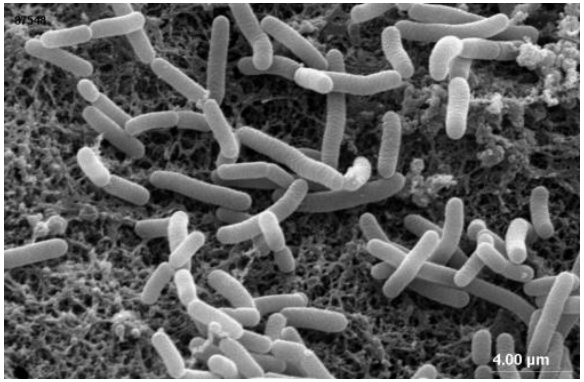
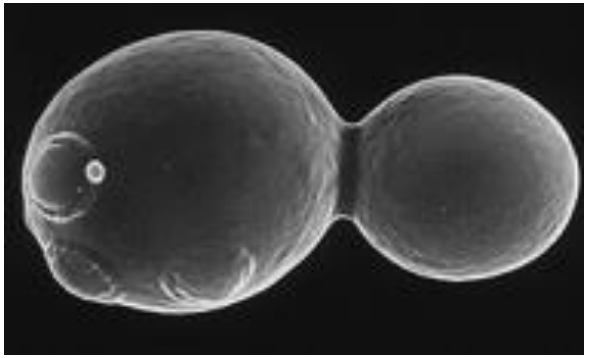
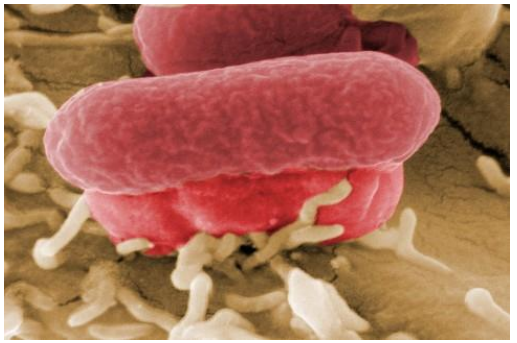
Techniques and –natural- solutions for strain optimization of lactic acid bacteria starter cultures

Dr. Wilbert Sybesma





Content



1. Introduction
2. Examples – Non GM approach
    1. Selection for more robust strains
    2. Selection for increased yield - acid tolerant strains
    3. Selection for increased yield – more energy efficient cells
    4. Selection for Low temperature yeast for Pizza
    5. Selection for “Sweety Yoghurt“
3. Screening and selection

4. Examples – GM approach
    1. Selection and screening for bacterial metabolite over producers
    2. Strain improvement via SS DNA recombineering
5. CRISPR –Technology and SS DNA recombineering

Strain improvement

The Science and technology for changing and improving microbial strains, in order to enhance their metabolic capacities for biotechnological applications

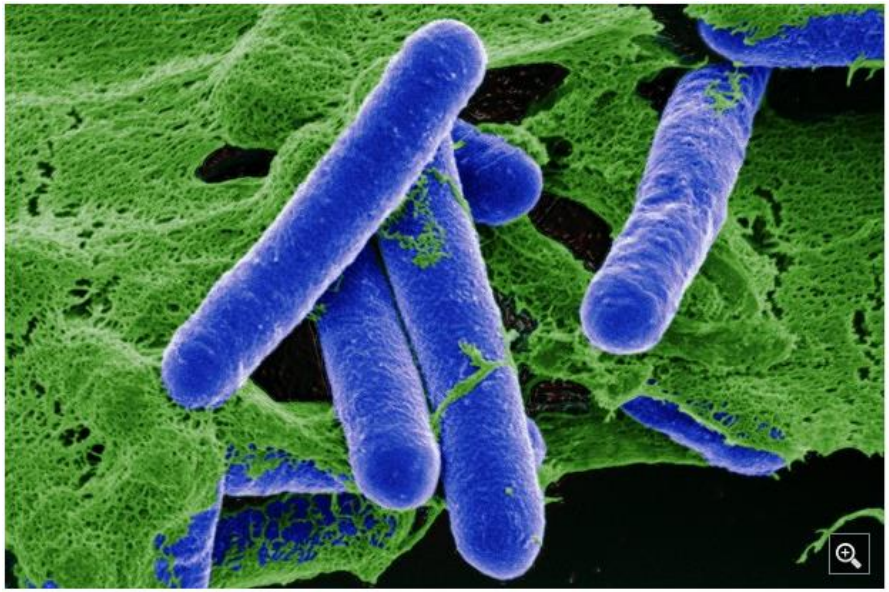
Targets of strain improvement

Targets of strain improvement

1. Rapid growth
2. Genetic stability
3. Non-toxicity to humans
4. Large cell size, for easy removal from the culture fluid
5. Ability to use cheaper substrates
6. Elimination of the production of compounds that may interfere with downstream processing
7. Increased yield and productivity

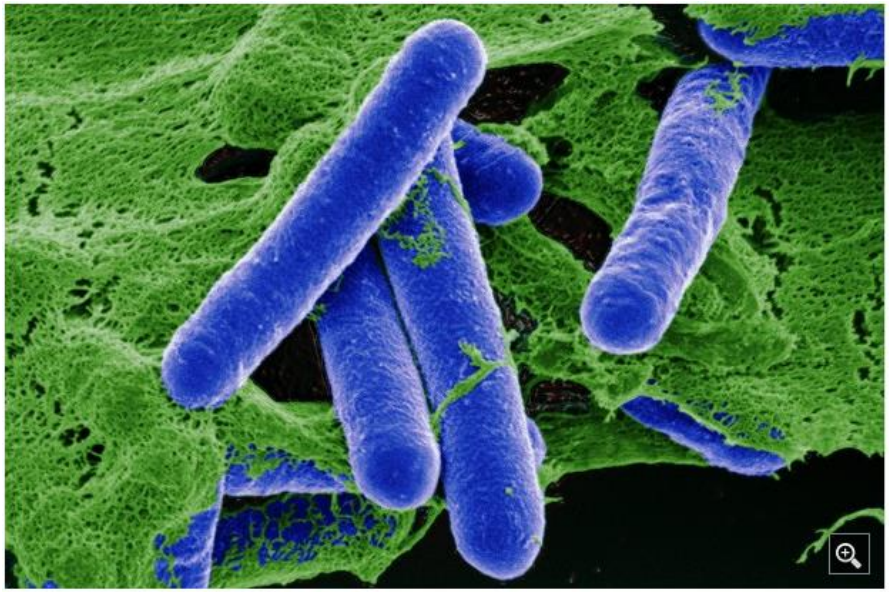
8. To improve the use of carbon and nitrogen sources
9. Production of
   - additional enzymes.
   - compounds to inhibit contaminant microorganisms.
10. Reduction of cultivation cost
    - lower price in nutrition
    - lower requirement for oxygen

Before strain improvement, optimize the growth conditions



Before strain improvement, optimize the growth conditions

- Modification of physical parameters (temperature, agitation, etc.)
- Modification of chemical parameter (pH, O_2 concentration)
- Optimization of nutrition of microorganisms
  - Carbon sources
  - Nitrogen sources
  - Minerals and vitamins
  - Precursors
  - Enzymes
- Apply sublethal stresses
- Find the right moment of harvesting



Random mutagenesis, Recombination, Screening and Selection

Not for exam

Mutagenesis

- Spontaneous mutations -Non GMO
- Induced mutation -Non GMO (addition, deletion, substitution, point)

Screening and Selection

- Directed/adaptive evolution
- Dominant selection
- Mechanical cell sorting

Site directed Mutagenesis -Non GMO/GMO

Methods involving Foreign DNA (Recombination)

- Transduction -Non GMO
- Conjugase -Non GMO
- Protoplast fusion -Non GMO
- Transformation -GMO
- Genetic engineering -GMO

Bacterial conjugation.

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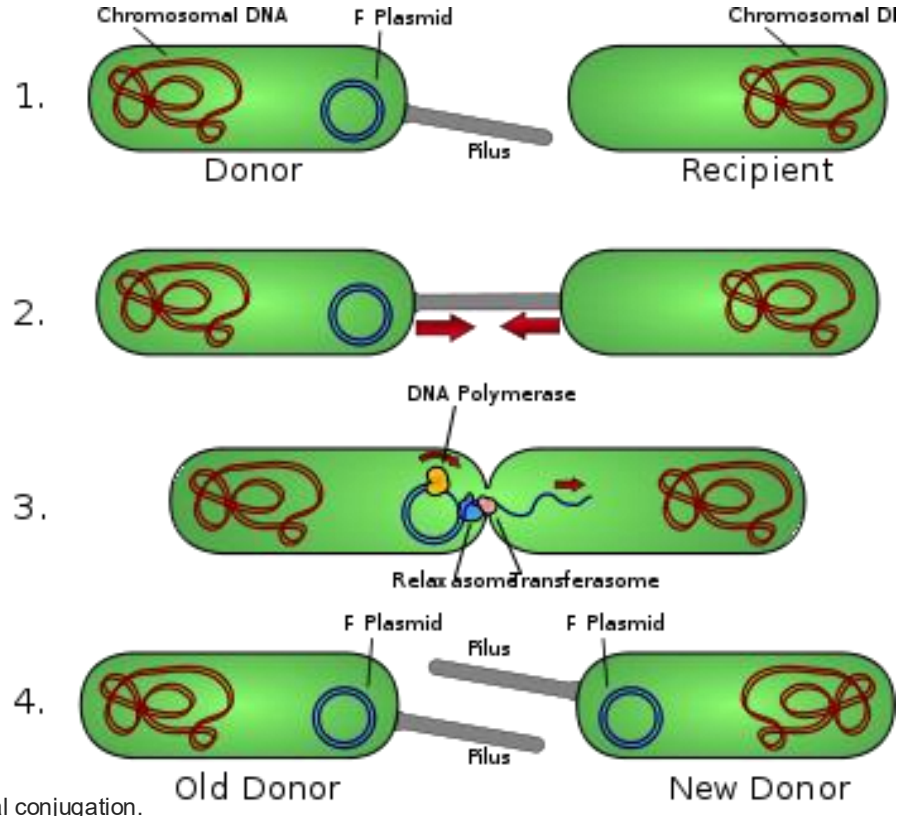
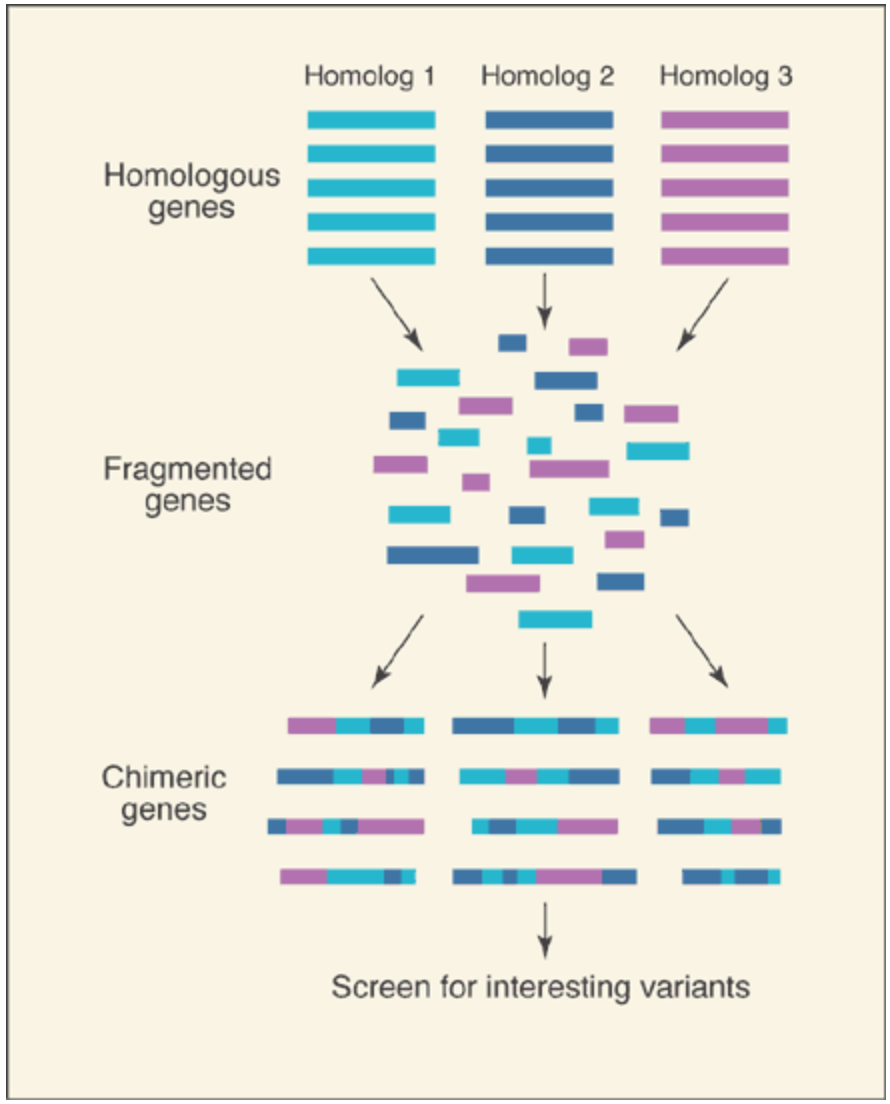


bacterial conjugation.

