Is Consolidated Bioprocessing a Reachable Goal?

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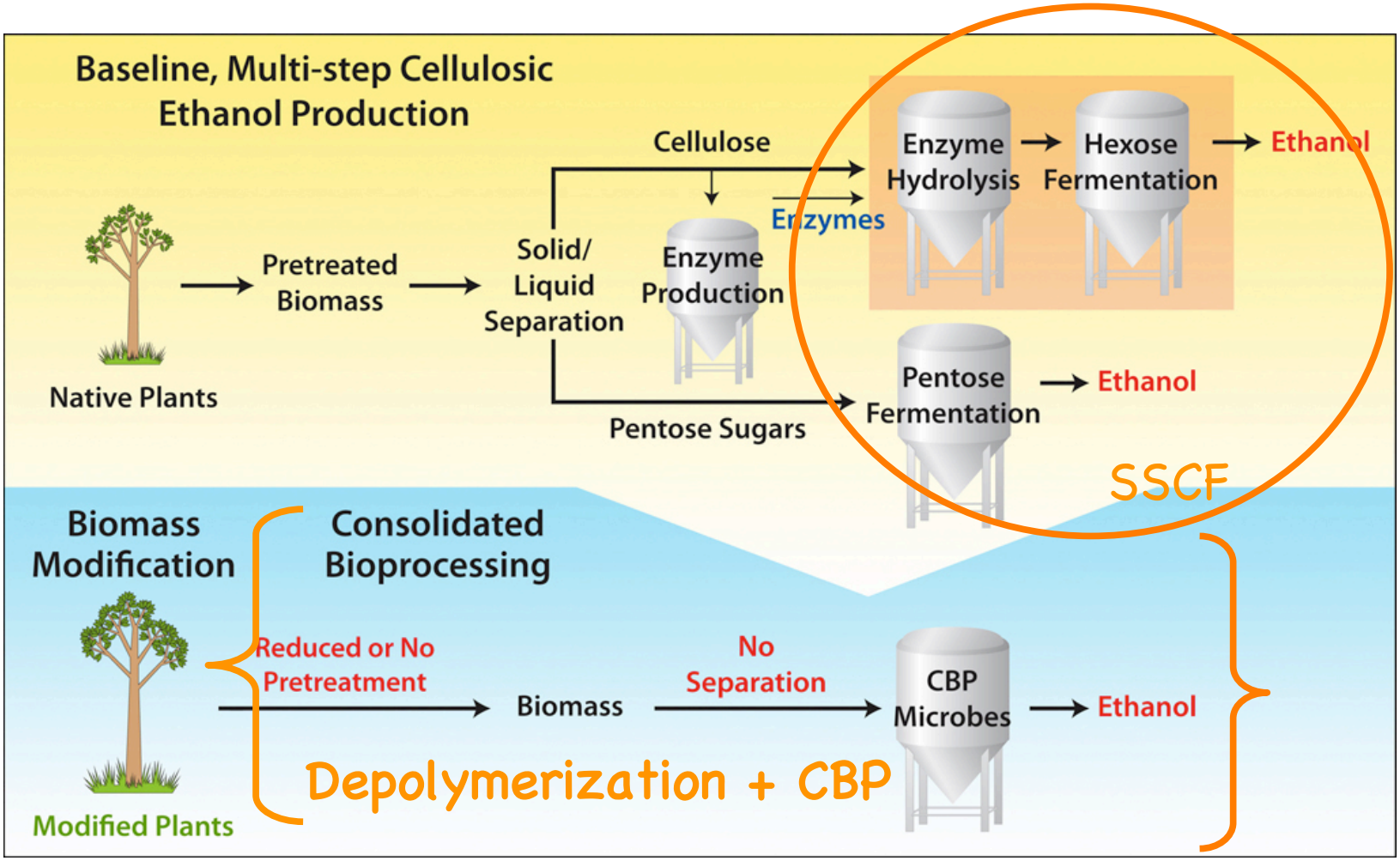
VIMSS Monthly Meeting

