *M. laboratorium* bacterium will replicate with its man-made DNA, making it the most synthetic organism to date, although the molecular machinery and chemical environment that would allow it to replicate is not synthetic

In December 2003, the Craig Venter team had reported a fast method of synthesizing a genome from scratch, producing the 5386-base genome of the bacteriophage Phi X 174 in about two weeks.

But the Mycobacterium genome is much bigger!

J. Craig Venter INSTITUTE

In January 2008, the team synthesized the complete 582,970 base pair chromosome of *M. genitalium*, with small modifications so that it won't be infectious and can be distinguished from the wild type.

They named this genome *Mycoplasma genitalium JCVI-1.0*

http://www.jcvi.org/

http://www.jcvi.org/cms/research/projects/first-self-replicating-synthetic-bacterial-cell/overview/

J. Craig Venter Institute (JCVI)

Mycoplasma genitalium genome (583 kb) was constructed from 101 pieces of DNA fragments, each 5–6 kb in length.

Construction of a synthetic *M. genitalium* genome.

Twenty-five different overlapping DNA segments (17–35 kb each) composing the genome were co-transformed into yeast followed by assembly of the entire genome in a single step

It is estimated that the synthetic genome cost US$40 million and took 20 people more than a decade of work

Understanding gene expression is crucial for the development of transcriptional gene circuits by chemically interconnecting multiple promoters, repressors, and activators for pathway engineering, multi gene expression and regulation.

Keasling JD. Synthetic biology for synthetic chemistry. ACS Chem Biol 2008, 3: 64–76.

Young E, Alper H. Synthetic biology: tools to design, build, and optimize cellular processes. J Biomed Biotechnol 2010, 12.

Mukherji S, van Oudenaarden A. Synthetic biology: understanding biological design from synthetic circuits. Nat Rev Genet 2009, 10: 859–871.

Yeh BJ, Lim WA. Synthetic biology: lessons from the history of synthetic organic chemistry. Nat Chem Biol 2007, 3: 521–525.

Tigges M, Fussenegger M. Recent advances in mammalian synthetic biology—design of synthetic transgene control networks. Curr Opin Biotechnol 2009, 20: 449–460.

Purnick PEM, Weiss R. The second wave of synthetic biology: from modules to systems. Nat Rev Mol Cell Biol 2009, 10: 410–422.

Benner SA, Sismour AM. Synthetic biology. Nat Rev Genet 2005, 6: 533–543.

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Hope you have enjoyed this 40 lecture series on

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