Application example.
J Appl Microbiol. 2002;92(2):238-46. Use of lacticin 481 to facilitate delivery of the bacteriophage resistance plasmid, pCBG104 to cheese starters. Mills S1, Coffey A, O'Sullivan L, Stokes D, Hill C, Fitzgerald GF, Ross RP.
https://onlinelibrary.wiley.com/doi/epdf/10.1046/j.1365-2672.2002.01527.x

Schematic drawing of bacterial conjugation. Conjugation diagram
1- Donor cell produces pilus.
2- Pilus attaches to recipient cell and brings the two cells together.
3- Mobile plasmid is nicked and a single strand of DNA is then transferred to the recipient cell.
4- Both cells synthesize a complementary strand to produce a double stranded circular plasmid and also reproduce pili; both cells are now viable donors.

Gene shuffling

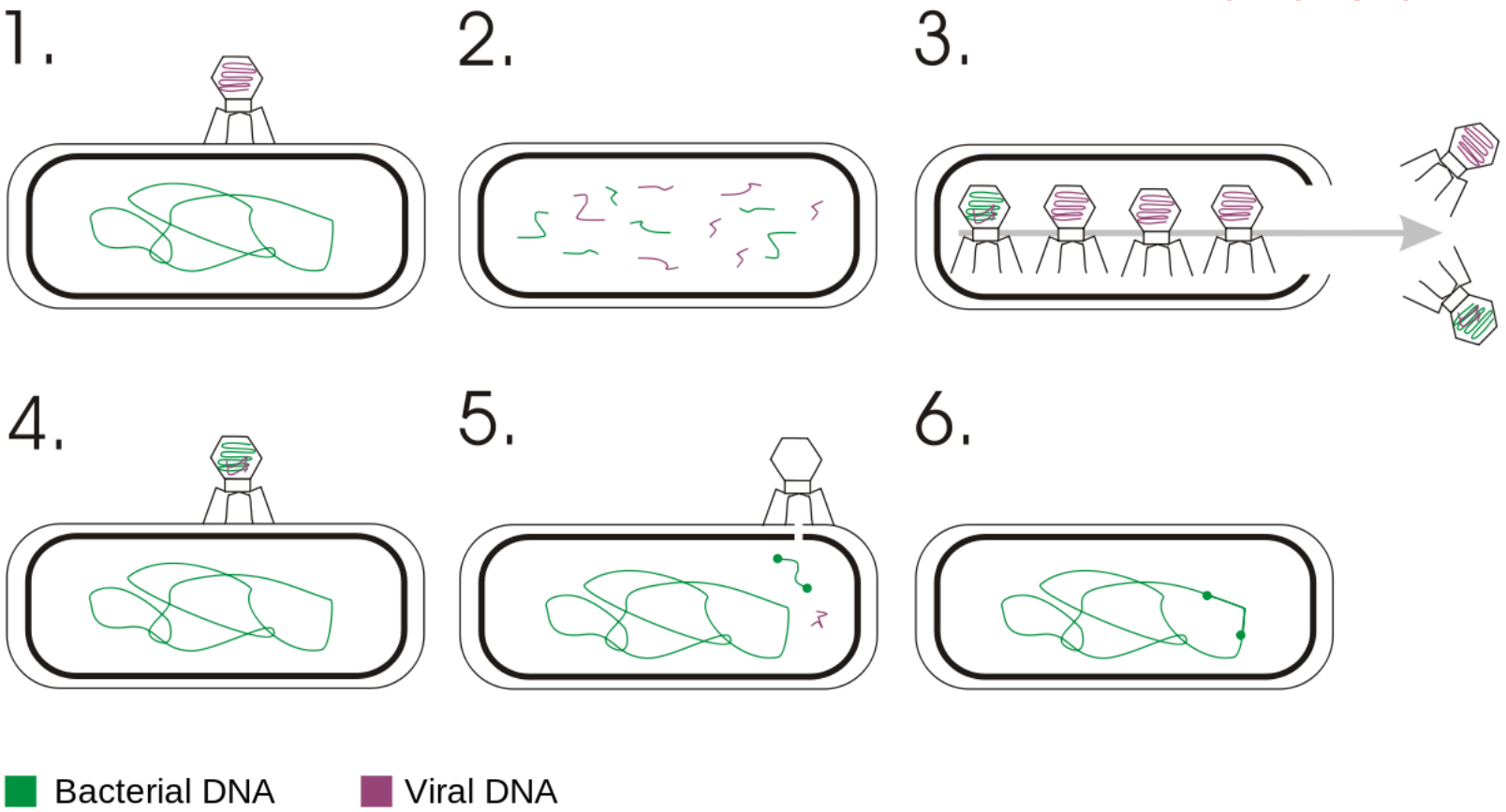


Not for exam

How DNA Shuffling Works. Jon Cohen. *Science* 13 Jul 2001: Vol. 293, Issue 5528, pp. 237. DOI: 10.1126/science.293.5528.237

Transduction

Not for exam



Conjugation and Transduction explained

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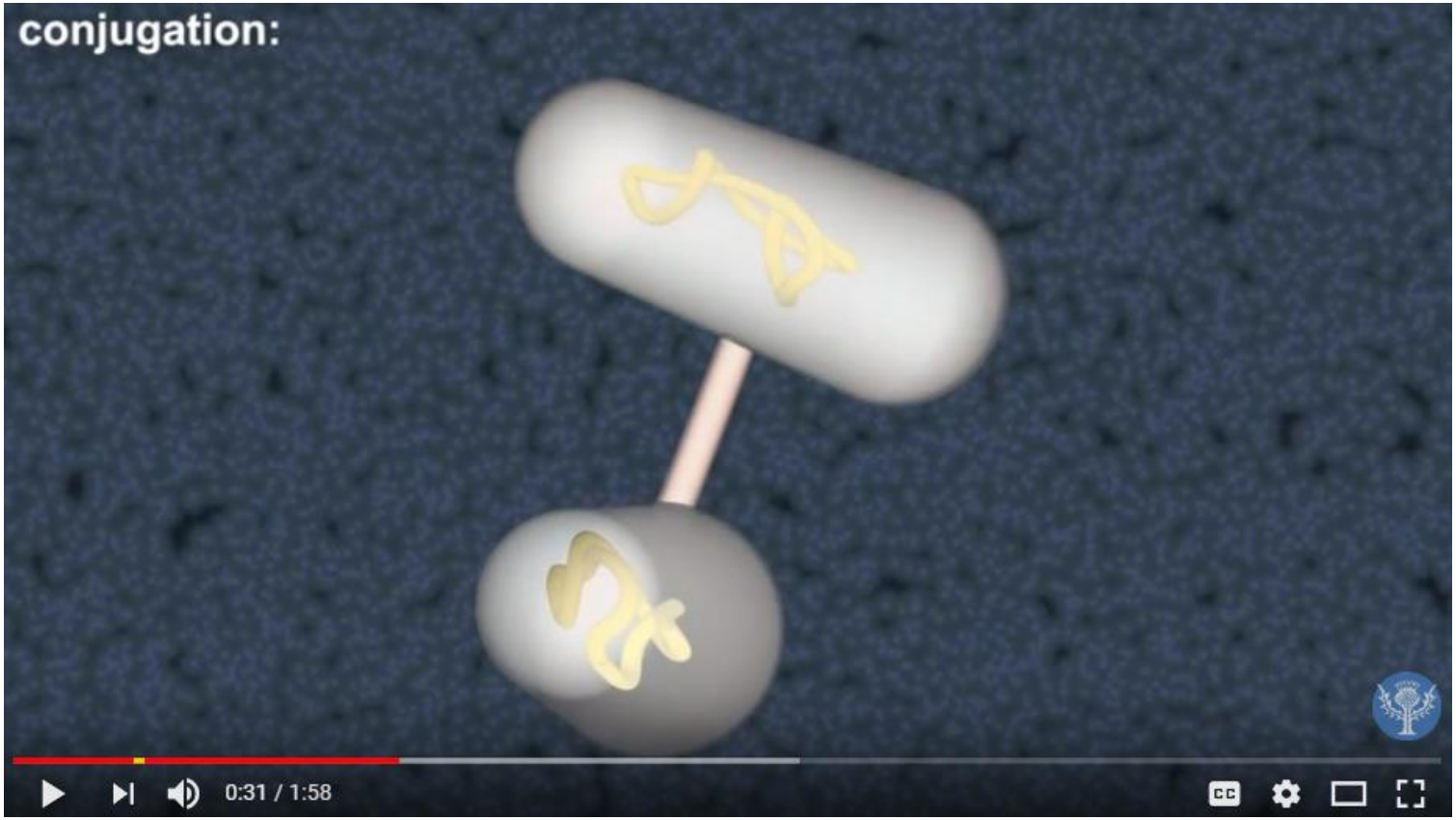
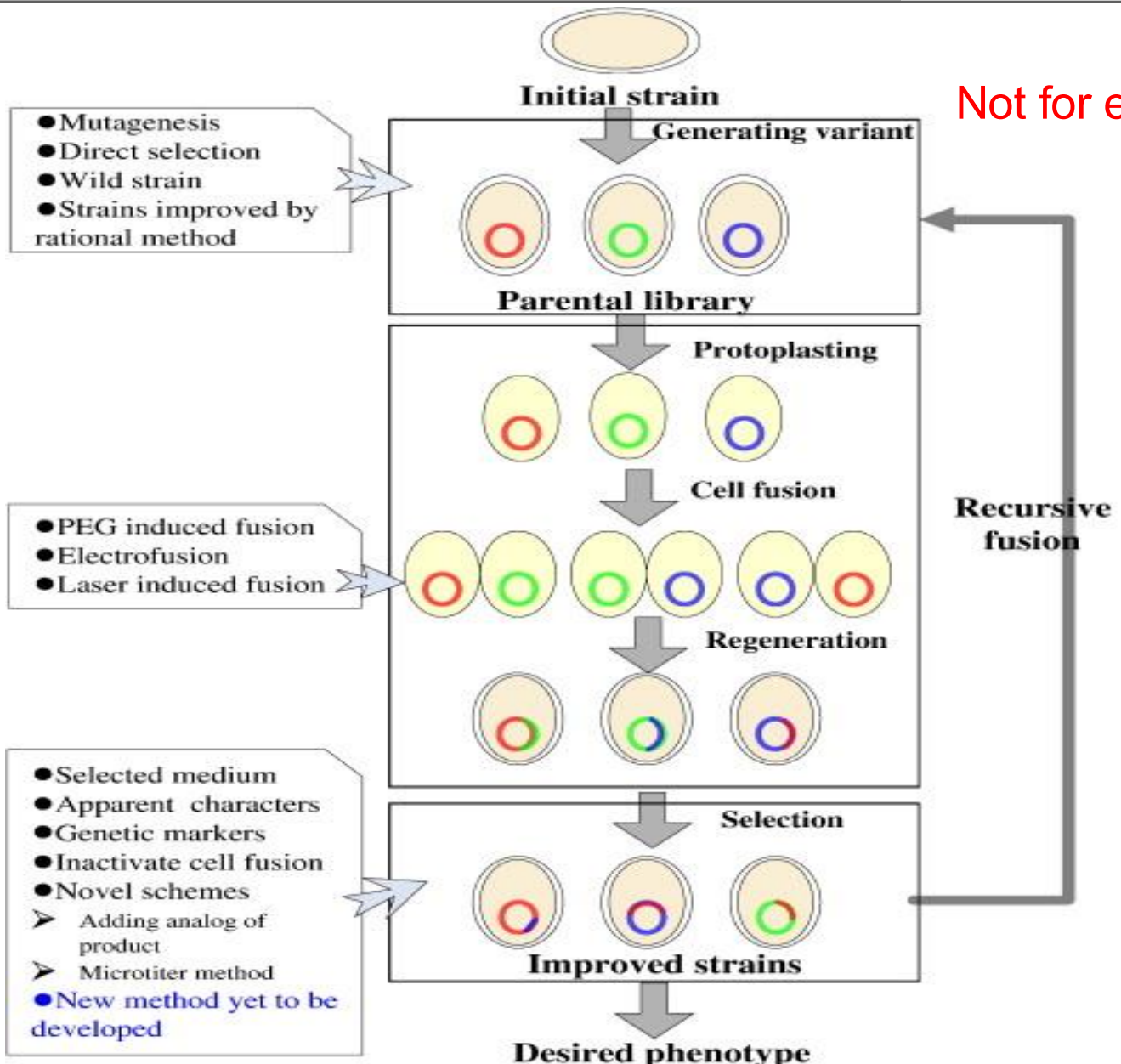