April 4, 2008

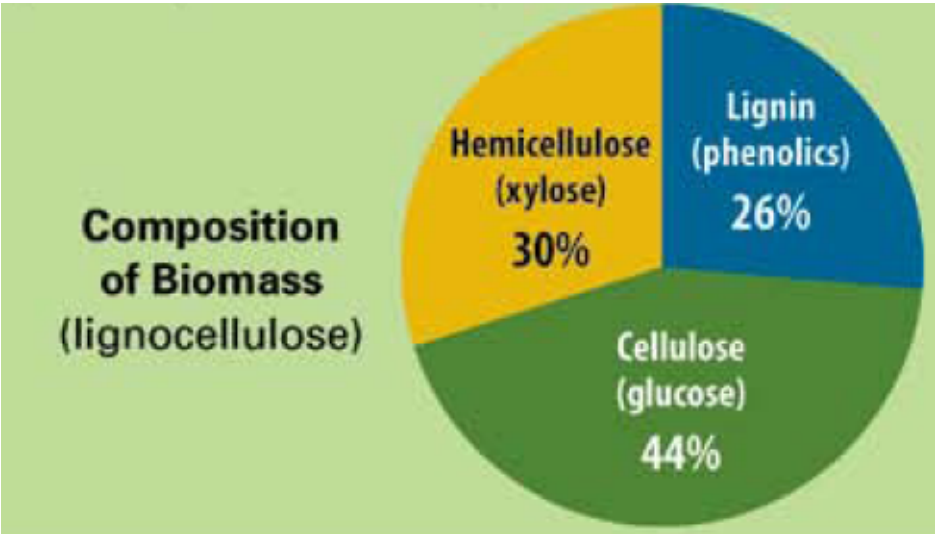
Seo et al., 2004

Biofuels Production Steps

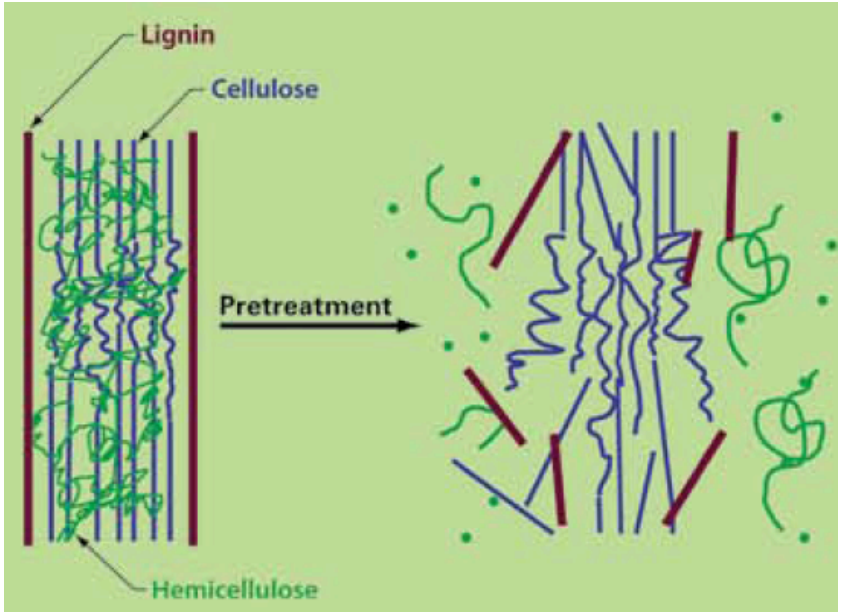


www.genomicsgtl.energy.gov/centers/center_ORNL.shtml

Plant Biomass Composition and Depolymerization



US DOE 2006. Biofuels Joint Roadmap

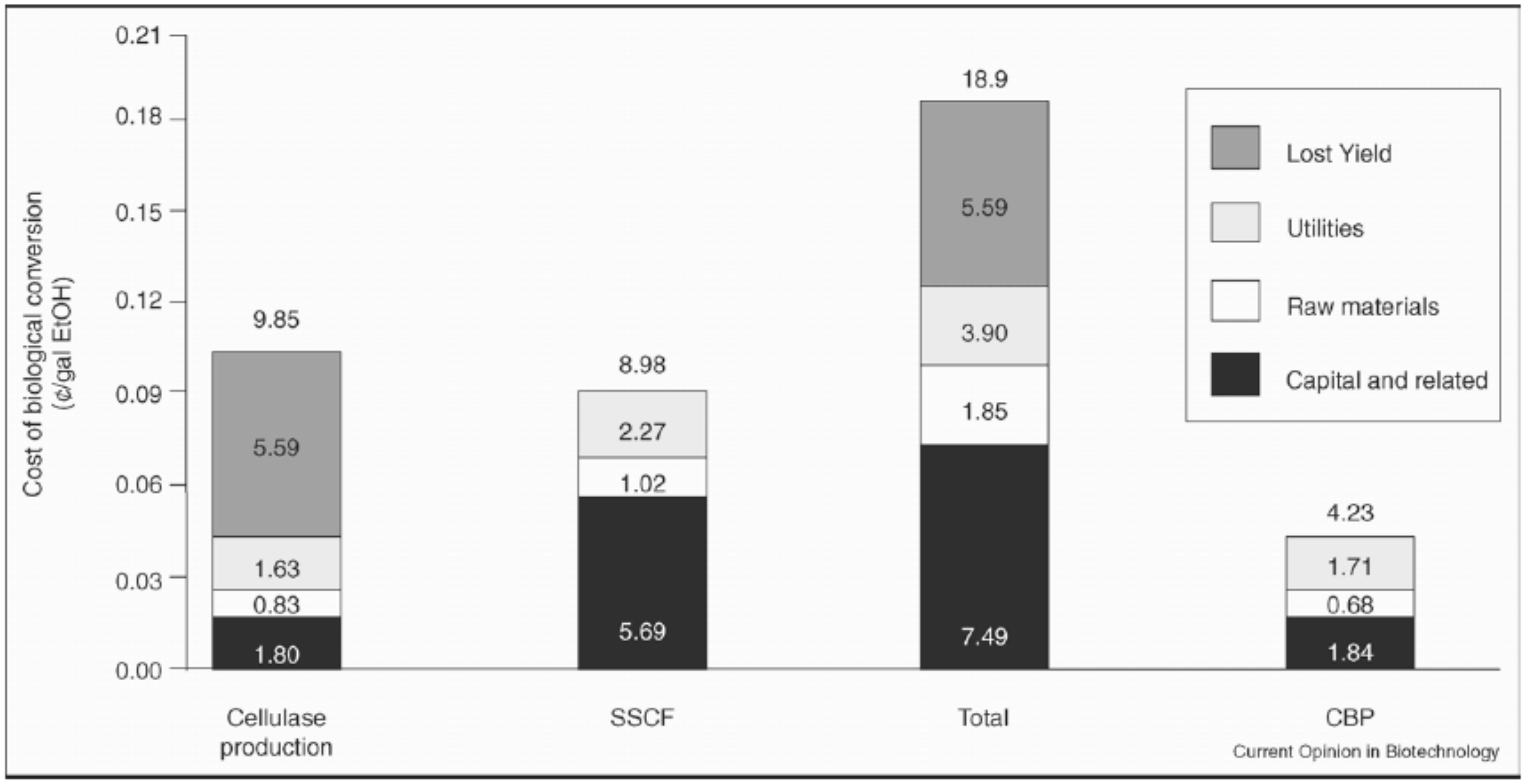


Thermochemical pretreatment methods include dilute acid, hot water, ammonia fiber explosion and lime applications.

Current Challenges

- Pretreatment of biomass
  - Difficulty in breaking down lignin to release hemicellulose and cellulose
  - Next to feedstock production, thermochemical pretreatment has the highest energy cost in the process
- Hydrolysis of cellulosic materials
  - Low efficiency in fermenting pentose sugar

Consolidated Bioprocess Benefits



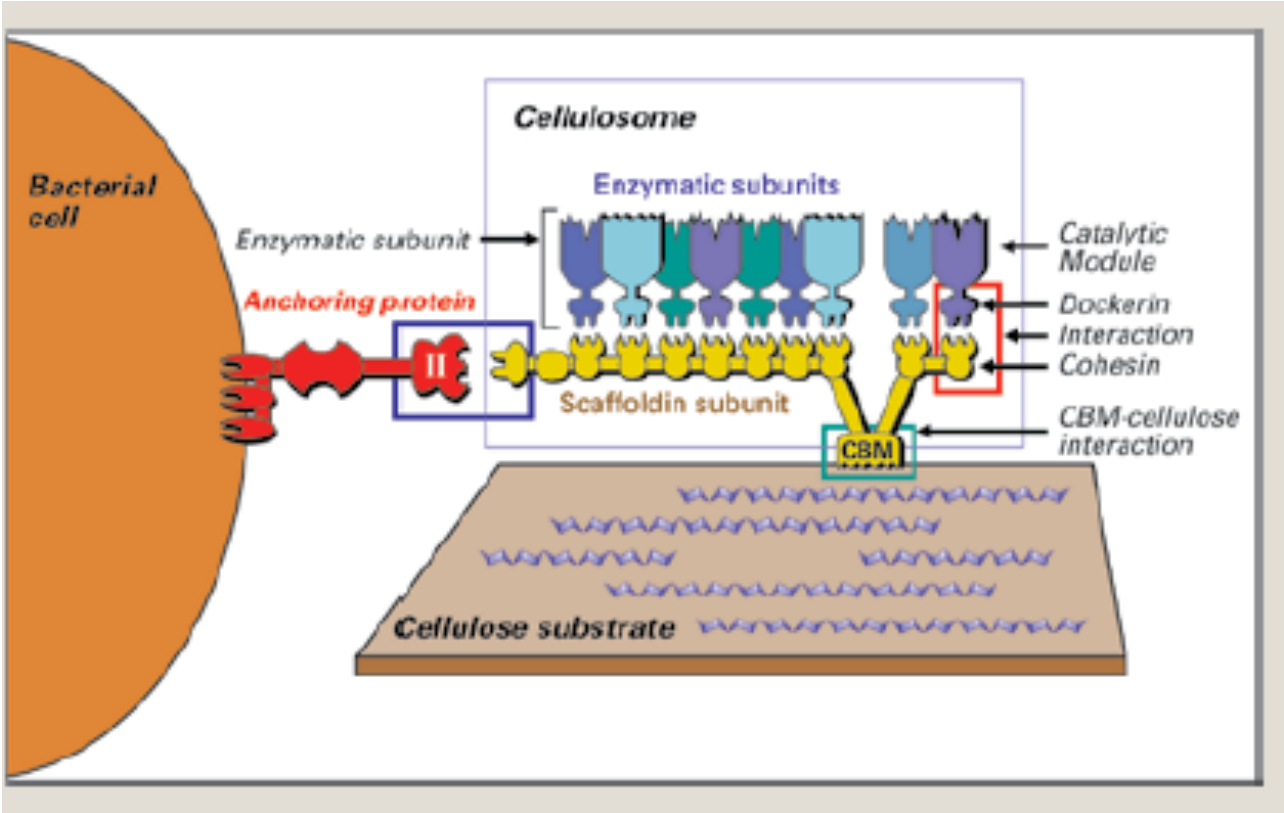
Simulations by ASPEN-based models developed at NREL

SSCF - simultaneous saccharification and co-fermentation

Time frame for cost calculation - SSCF - 7 days; CBP - 3 days

CBP - has the potential for higher hydrolysis rates, lower reactor volume and capital investment

Strategy - cellulosomes from *Clostridum thermocellum*

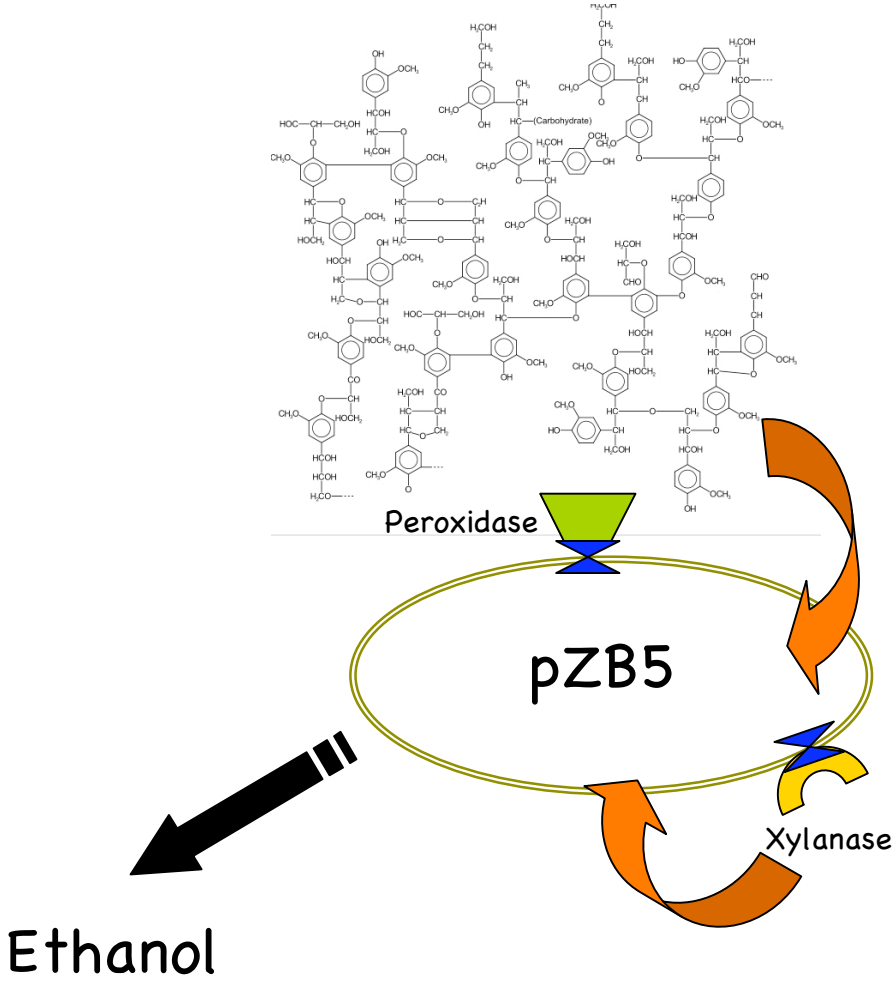


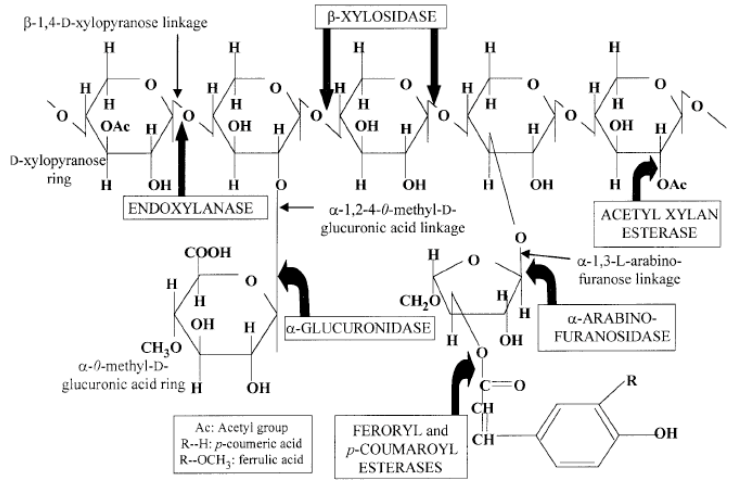
US DOE 2006. Biofuels Joint Roadmap

Concerted cellulolytic enzyme activity on cell surface

- ✓ Mechanisms of assembly still under investigation
- ✓ Unclear if enzymes can degrade lignin
- ✓ What are the sizes of the surface enzymes?

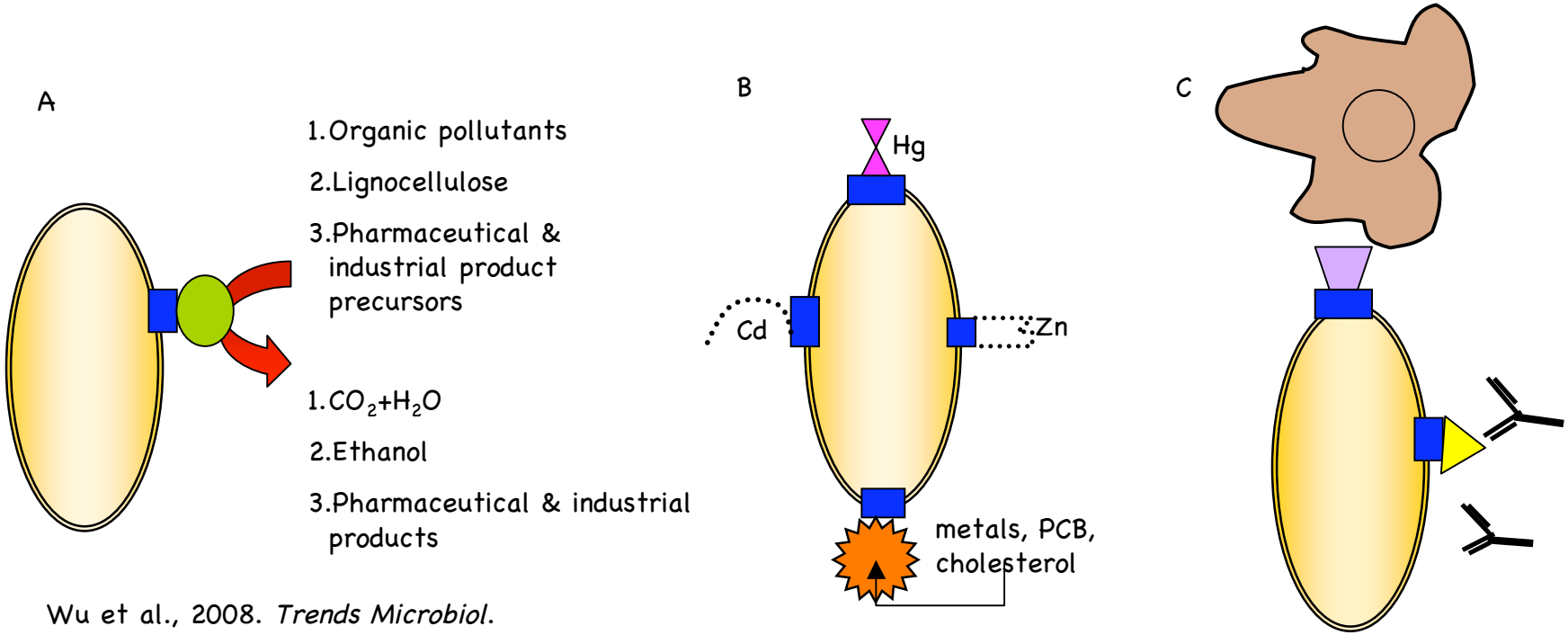
Strategy- surface expression of peroxidase and xylanase on xylose fermenting *Z. mobilis*