https://www.youtube.com/watch?v=7stZk6TesKk

Genome shuffling– by protoplast fusion



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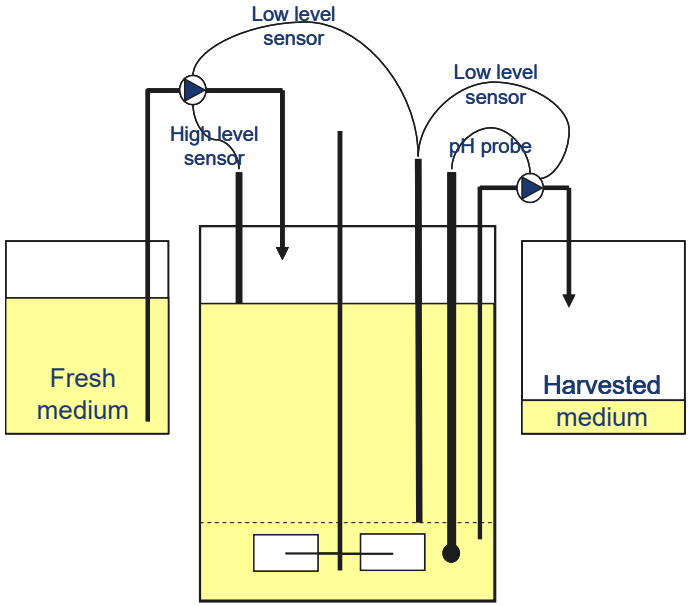
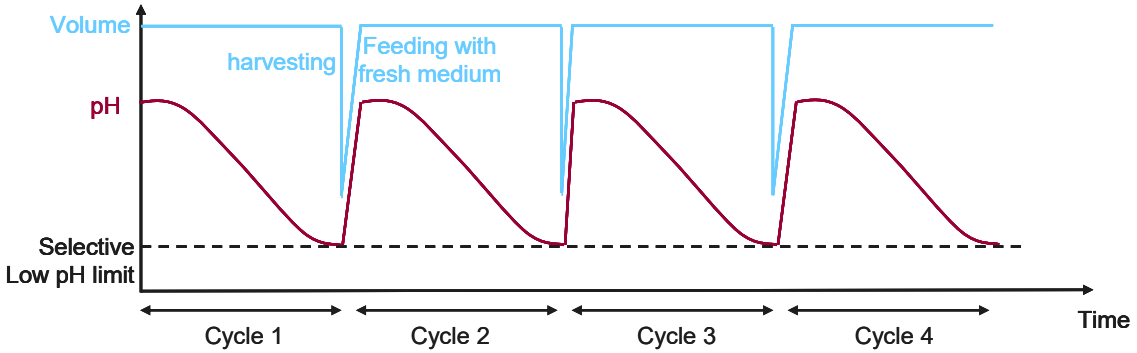
pH-Stat system - Selection of acid tolerant strain, to improve yield and stability

pH-Stat system = Succession of pH-limited batches

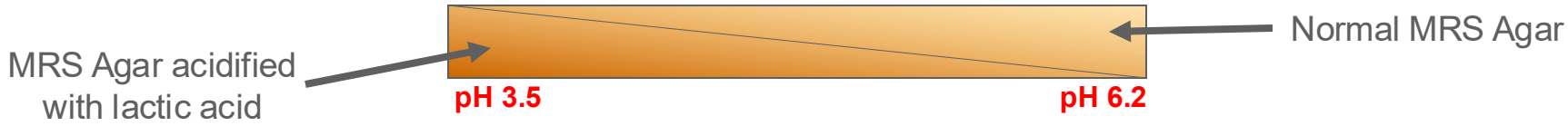


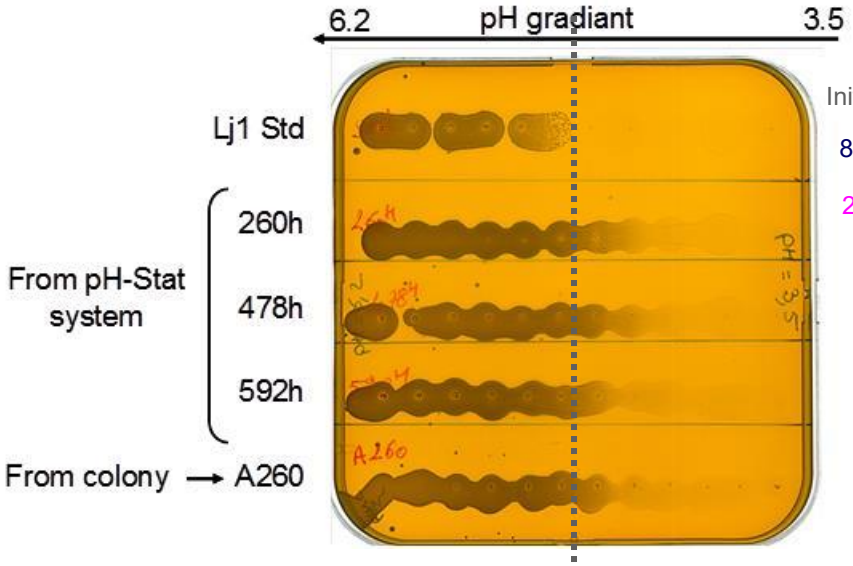


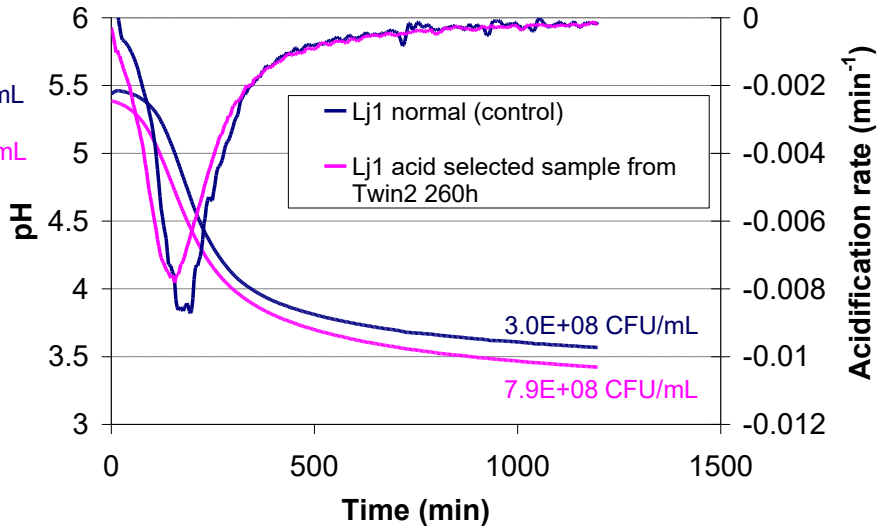
Select acid tolerant strain

Sybesma, Servaes unpublished results

Selection of an acid tolerant strain







Selected strains are able to grow at a lower pH than the WT *L. Johnsonii* resulting in more biomass

Sybesma, Servaes unpublished results

Examples genome shuffling. Increase of yield at low pH. 3 circles of fusion

Not for exam

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DOI: 10.5454/mi.5.1.4

Genome-Shuffling-Improved Acid Tolerance and Lactic Acid Production in *Lactobacillus plantarum* for Commercialization

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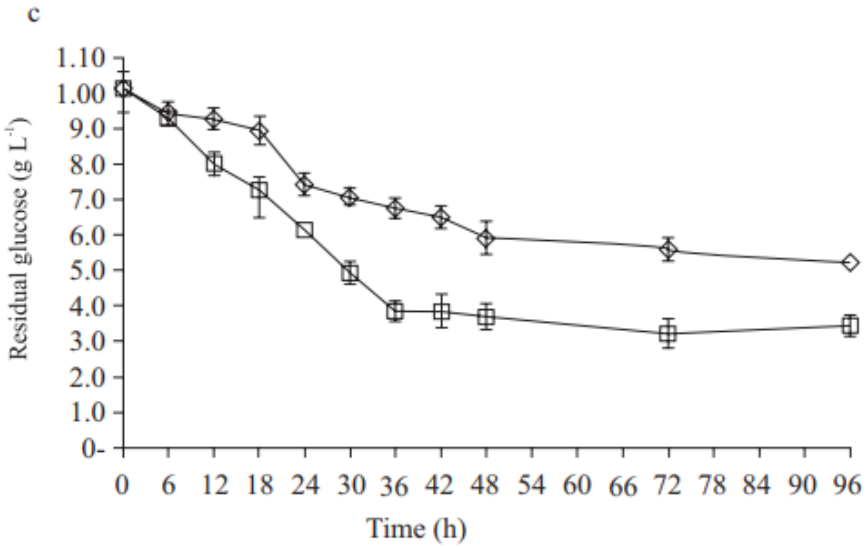


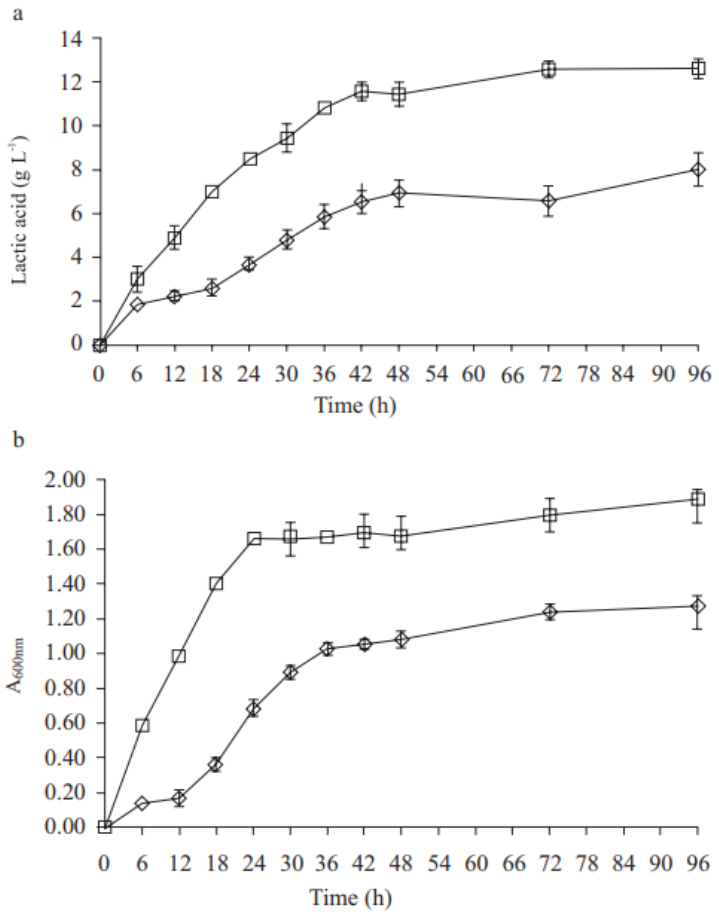


Fig 4 Characterization of lactic acid production: a, cell growth; b, and residual sugar (glucose) of fusant F3-33 compared to that of wild type (WT) *Lactobacillus plantarum*. ◇, WT; □, F3-33.

A general scheme of the genome shuffling process

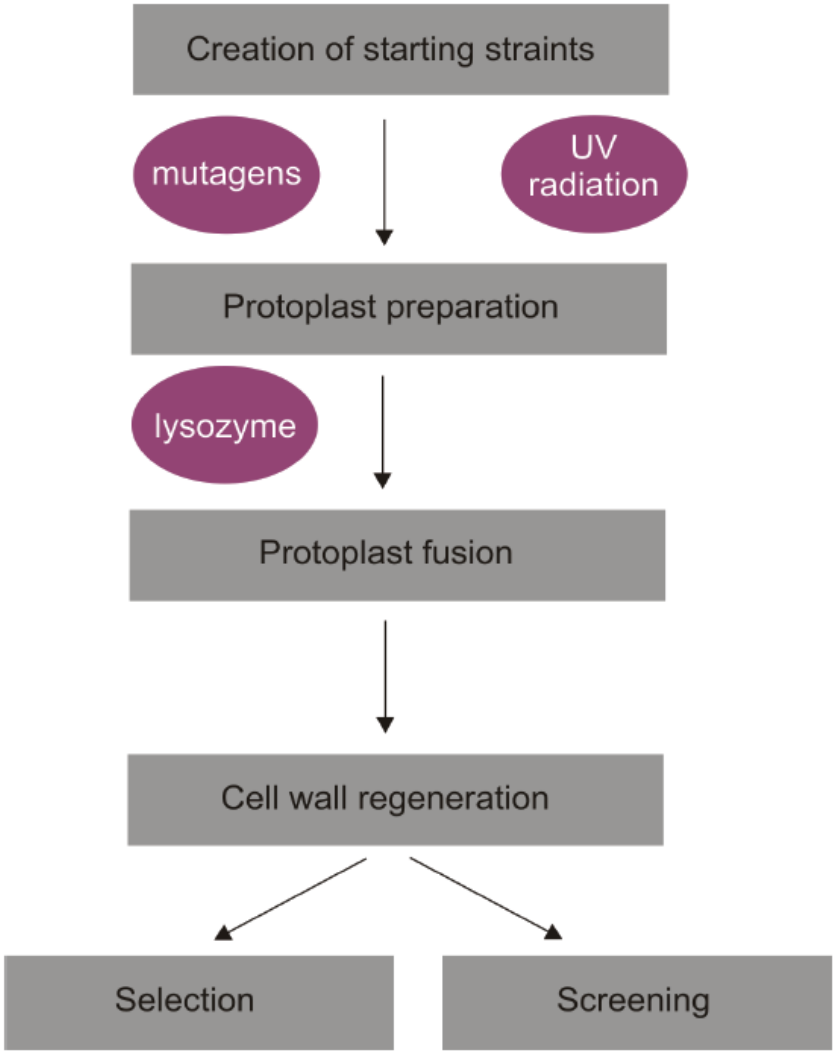


Fig. 1. A general scheme of the genome shuffling process

Not for exam

BioTechnologia vol. 92(4) C pp. 345-351 C 2011
Journal of Biotechnology, Computational Biology and Bionanotechnology