Beg et al., 2001

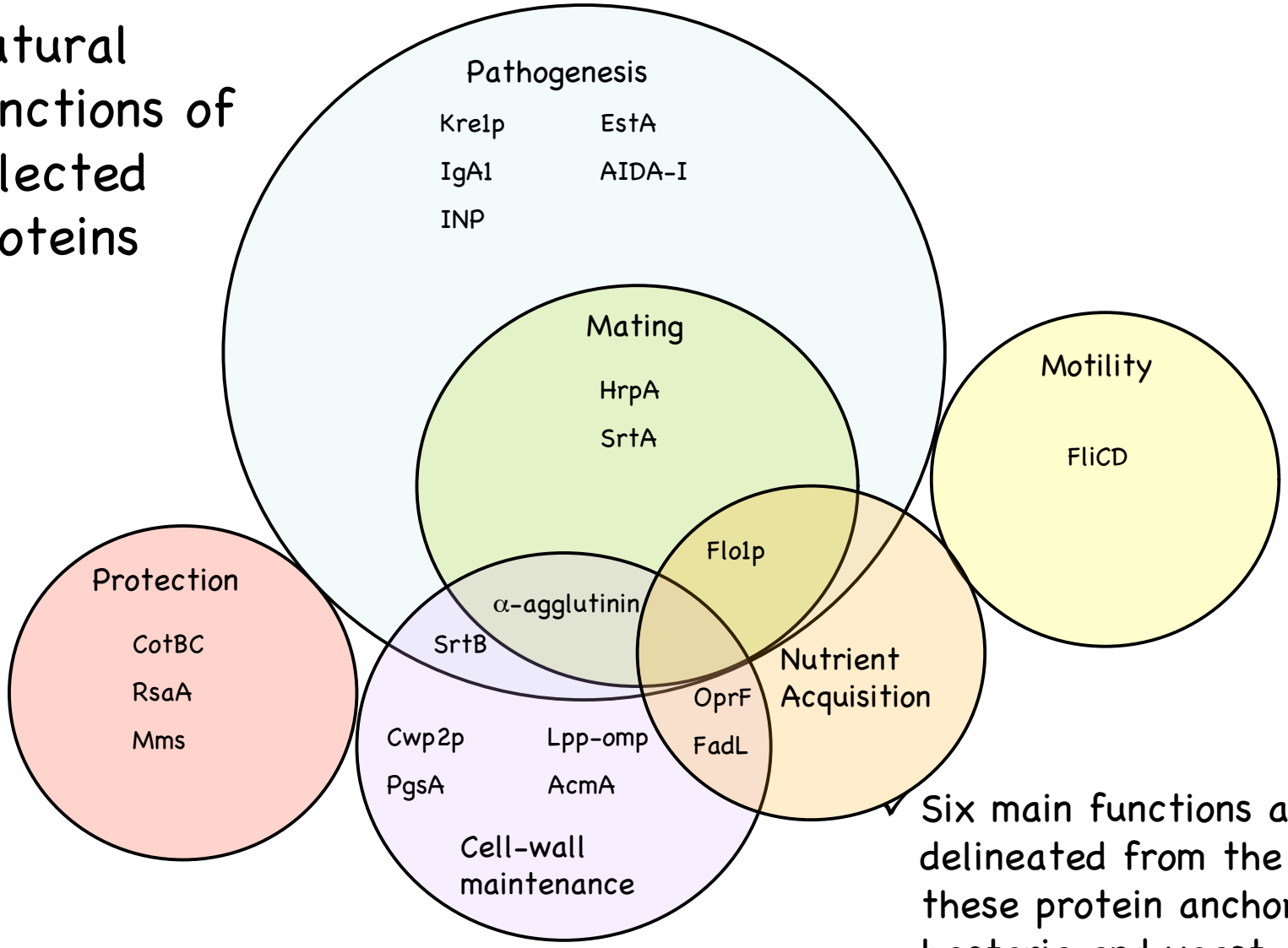
Applications of Surface Expressed Proteins



Wu et al., 2008. *Trends Microbiol.*

- ✓ Biocatalysts have usages in organic pollutant degradation, biofuel production and production of enantiomerically selective organic compounds.
- ✓ Biosorbents displaying peptides, proteins and cyclodextrin can be used to remove metals, organic pollutants and cholesterol.
- ✓ Biostimulants with surface-displayed antigens or surface adhesion proteins induce antibody production, and attach to specific sites in eukaryotic cells which is useful for vaccine development.

Natural functions of selected proteins



Wu et al., 2008. *Trends Microbiol.*

- ✓ Six main functions are delineated from the roles of these protein anchors from bacteria and yeast.
- ✓ Most of the proteins fall under the functions pathogenesis and cell-wall maintenance.

Surface expression used for biofuels production

Target	Anchor	Passenger	Host Cell	Reference
Cellobiose	α-agglutinin	β-glucosidase	Yeast	Ehsan et al 2007
Glucan	α-agglutinin	Endoglucanase II and β-glucosidase	Yeast	Pack et al., 2002; Fujita et al 2002
Starch	α-agglutinin	Glucoamylase	Yeast	Katahira et al., 2006
starch	α-agglutinin	Glucoamylase and amylase	Yeast	Kondo et al., 2002; Shigechi et al., 2002
Xylan	α-agglutinin	Xylanase II and xylosidase	Yeast	Shigechi et al., 2004; Fujita et al., 2002
Starch	α-agglutinin	Cyclodextrin glycosyl transferase	Yeast	Katahira et al., 2004
Starch	Flo1p	Amylase	Yeast	Shigechi et al., 2004

Some bacteria has higher ethanol and temperature tolerance than yeast, as well as lower production cost.

Advantages of using *Zymomonas mobilis*

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Alpha Proteobacteria
Order:	Sphingomonadales
Family:	Sphingomonadaceae
Genus:	Zymomonas
Species:	Z. mobilis

- ✓ High ethanol yield and tolerance
- ✓ High fermentation selectivity and specific productivity
- ✓ Can ferment sugars at low pH
- ✓ Has high tolerance to inhibitors found in lignocellulosic hydrolysates
- ✓ Facultative anaerobe
- ✓ Xylose fermenting recombinant strain (Zhang et al., 1995)
- ✓ Acetate tolerant strain exist (Z. mobilis ZM4/Ac^R (pZB5), Mohagheghi et al., 2004)

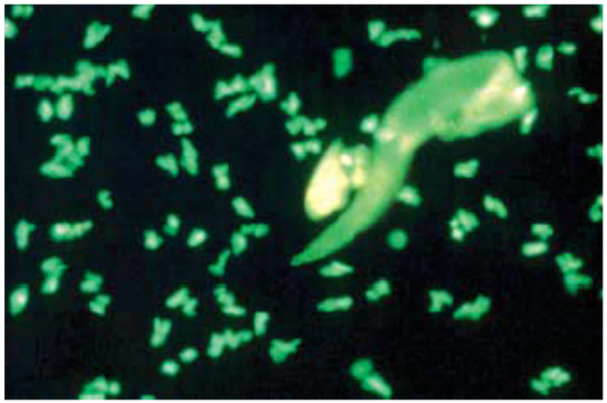


Fig. 2. *Zymomonas mobilis*, a metabolically engineered bacteria used for fermenting both glucose and xylose to ethanol. [Credit: Zhang, Min; DOE/NREL] Stephanopoulos, 2007

Zymomonas mobilis ZM4 (pZB5)

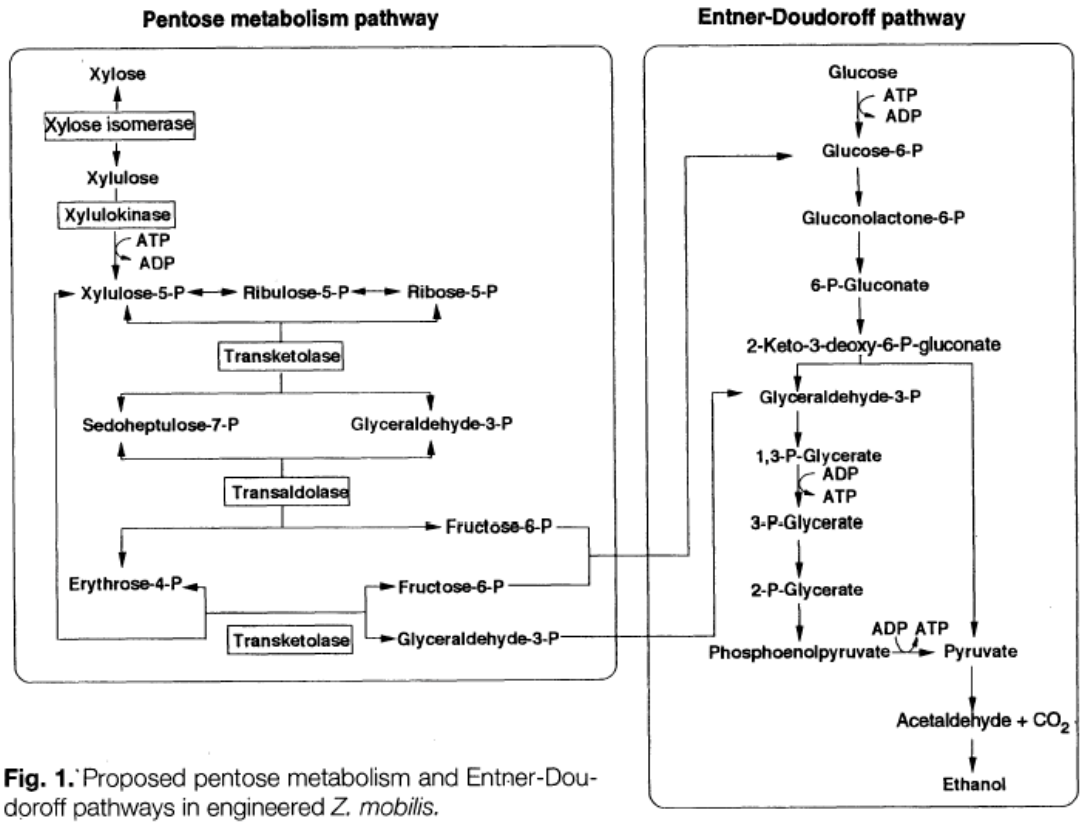
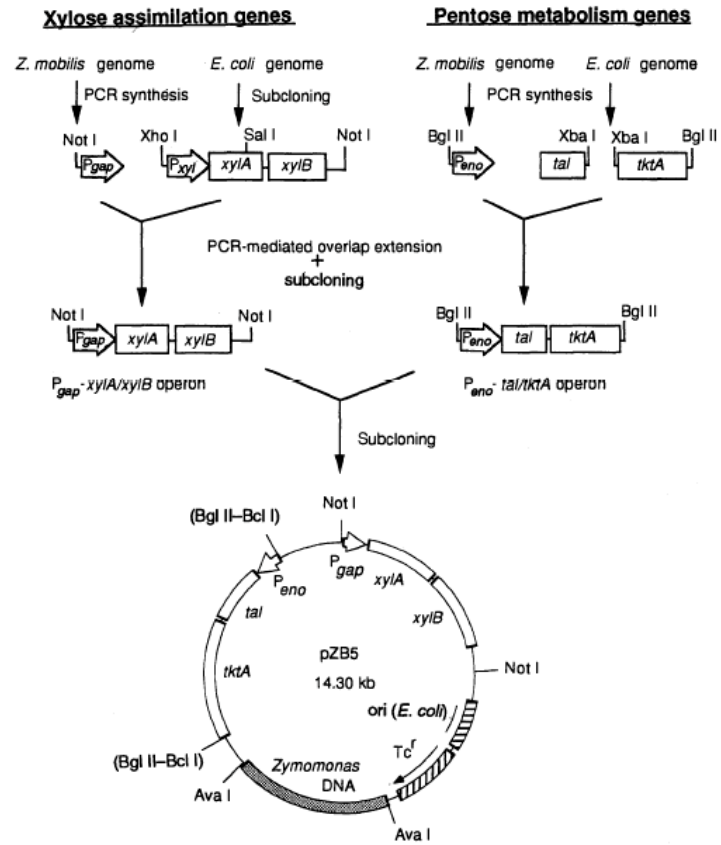


Fig. 1. Proposed pentose metabolism and Entner-Doudoroff pathways in engineered *Z. mobilis*.

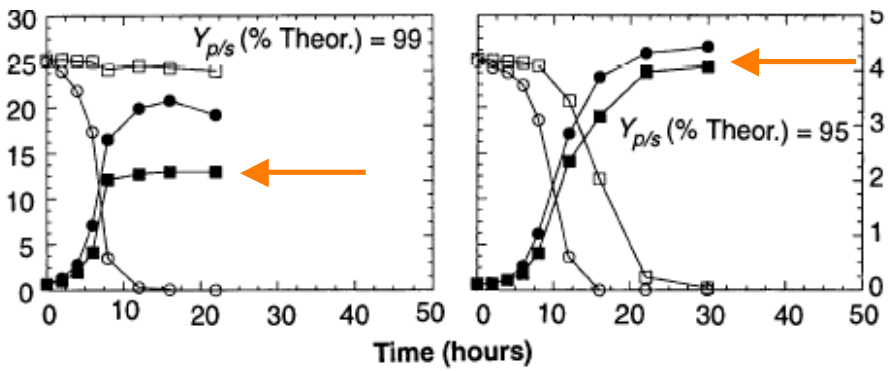


Zhang et al 1995. Science. 267:240

Operons encoding xylose assimilation and pentose phosphate pathway enzymes were transferred into ZM

Evaluation of Z. mobilis pZB5 ethanol production

100 ml shake flask



Fermentation performance of Z.mobilis control strain (pZB186) vs Z. mobilis (pZB5) grown in xylose and glucose medium. Zhang et al., 1995.

- Ethanol yield 0.51 g/g
- cell growth on Xylose - 0.057 per h

1.5 L batch fermenter

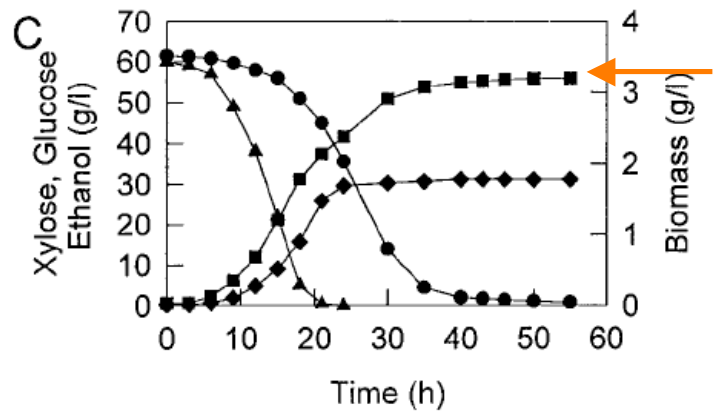


FIG. 1. Fermentation characteristics of recombinant strain ZM4(pZB5) using xylose (60.5 g/liter) (A), glucose (64.8 g/liter) (B), and a mixture of glucose (60.0 g/liter) and xylose (61.5 g/liter) (C). ●, Xylose; ▲, glucose; ■, ethanol; ◆, biomass. Kim et al., 2000

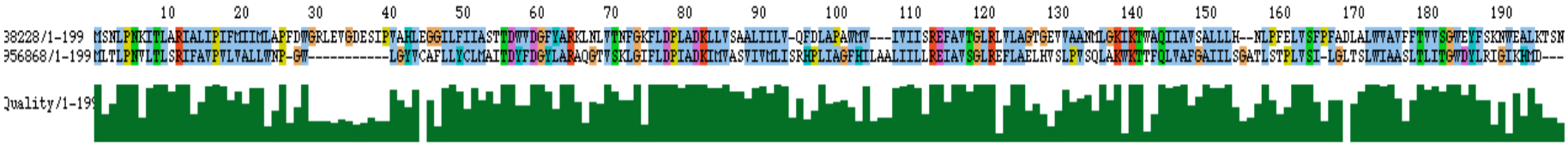
- Ethanol yield 0.46 g/g
- cell growth on xylose - 0.11 - 0.13 per h