Spontaneous and induced mutations - Choice of mutagen

1. Spontaneous mutation: Occur spontaneously at the rate of ~>10^{-7} per generation.

2. Induced mutation: Mutagenic agents are numerous but not necessarily equally effective in all organisms.

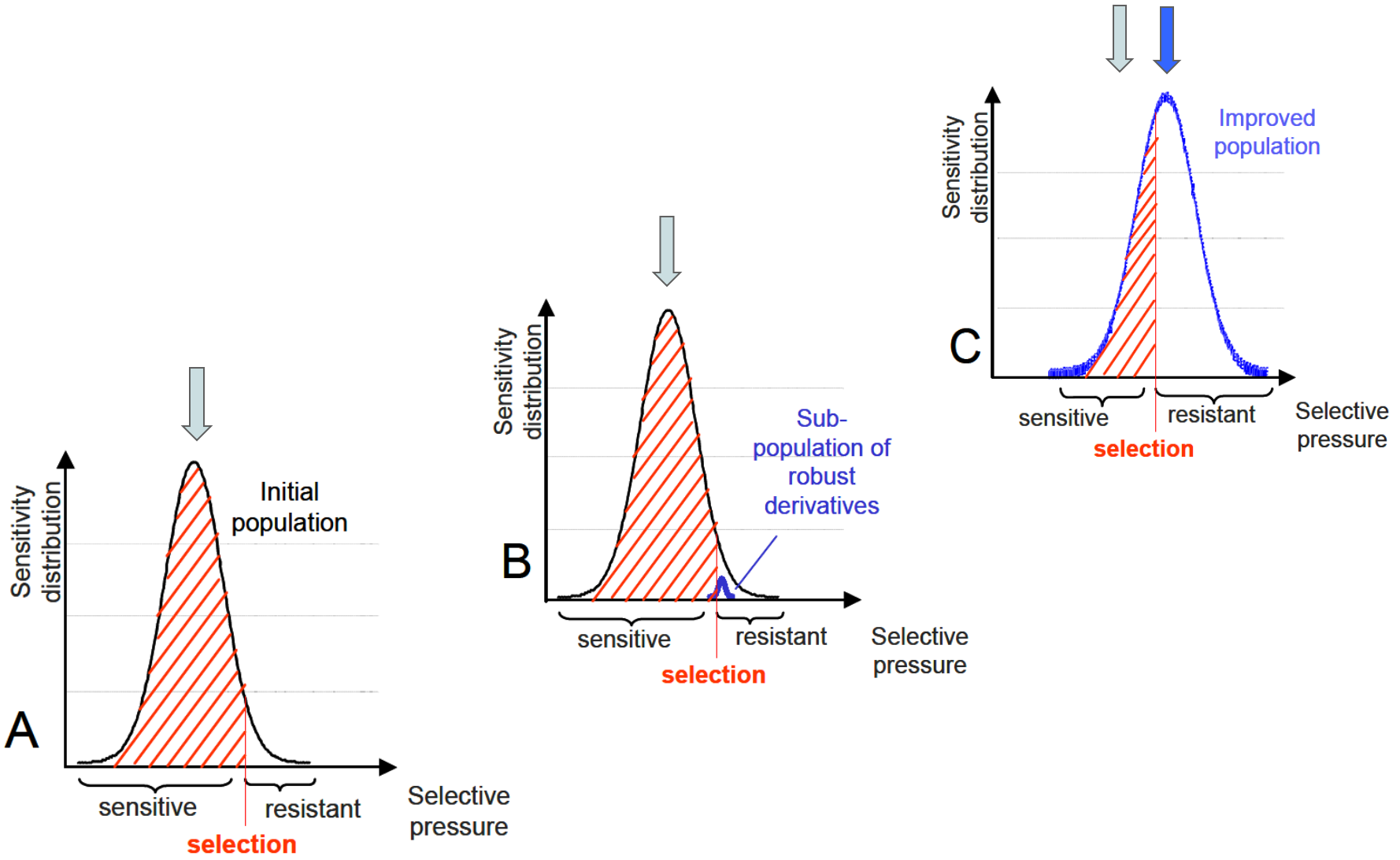
- Ionizing Radiations-X Rays, gamma rays UV rays,
- chemicals
- Hydroxylamine(NH2OH
- N-Methyl –N'-Nitro N-Nitrosoguanidine
- Nitrous acid(HNO2)
- Ethylmethane-sulfonate

Other factors to consider

- *the safety of the mutagen*
- *simplicity of technique*
- *availability of the necessary equipment and chemicals rays*

→ *There is a risk of accumulation of unintended mutations*

Example: Directed evolution and natural selection of “resistant” strain derivatives

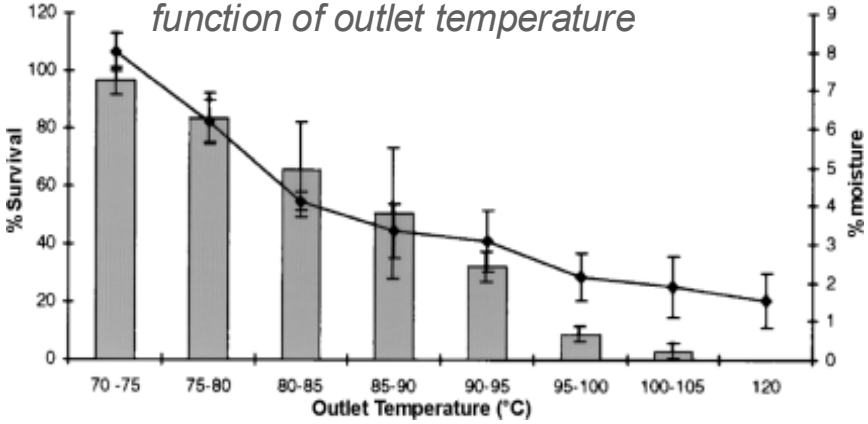


B. Berger, et al. 2010, J. Bact

The main challenge is to keep the probiotics viable during processing and storage

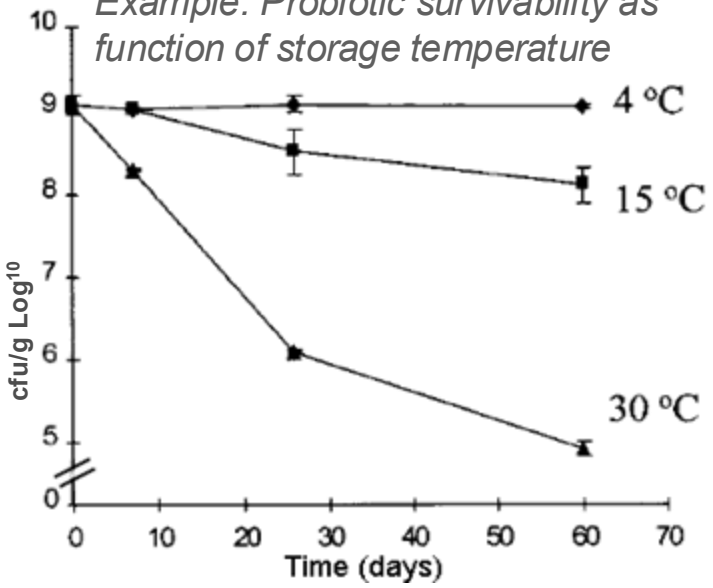
- Production
  - Survivability
  - Extreme temperature changes
  - Osmotic pressure changes
  - Oxygen stress

Example: Probiotic survivability as function of outlet temperature



- Consumption
  - Storage stability
  - Remain viable during gastric transit
  - pH stress

Example: Probiotic survivability as function of storage temperature



I. Jankovic (2010), Ananta *et al.* (2005), R.P. Ross *et al.* (2005), C. Stanton *et al.* (2003), T. Mattila-Sandholm (2002), G.E. Gardiner *et al* (2000).

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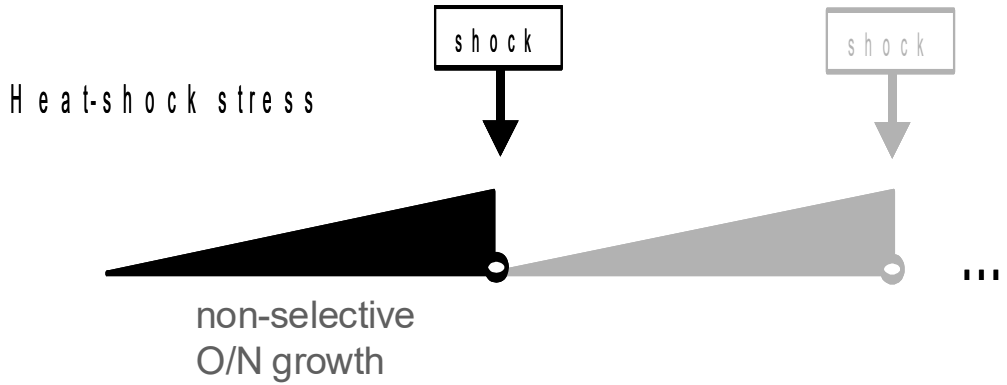
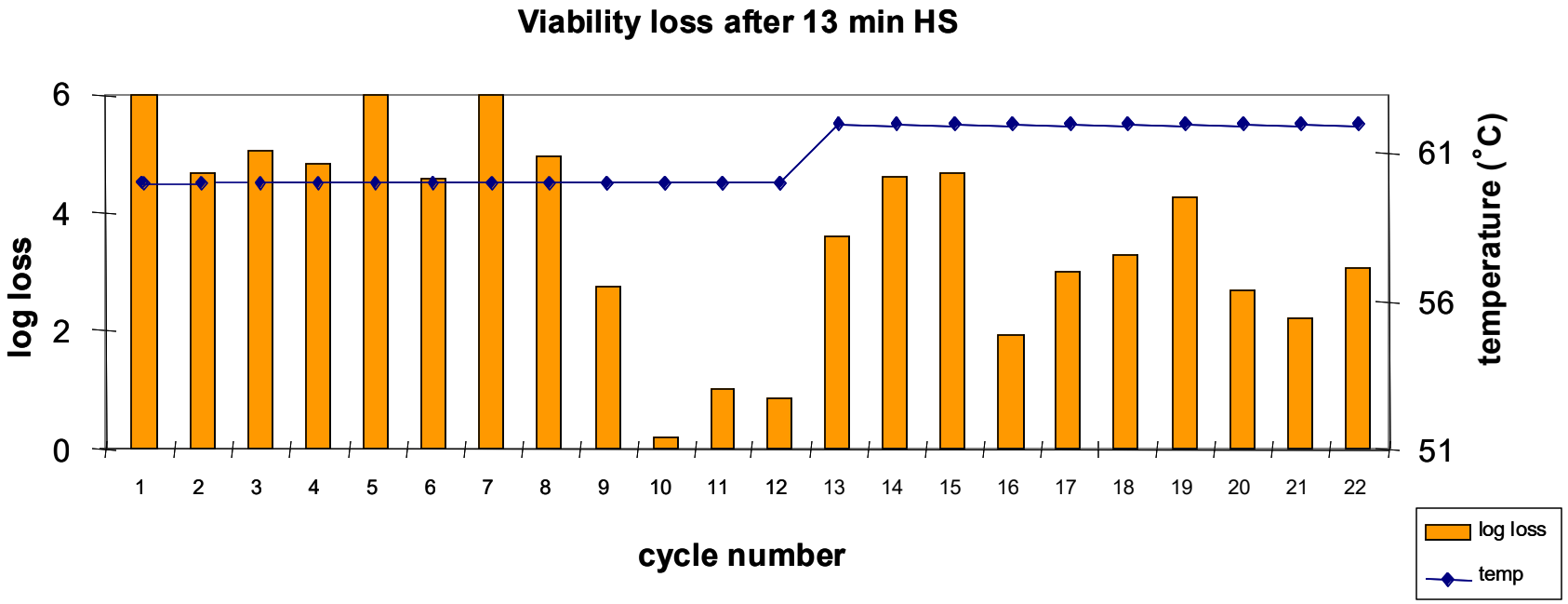
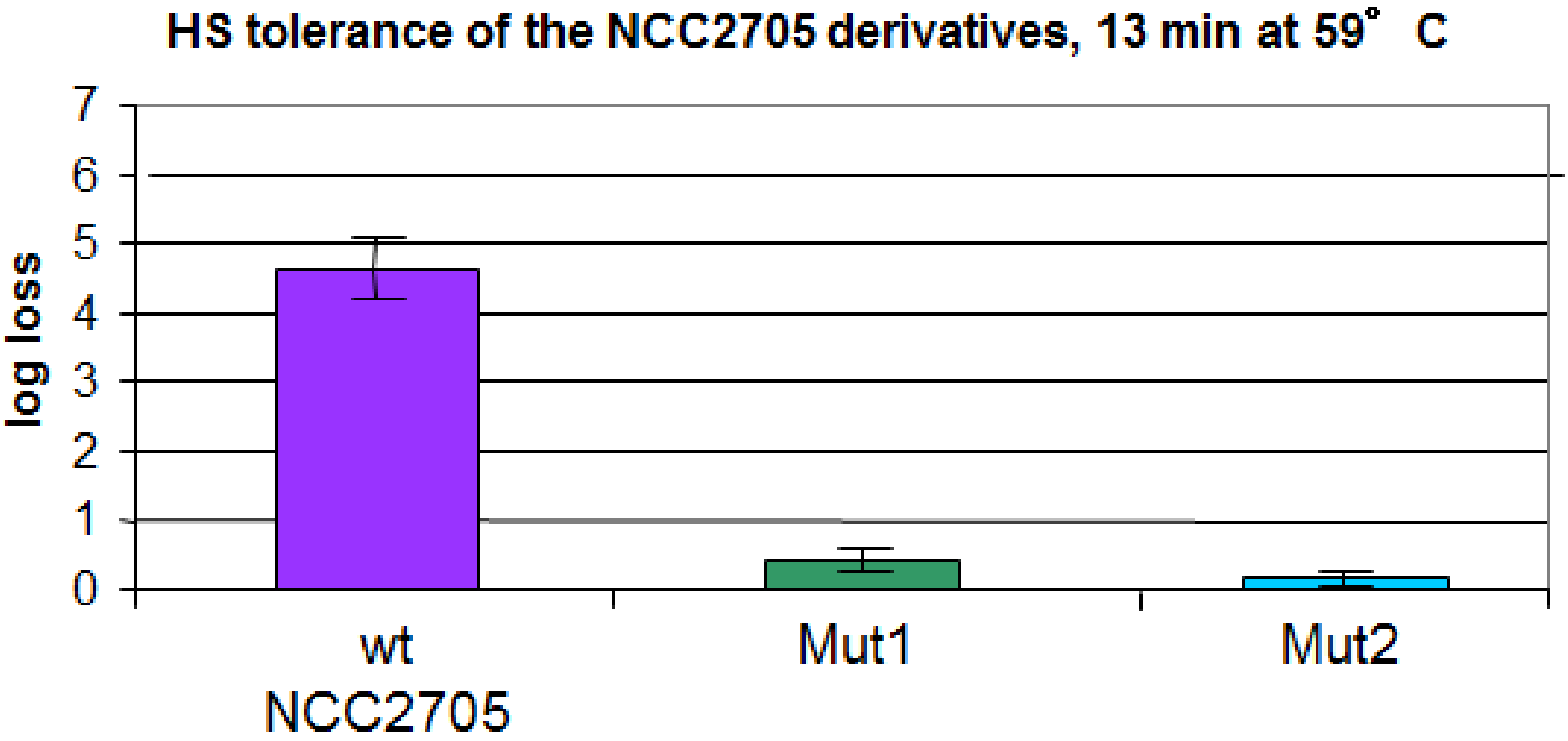
Cyclic selection of naturally occurring Heat-Shock tolerant mutants





B. Berger, et al. 2010, J. Bact

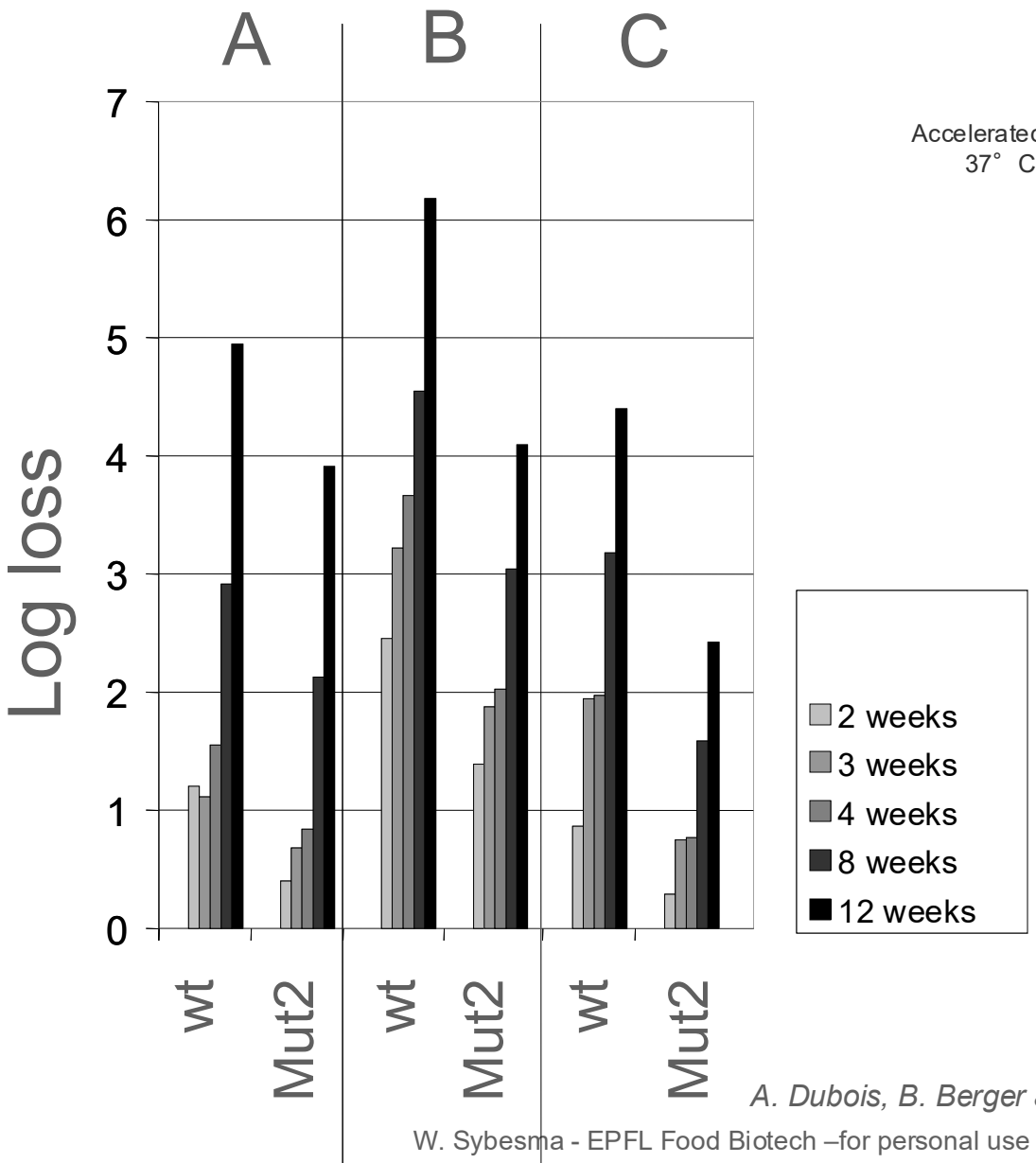
Isolation of two independent *B. longum* Heat-Shock tolerant mutants



Mut1 and Mut2 were isolated from separate batches.

B. Berger, et al. 2010, J. Bact

Heat resistant mutants are more stable after spray-drying and storage compared to WT(N=3)

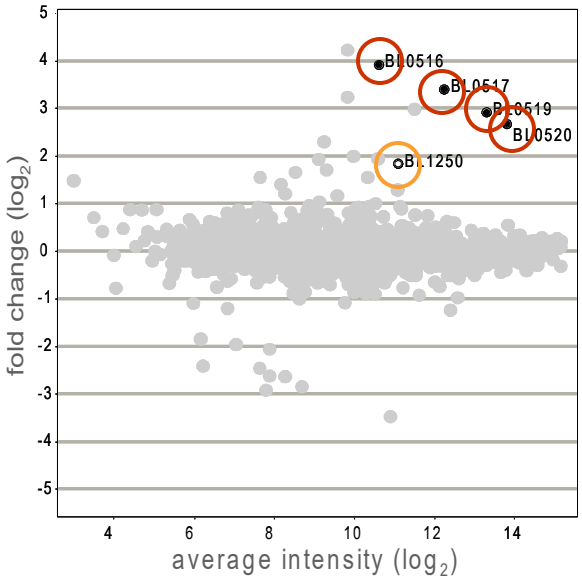


Accelerated Storage Test
37° C, Aw 0.22

A. Dubois, B. Berger and W. Sybesma (unpublished)

Heat-Shock tolerant mutants have constitutive expression of “stress genes”

Mut / wt

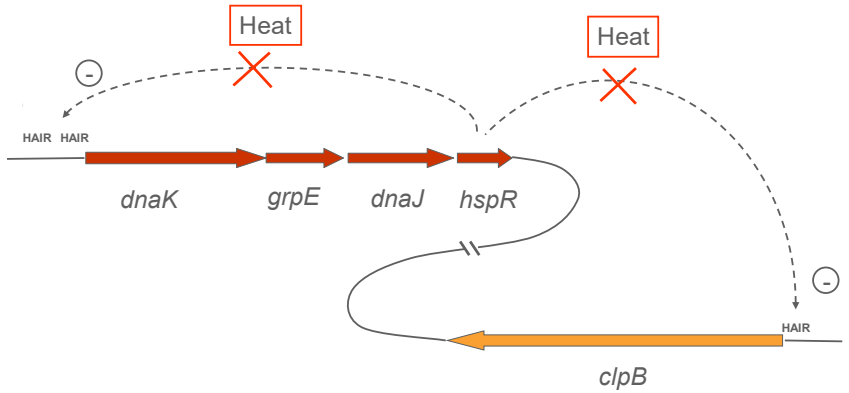


dnaK operon

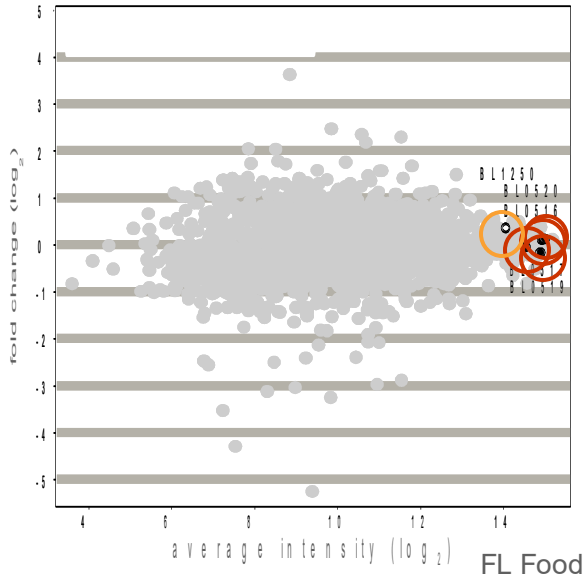
*clp*B

Just for illustration, no need to study

before HS



c/o *Str. coelicolor* (Bucca et al., 1997, 2000, 2003)



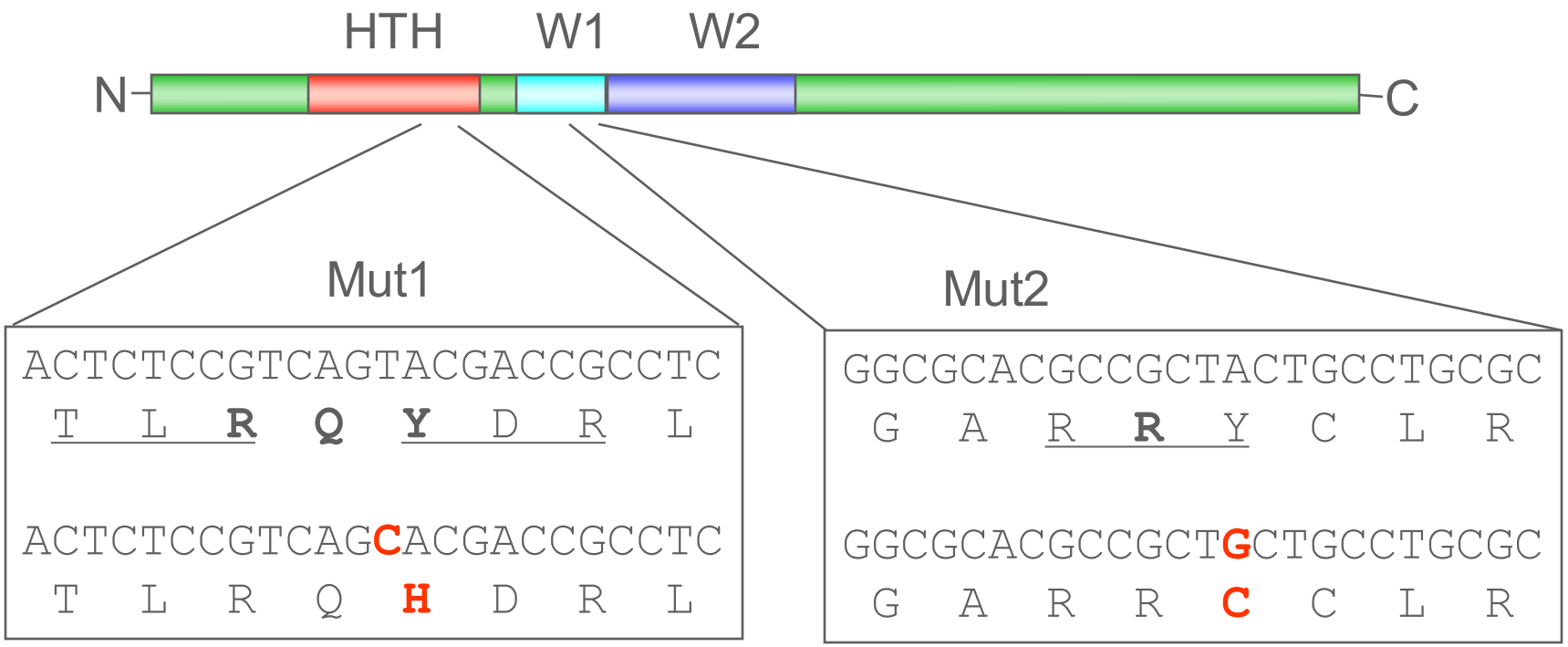
7 min
HS 50°

Possible explanation of improved heat tolerance:
Mutation on a potential dnaK-clpB regulator