Large functional surface protein expression in literature

Protein Name	Original organism	Passenger protein&size	Host Organism	Found in ZM?	Ref
INP	*P. syringae*	LevU from *Z. mobilis* (47kDa) OPH from *P. diminuta* (35 kDa)	*E. coli*	No	Jung et al., 1998; Cho et al., 2004
PgsA	*B. subtilis*	Amylase from *Streptococcus bovis* (77kDa), Lipase B from *Candida antarctica* (34 kDa)	*E. coli* *Lactobacillus*	Yes	Narita et al (2006) a, b
EstA	*P. aeruginosa*	LipA from *B. subtilis* (~40kDa) Cutinase from *F. solani* (~40kDa) lipase from *Serratia marcescens* (62kDa) LipH from *P. aeruginosa* (~30kDa)	*E. coli*	No	Becker et al., 2005; Wilhelm et al., 2007
OprF	*P. aeruginosa*	Lipase from *P. fluorescens* (~49.1kDa)	*P. putida*	No	Lee et al., 2005
FadL	*E. coli*	lipase from B. subtilis (44.5kDa)	*E. coli*	No	Lee et al., 2004
CPX	*de novo*	GFP (27 kDa) and streptavidine binding peptide	*E.coli*	No	Rice et al., 2006; Oh et al., 2006

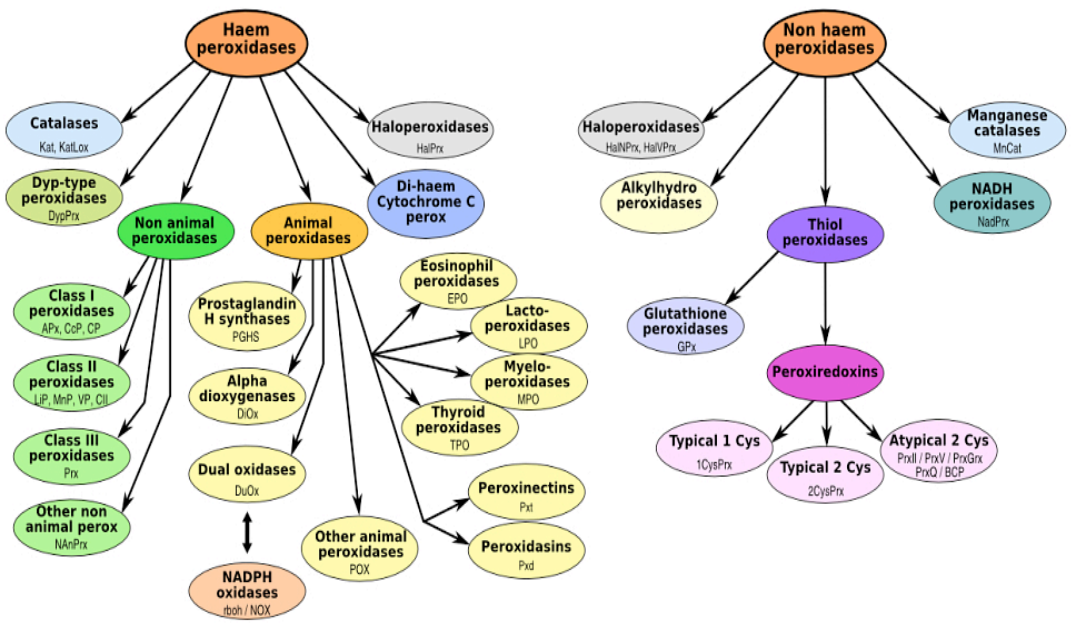
PgsA comparison

VIMSS Id	Name	Organism
38228	pgsA	Bacillus subtilis
956868	pgsA	Zymomonas mobilis subsp. mobilis ZM4



www.microbesonline.org Alm et al., 2005

Enzymes - Peroxidases, Manganases,



Taxonomic group	# Organisms	# Peroxidases
Bacteria		
Actinobacteria	42	191
Bacteroidetes/Chlorobi	27	76
Chlamydiae/Verrucomicrobia	0	0
Cyanobacteria	16	64
Firmicutes	46	134
Proteobacteria		
Alphaproteobacteria	57	139
Betaproteobacteria	45	144
Deltaproteobacteria	12	28
Epsilonproteobacteria	10	12
Gammaproteobacteria	103	318
Other Proteobacteria	2	2
Other Bacteria	22	42
Archaea	21	41
Fungi/Metazoa		
Choanoflagellida	1	1
Fungi		
Ascomycota	70	366
Basidiomycota	50	156
Chytridiomycota	2	5
Microsporidia	1	2
Zygomycota	2	10
Other Fungi	1	1

http://peroxibase.isb-sib.ch
Passardi et al. 2007. Phytochemistry 68(12):1605-11.

Fungal LiP (~45-50 kDa)

24 peroxidase(s) match

Select: All | Invert | None

Retrieve as fasta

Name	Taxonomic group	Organism	Class	Sequence Status	Action	Select
BaLiP	Basidiomycota	Bjerkandera adusta	Lignin peroxidase	complete	View	
CsuLiP01 (Csg1)	Basidiomycota	Ceriporiopsis subvermispora	Lignin peroxidase	partial	View	
CsuLiP02 (Csg2)	Basidiomycota	Ceriporiopsis subvermispora	Lignin peroxidase	partial	View	
PcLiPA (LPOA, Ligninase H8)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPA_SC26 (rLDM(TM)6)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	partial	View	
PcLiPB (LPOB, Ligninase B)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPC (GLG5, LIP6)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPD (GLG4, LiP2)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPE (lpo)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPE_OGC101 (LiP2, GLG2)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPF (GLG6)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPG	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPI (LIG3, GLG3)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPI_ME446 (LIG1)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PcLiPJ (lipJ)	Basidiomycota	Phanerochaete chrysosporium	Lignin peroxidase	complete	View	
PsoLiP01	Basidiomycota	Phanerochaete sordida	Lignin peroxidase	partial	View	
PsoLiP02	Basidiomycota	Phanerochaete sordida	Lignin peroxidase	partial	View	
PrLiP01 (lip1)	Basidiomycota	Phlebia radiata	Lignin peroxidase	complete	View	
PrLiP03 (lip3)	Basidiomycota	Phlebia radiata	Lignin peroxidase	complete	View	
PrLiP04 (lip4)	Basidiomycota	Phlebia radiata	Lignin peroxidase	complete	View	
PcocLiP	Basidiomycota	Pycnoporus coccineus	Lignin peroxidase	partial	View	
TvLiP (LPGIV, LPG1, Ligninase C)	Basidiomycota	Trametes versicolor (Coriolus versicolor)	Lignin peroxidase	complete	View	
TvLiP07 (LP7, LPGVI)	Basidiomycota	Trametes versicolor (Coriolus versicolor)	Lignin peroxidase	complete	View	
TvLiPGIII	Basidiomycota	Trametes versicolor (Coriolus versicolor)	Lignin peroxidase	complete	View	

Select: All | Invert | None

Fungal MnP (~ 23-32kDa)

Select: All | Invert | None

Retrieve as fasta

Name	Taxonomic group	Organism	Class	Sequence Status	Action	Select
AbMnP01	Basidiomycota	Agaricus bisporus	Manganese peroxidase	complete	View	
AarMnP01	Basidiomycota	Amylostereum areolatum	Manganese peroxidase	partial	View	
AchMnP01	Basidiomycota	Amylostereum chailletii	Manganese peroxidase	partial	View	
AfeMnP01	Basidiomycota	Amylostereum ferreum	Manganese peroxidase	partial	View	
AlMnP01_B1371	Basidiomycota	Amylostereum laevigatum	Manganese peroxidase	partial	View	
AMspMnP01	Basidiomycota	Amylostereum sp.	Manganese peroxidase	partial	View	
BmoMnP01	Basidiomycota	Bondarzewia montana	Manganese peroxidase	partial	View	
BmoMnP02_DAOM415	Basidiomycota	Bondarzewia montana	Manganese peroxidase	partial	View	
CsuMnP01 (mnp1)	Basidiomycota	Ceriporiopsis subvermispora	Manganese peroxidase	complete	View	
CsuMnP02a (mnp2)	Basidiomycota	Ceriporiopsis subvermispora	Manganese peroxidase	complete	View	
CsuMnP02b (mnp2B)	Basidiomycota	Ceriporiopsis subvermispora	Manganese peroxidase	complete	View	
CsuMnP03	Basidiomycota	Ceriporiopsis subvermispora	Manganese peroxidase	complete	View	
CsuMnP04	Basidiomycota	Ceriporiopsis subvermispora	Manganese peroxidase	complete	View	
CgalMnP01	Basidiomycota	Coriolopsis gallica	Manganese peroxidase	partial	View	