Berger et al, J. Bacteriol. 2010

HspR, the *dnaK-clpB* regulator, is mutated in potential DNA-binding motifs

Just for illustration, no need to study

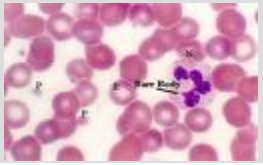


HTH : helix-turn-helix; W : winged helix
underlined : conserved in Actinobacteridae
bold : interaction with DNA molecule
red : mutation

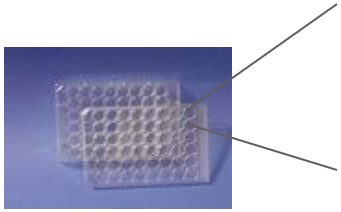
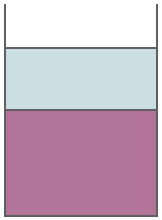
B. Berger, et al. 2010, J. Bact

Confirming functionality by Peripheral Blood Mononuclear Cell Assay

Human blood → Peripheral Blood Mononuclear Cells (PBMC) → Samples (36h) → Cytokines IL-10, TNF-α, IFN-γ

→ Allows to investigate the effect of bacterial strains on immune blood cells

50µl samples containing $5x10^6$ bacteria (10^8 cfu/ml)

450µl blood cells ($6.7x10^5$ cells)

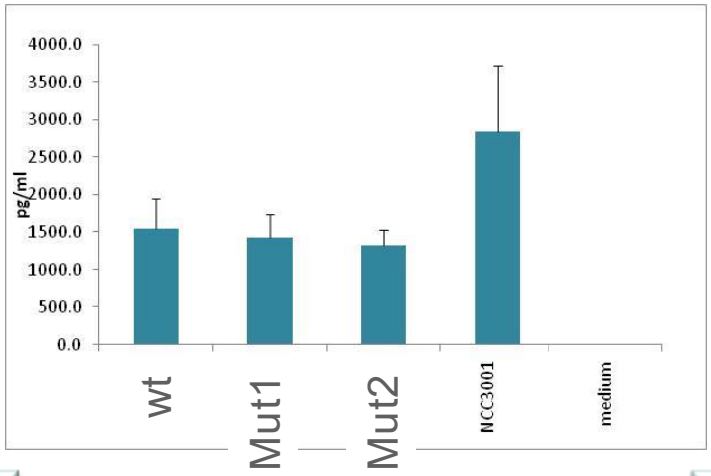
Optimal ratio: ~ 8 bacteria / human cell

Blood from 4 donors at least

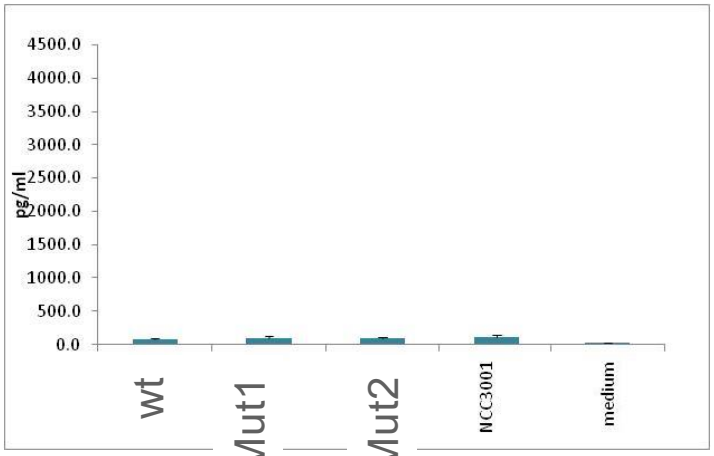
Just for illustration, no need to study

No difference between the mutants and the wt strain on PBMC assay

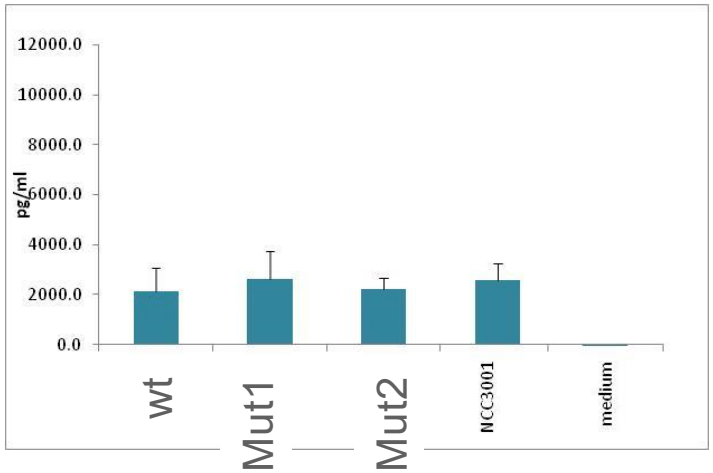
IL-10



IFN-g



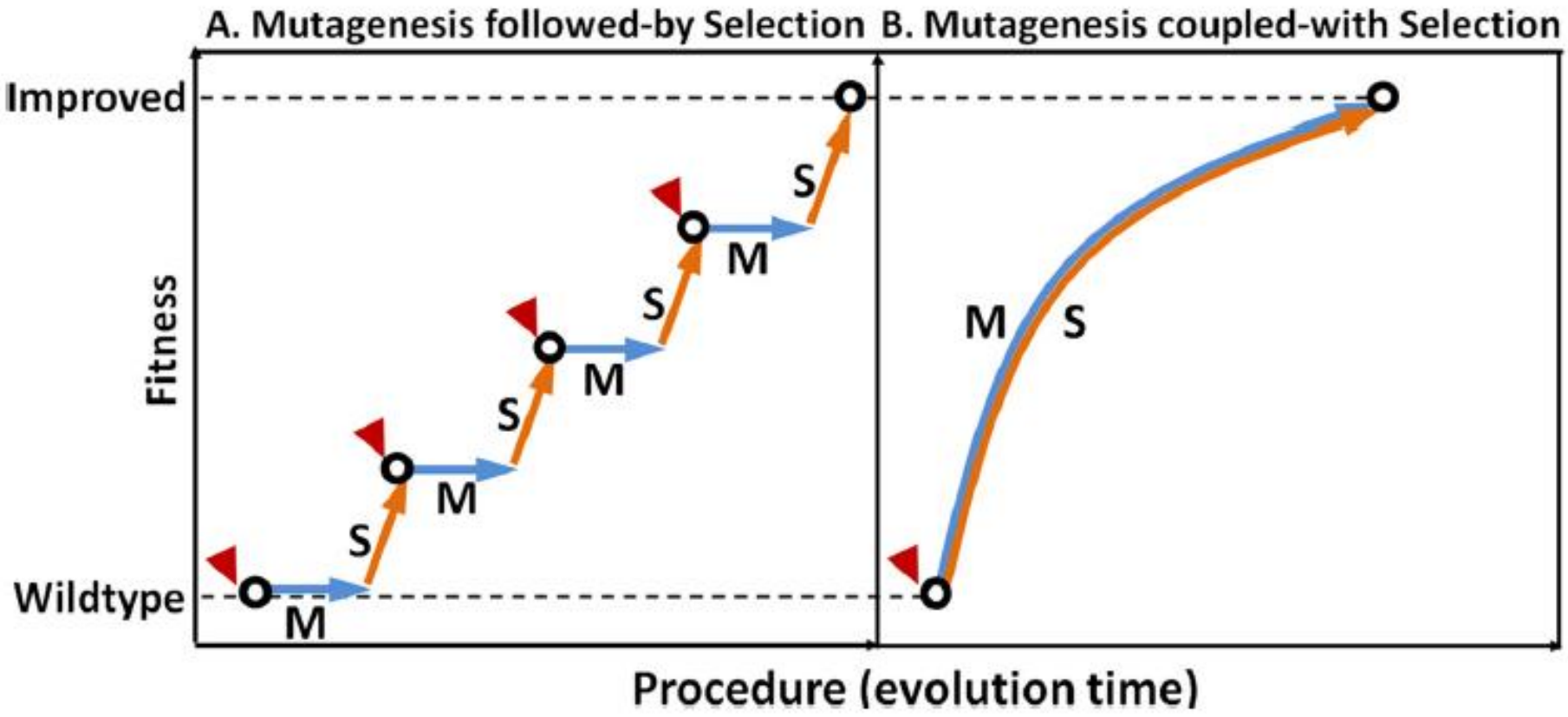
TNF-a



G. Prioult, A.Demont, and A. Mercenier (unpublished)

GREACE: Genome replication engineering assisted continuous evolution

Not for exam

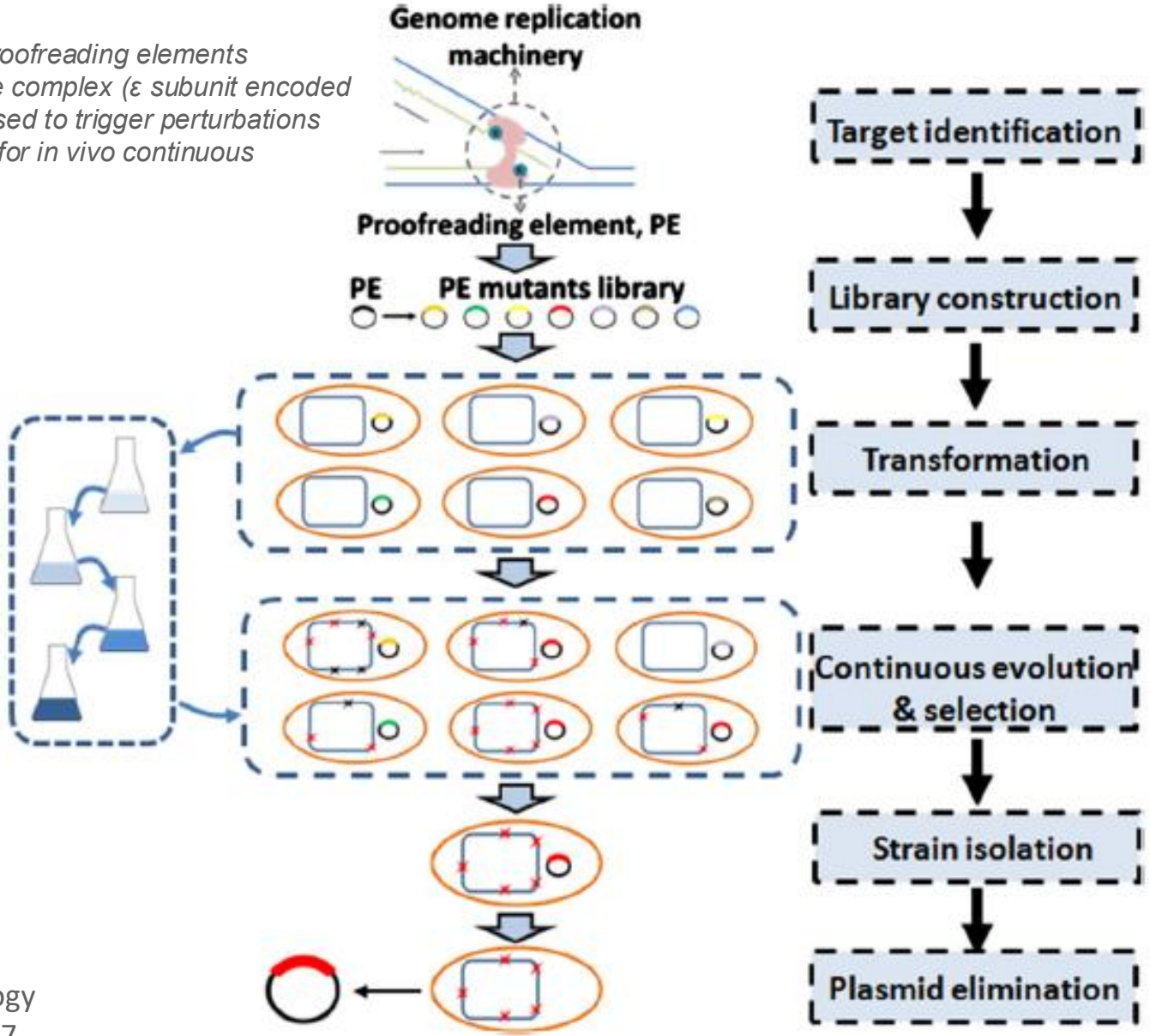


Luan et al. Biotechnology for Biofuels 2013, 6:137

GREACE: Genome replication engineering assisted continuous evolution

Not for exam

Genetically modified proofreading elements of the DNA polymerase complex (ε subunit encoded by dnaQ gene) were used to trigger perturbations on genome replication for in vivo continuous mutagenesis.