Extracellular Peroxidases from Actinomycetes (~56.5 kDa)

TABLE 1. Extracellular peroxidase activity produced by actinomycete strains grown on ball-milled wheat straw medium

Organism	Strain[a]	Incubation temp (°C)	Mean peroxidase activity (pg/ml of HRP/mg of protein)[b]
T. mesophila	BD123, T-1, DSM43048, CUB1001	37	17.04
A. autotrophica	DSM43099	30	16.13
A. autotrophica	DSM43089	30	5.36
Streptomyces sp.	EC22	37	14.47
Streptomyces sp.	EC1	30	11.89
S. viridosporus	T7A, DSM40243, ATCC27479	37	10.24
Streptomyces sp.	EC10	30	8.21
Streptomyces sp.	EC55	37	8.09
Thermomonospora curvata	BD3, B9, CBS162.64, CUB467	45	8.01
Saccharomonospora viridis	BD127, P101, NCIB9602, CUB1003, DSM43017	37	7.94
Saccharomonospora viridis	BD42, MT802	37	7.14
Streptomyces sp.	11	30	7.11
Streptomyces sp.	EC54	37	7.11
T. curvata	BD117	50	7.11
Thermomonospora chromogena	EC12	50	6.49
Streptomyces sp.	LL6	37	6.15
Thermomonospora sp.	RW4	50	6.14
Thermomonospora alba	BD126, IPV1900, NCIB10169, CUB1002	45	5.76
Thermomonospora sp.	LL14	37	5.34
T. chromogena	BD113, N7, CUB991	45	5.04
Streptomyces sp.	EC3	37	4.92
S. badius	252	30	4.76
Streptomyces lividans	TK64, NCIMB11416	30	4.73
S. lividans + pIJ702	TK64, NCIMB11416	30	4.13
Streptomyces sp.	EC37	30	4.61
Thermomonospora mesouviformis (*alba*)	BD12, T-3, ATCC27644, CUB689, NCIB11543, DSM43185	30	4.22
Streptomyces sp.	EC5	37	3.74
Streptomyces sp.	119	37	3.74
Thermomonospora sp.	RW7	50	3.72
Streptomyces sp.	EC43	30	3.08
T. chromogena	BD8	45	2.94
Actinomadura flexuosa	BD118, Agre435, DSM43186, CUB999	50	2.90
Thermomonospora fusca	MC28, MT800	50	2.90
Streptomyces sp.	EC31	30	2.14
Streptomyces sp.	0	30	1.34
Thermoactinomyces candidus	BD28, T-106, ATCC27868, NCIB11249, CUB749	50	<1
T. fusca	MC8	37	<1
T. fusca	MC10	37	<1
T. fusca	MC12	37	<1
Pseudonocardia sp.	EC20	50	<1
Saccharomonospora viridis	30 Urban K	30	<1

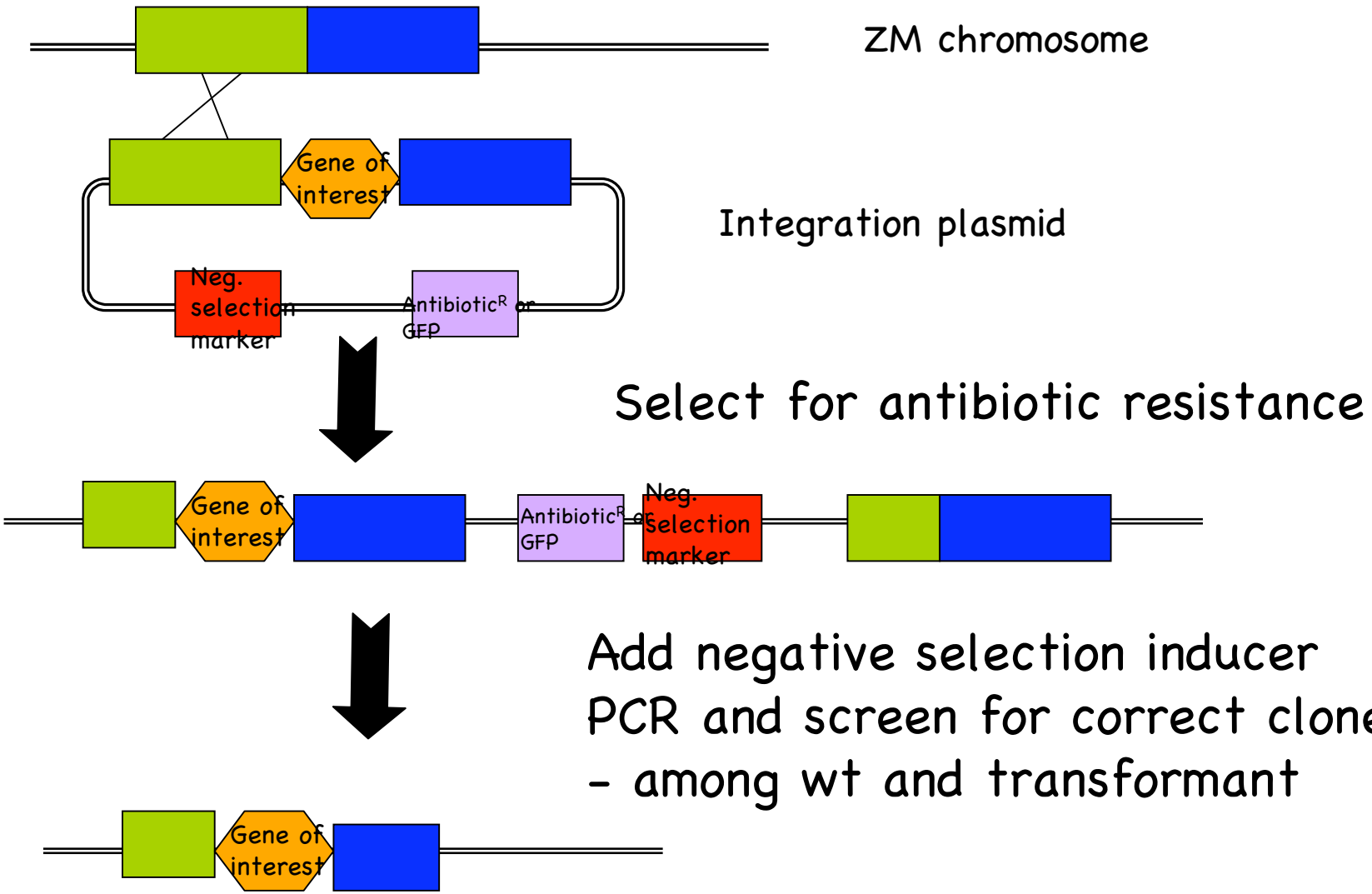
Mercer et al. 1996

Microbial Xylanases

Beg et al. 2001

Microorganism	Molecular weight (kDa)	Optimum		Stability		pI	K_m (mg/ml)	V_{max} (μM/mine per mg)	References
		pH	Temperature (°C)	pH	Temperature (°C)				
Aspergillus sydowii MG 49	30	5.5	60	–	–	–	–	–	Ghosh and Nanda 1994
Cephalosporium sp.	30, 70	8	40	8–10	–	–	0.15	–	Bansod et al. 1993
Fusarium oxysporum	20.8, 23.5	6	60, 55	7–10	30	–	9.5; 8.45, 8.7	0.41, 0.37	Christakopolous et al. 1996
Geotrichum candidum	60–67	4	50	3–4.5	45	3.4	–	–	Radionova et al. 2000
Paecilomyces varioti	20	4	50	–	–	5.2	49.5	–	Kelly et al. 1989
Penicillium purpurogenum	33, 23	7, 3.5	60, 50	6–7.5, 4.5–7.5	40	8.6, 5.9	–	–	Belancic et al. 1995
Thermomyces lanuginosus DSM 5826	25.5	7	60–70	5–9	60	4.1	7.3	–	Cesar and Mrsa 1996
Thermomyces lanuginosus–SSBP	23.6	6.5	70–75	5–12	60	3.8	3.26	6300	Lin et al. 1999
Trichoderma harzianum	20	5	50	–	40	–	0.58	0.106	Tan et al. 1985
Trichoderma reesei	20, 19	5–5.5, 4–4.5	45, 40	3–8.5, 2.5–8.5	–	9, 5.5	3–6.8, 14.8–22.3	–	Tenkanen et al. 1992
Yeast									
Aureobasidium pullulans Y-2311–1	25	4.4	54	4.5	55	9.4	7.6	2650	Li et al. 1993
Cryptococcus albidus	48	5	25	–	–	–	5.7, 5.3	–	Morosoli et al. 1986
Trichosporon cutaneum SL409	–	6.5	50	4.5–8.5	50	–	–	–	Liu et al. 1998
Actinomycete									
Streptomyces sp. EC 10	32	7–8	60	–	–	6.8	3	–	Lumba and Pennickx 1992
Streptomyces sp. B–12–2	23.8–40.5	6–7	55–60	–	–	4.8–8.3	0.8–5.8	162–470	Elegir et al. 1994
Streptomyces T7	20	4.5–5.5	60	5	37–50	7.8	10	7610	Kesker 1992
Streptomyces thermoviolaceus OPC–520	33, 54	7	60–70	–	–	4.2, 8	–	–	Tsujibo et al. 1992
Streptomyces chattanoogensis CECT 3336	48	6	50	5–8	40–60	9	4, 0.3	78.2, 19.1	Lopez-Fernandez et al. 1998
Streptomyces viridisporus T7A	59	7–8	65–70	5–9	70	10.2–10.5	–	–	Magnuson and Crawford 1997
Streptomyces sp. QG-11-3	–	8.6	60	5.4–9.2	50–75	–	1.2	158.85	Beg et al. 2000a
Thermomonospora curvata	15–36	6.8–7.8	75	–	–	4.2–8.4	1.4–2.5	–	Stutzenberger and Bodine 1992

Double Homologous Recombination



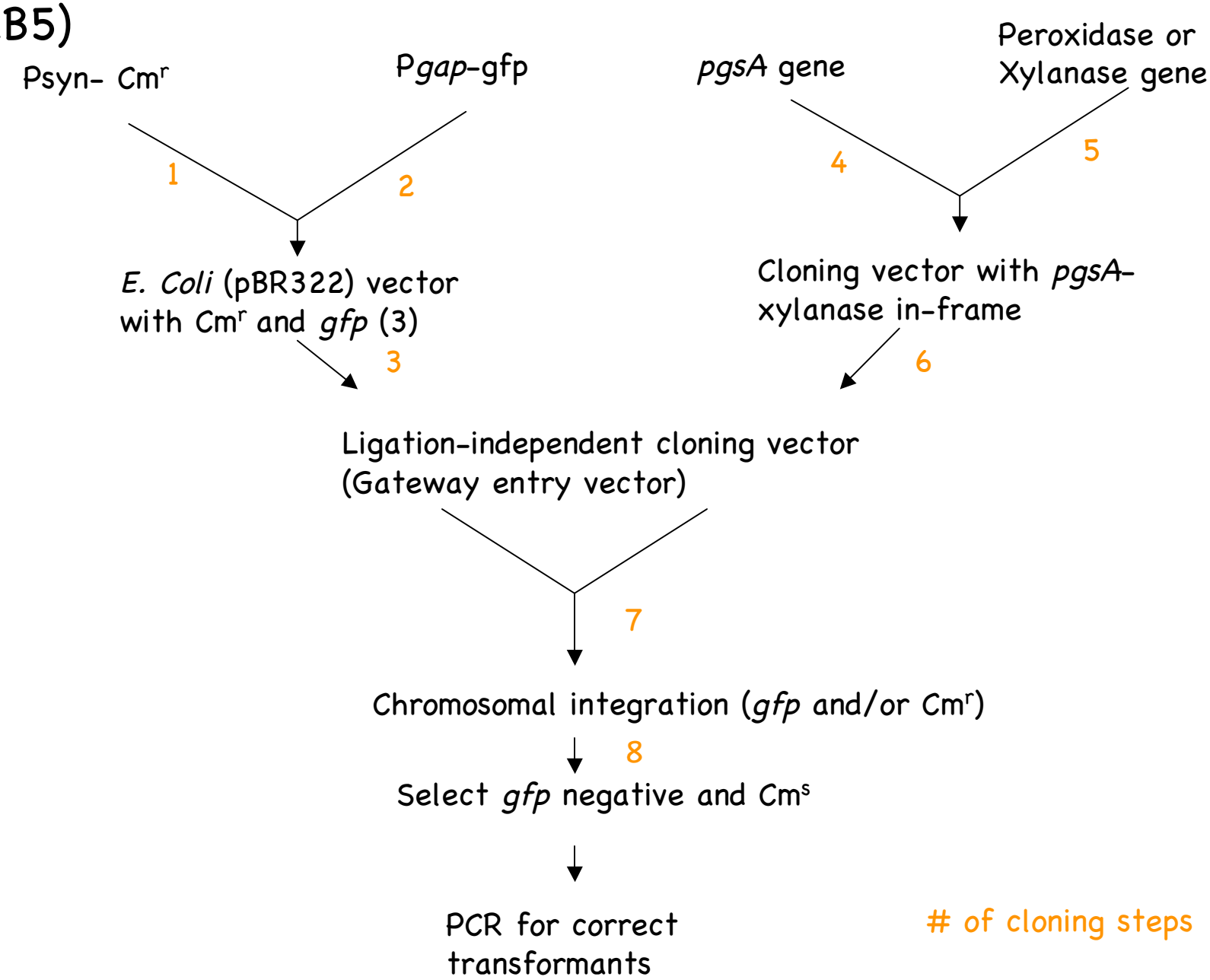
Negative selection makers considered:

- ✓ *sacB*: makes levan when grown on sucrose, resulting in cell death
  - ✓ Zymomonas has native *sacB*, *sacA*, *sacC*
  - ✓ Zymomonas can make levan
- ✓ *galK*: converts 2-Deoxy-galactose into product that cell cannot use
  - ✓ Zymomonas may not be able to take up galactose
  - ✓ Does this mechanism kill ZM?
  - ✓ Not sure whether *galM* from *E. coli* could uptake 2-DOG in ZM
- ✓ Are there other negative selection strategy that may work for ZM?

Positive selection strategy

- ✓ Fluorescence activated cell sorter (FACS) screening for *gfp* positive clones after the first HR
- ✓ FACS screen for *gfp* negative clones, PCR screen for correct transformant

Cloning strategies for chromosomal integration into ZM (pZB5)



Selection and Functionality Characterization

- ✓ Immunofluorescence staining of FLAG tagged surface expressed enzymes
  - ✓ FACS
  - ✓ microscopy
  - ✓ Western Blotting
- ✓ Functionality
  - ✓ Peroxidase activity - measure activity against hydrogen peroxide using luminescence reader for ELISA or spectrophotometric assays
  - ✓ Lignocellulose degradation - GCMS? microscopy?
  - ✓ Ethanol production - HPLC

Thank You

- Collaborators
  - Ping Hu (Andersen Lab)
  - Hazen Lab
  - Arkin Lab
  - M. Zhang (NREL)?

And yes it's reachable...

Questions about ZM functional genomics...

- *Zymomonas* promoters?
- *Zymomonas* PgsA structure? Is the C-terminus outside the cell wall?
- Are there other membrane proteins from ZM that can be used?
- Homologous recombination rate in ZM?
- Will expression of peroxidase or xylanase be toxic to ZM?