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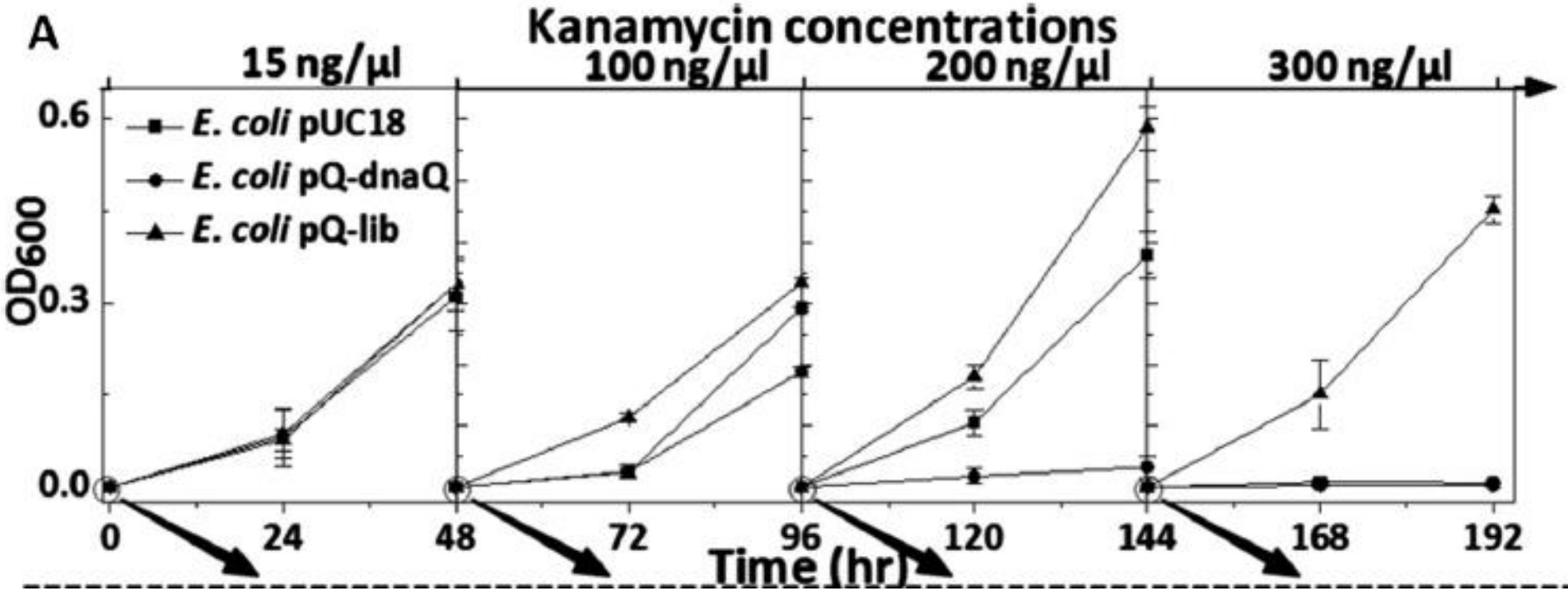
Engineering kanamycin resistance of E. coli with GREACE

Not for exam



Luan et al. Biotechnology for Biofuels 2013, 6:137

Chilled Pizza Dough and Baker's Yeast
The Problem and the Solution

The Problem:
Too high activity even under refrigeration

The solution:
Very little activity under refrigeration but normal activity at higher temperatures

A need for Low Temperature Inactive (LTI) Baker's Yeast

Requirements:

"Wild" Baker's yeast"
(Saccharomyces cerevisiae)

- **Activity** – to guarantee an open dough texture a residual yeast activity during storage or immediately prior to baking must remain.
- **Consumer acceptance**– the new baker's yeast variety had to be obtained purely by classical strain development methods.

Yeast producers → **Nestlé Research**

Selection für strong CO_2 development

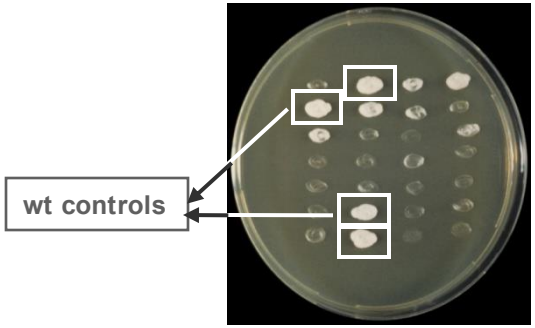
Selection for strongly reduced CO_2 development under refrigeration

"Ordinary" Baker's yeast varieties

LTI Baker's yeast variety

Strain construction and selection

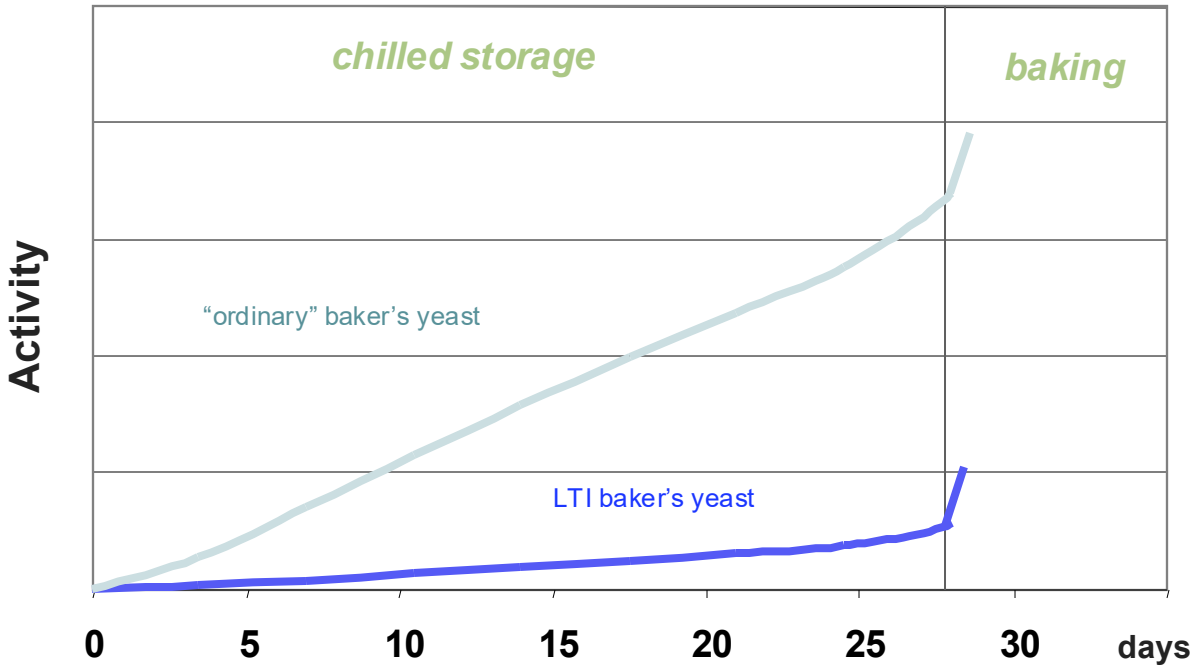
1) EMS mutagenesis and selection for desired phenotype:



28d 8° C, 1d 30C

4 weeks survival under refrigeration

Original strain : **YH-G3**
(a arg4-17 his4-38 lys1-1 met8-1 trp1-1)



Example: multi step classical strain improvement for sweety yoghurt

problem:

yoghurt is considered a healthy natural product; but often contains added sucrose to boost sweetness

CHR HANSEN

Improving food & health

How to make a natural sweet yoghurt?

1. Yoghurt starter cultures typically contain 90% *S. thermophilus* and 10% *Lb. delbrueckii* subsp. *bulgaricus*

2. *S. thermophilus* grown on lactose, metabolize galactose and excrete glucose

3. Enabling & boosting the fermentation of galactose by *S. thermophilus*

4. Dominant selection using galactose as sole carbon source

Result

1. *G* to *A* mutation at -10 position in the *galK* promoter results in a 2.5 to 3.7 fold increase of transcription of the genes from the galactose operon (*galK, galT, galE, and galM*)

AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Enhancing the Sweetness of Yoghurt through Metabolic Remodeling of Carbohydrate Metabolism in *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

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1. Select for growth on galactose

Citation Sørensen KI, Curic-Bawden M, Junge MP, Janzen T, Johansen E. 2016. Enhancing the sweetness of yoghurt through metabolic remodeling of carbohydrate metabolism in *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. Appl Environ Microbiol 82:3683–3692. doi:10.1128/AEM.00462-16.

Relevant Summary

ABSTRACT

- *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are used in the fermentation of milk to produce yoghurt. These species normally metabolize only the glucose moiety of lactose, secreting galactose and producing lactic acid as the main metabolic end product. We used multiple serial selection steps to isolate spontaneous mutants of industrial strains of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* that secreted glucose rather than galactose when utilizing lactose as a carbon source. Sequencing revealed that the *S. thermophilus* strains had mutations in the *galKTEM* promoter, the glucokinase gene, and genes encoding elements of the glucose/mannose phosphotransferase system (PTS). These strains metabolize galactose but are unable to phosphorylate glucose internally or via the PTS. The *L. delbrueckii* subsp. *bulgaricus* mutants had mutations in genes of the glucose/mannose PTS and in the pyruvate kinase gene. These strains cannot grow on exogenous glucose but are proficient at metabolizing internal glucose released from lactose by β-galactosidase. The resulting strains can be combined to ferment milk, producing yoghurt with no detectable lactose, moderate levels of galactose, and high levels of glucose. Since glucose tastes considerably sweeter than either lactose or galactose, the sweetness of the yoghurt is perceptibly enhanced. These strains were produced without the use of recombinant DNA technology and can be used for the industrial production of yoghurt with enhanced intrinsic sweetness and low residual levels of lactose.

- **IMPORTANCE** Based on a good understanding of the physiology of the lactic acid bacteria *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, we were able, by selecting spontaneously occurring mutants, to change dramatically the metabolic products secreted into the growth medium. These mutants consume substantially more of the lactose, metabolize some of the galactose, and secrete the remaining galactose and most of the glucose back into the milk. This allows production of yoghurt with very low lactose levels and enhanced natural sweetness, because humans perceive glucose as sweeter than either lactose or galactose.

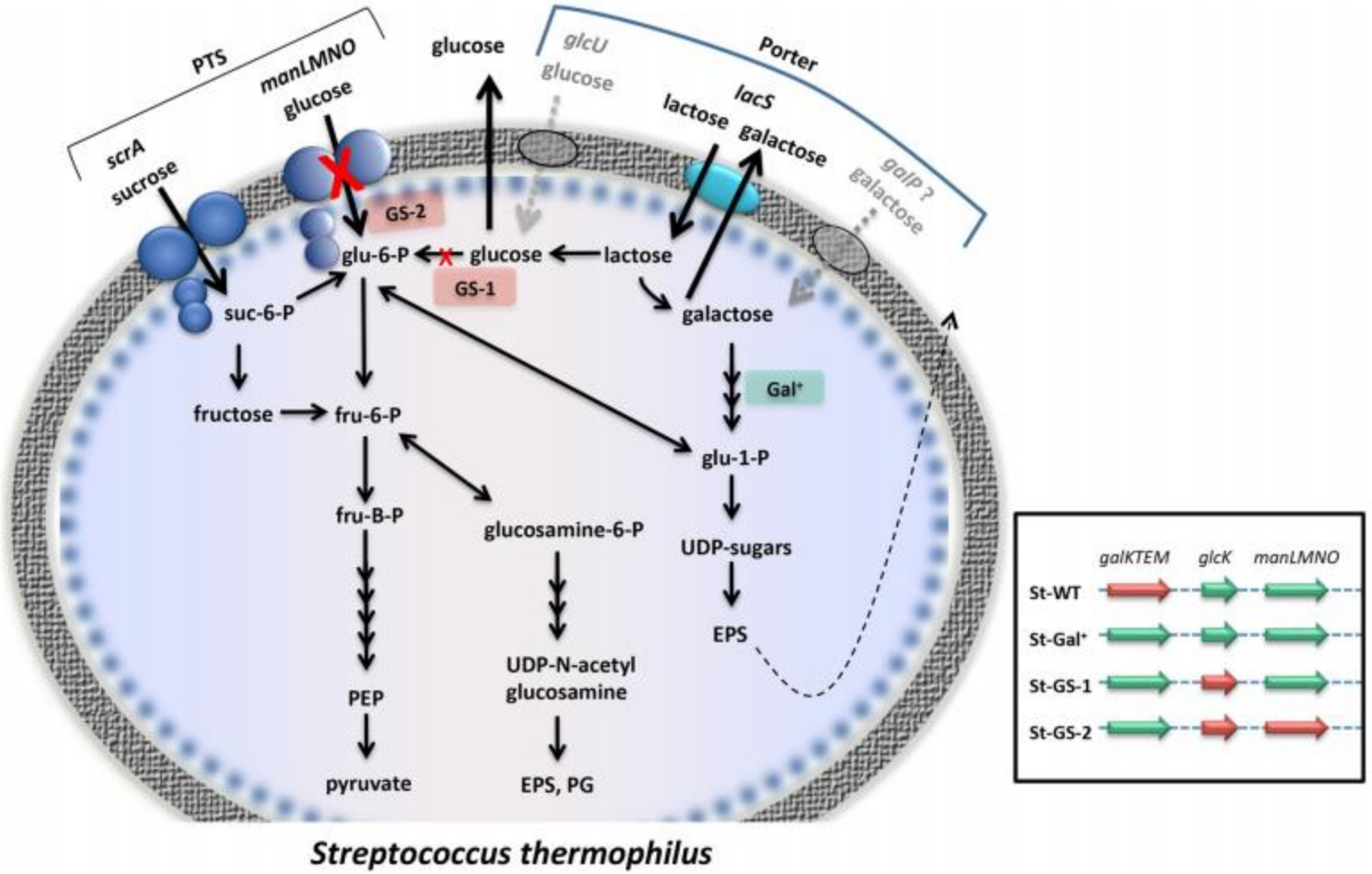
DOI: https://doi.org/10.1128/AEM.00462-16

How to make a natural sweet yoghurt?

1. Selection of mutants with impaired glucose metabolism resistant to 2-deoxy glucose
2. Glucokinase (glcK) knock-out mutants give resistance to 2-deoxy glucose
3. To circumvent bypass of glcK, the glucose transporting PTS system has to be inactivated
4. Selection of mutants resistant to higher levels of 2-deoxy-glucose on plates with galactose as sole carbon source gives mutations in PTS system transporting glucose
5. Glucokinase-negative galactose-fermenting PTS-negative ST resistance to 2- deoxy glucose

Inhibit the growth on glucose

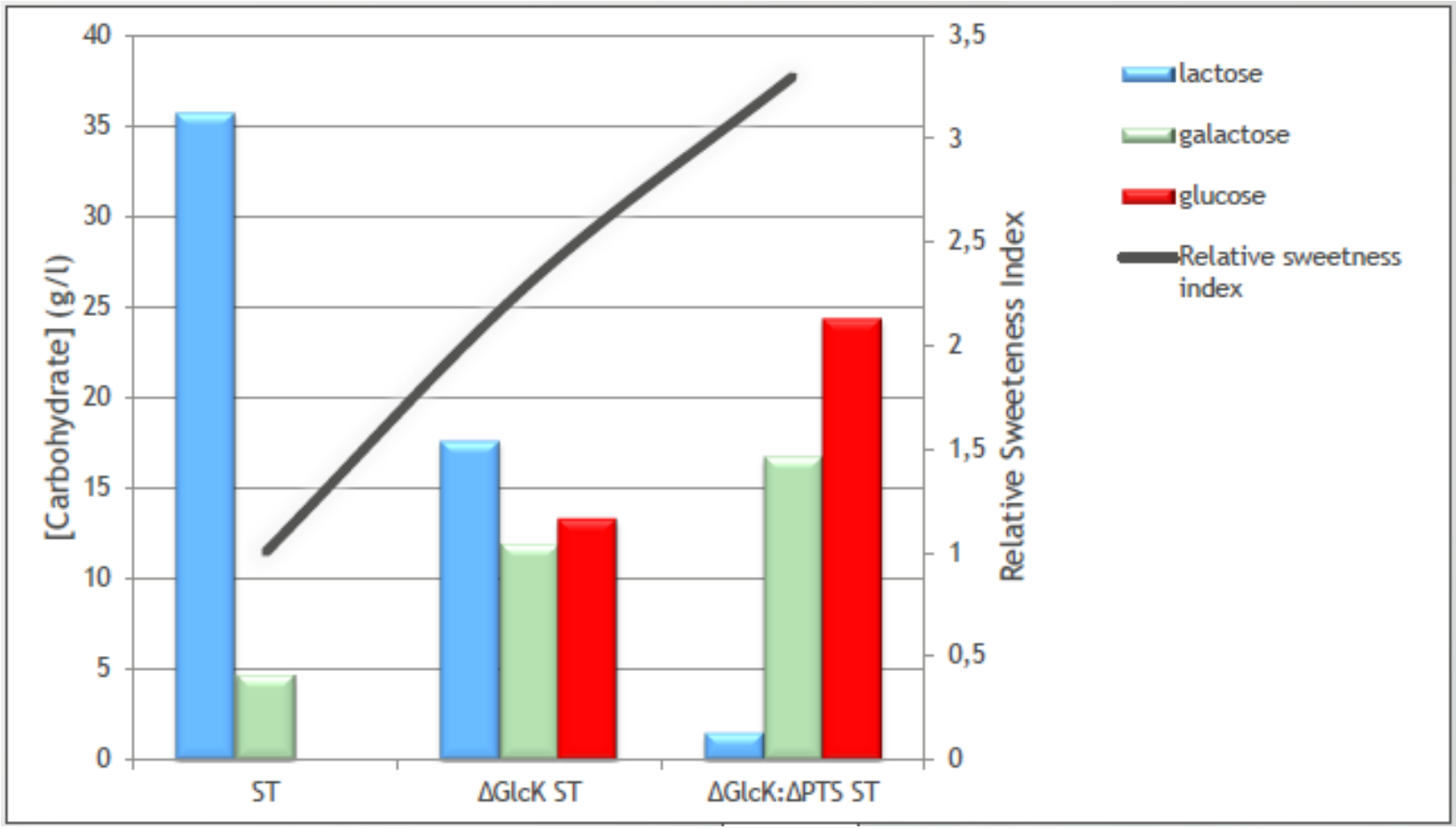
No need to study details



CHR. HANSEN

Improving food & health

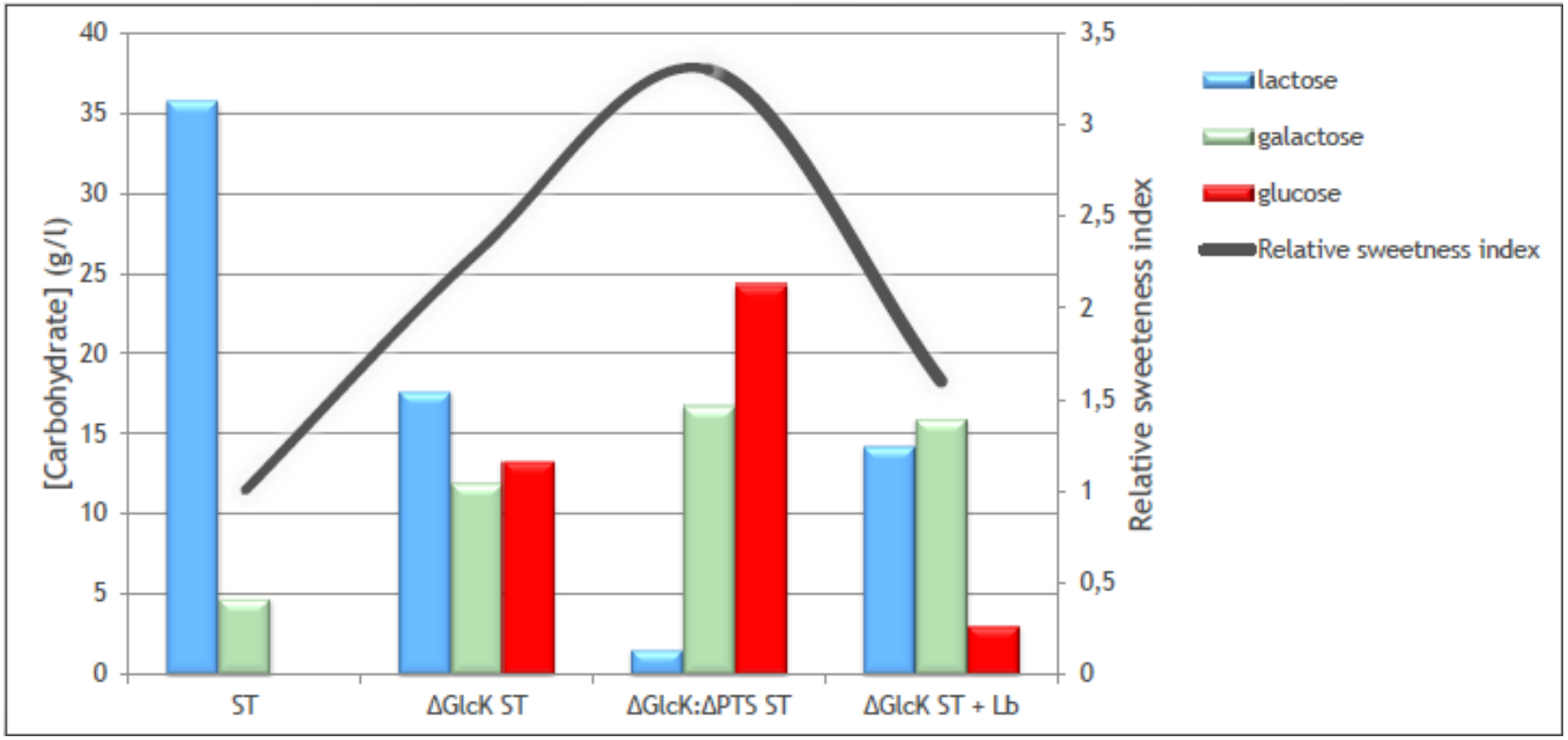
Sweety ST enhances sweetness of fermented milk by excretion of glucose



CHR HANSEN

Improving food & health

Sweety ST enhances sweetness of fermented milk by excreting glucose which gets consumed by *Lb. delbrueckii* subsp. *bulgaricus*



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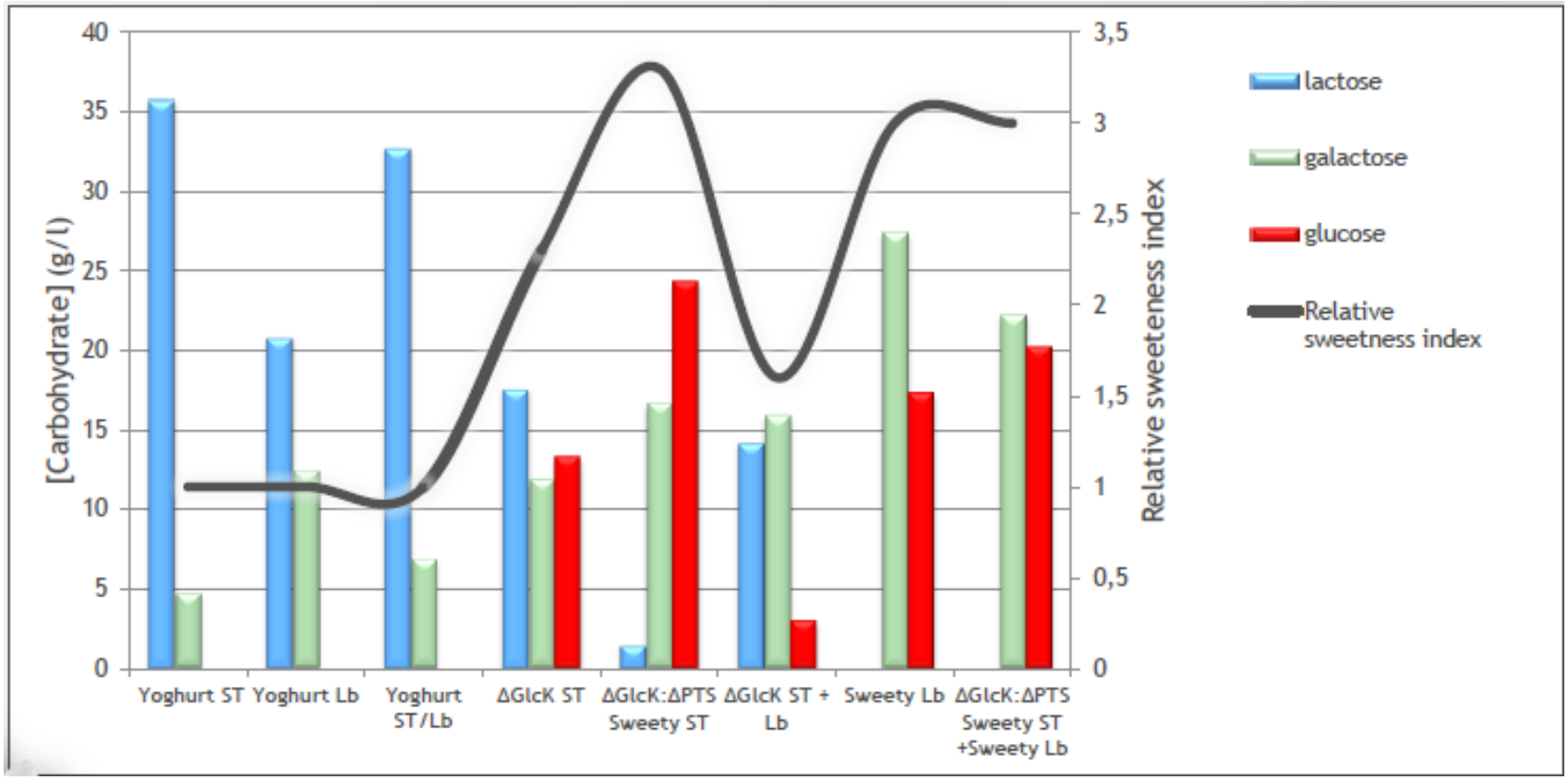
Patrick M.F. Derkx, LAB 11 conference, August 29 2014,Egmond aan Zee, NL

A Combination of Sweety ST & Sweety LB enhances Sweetness of plain Yoghurt by 3-fold



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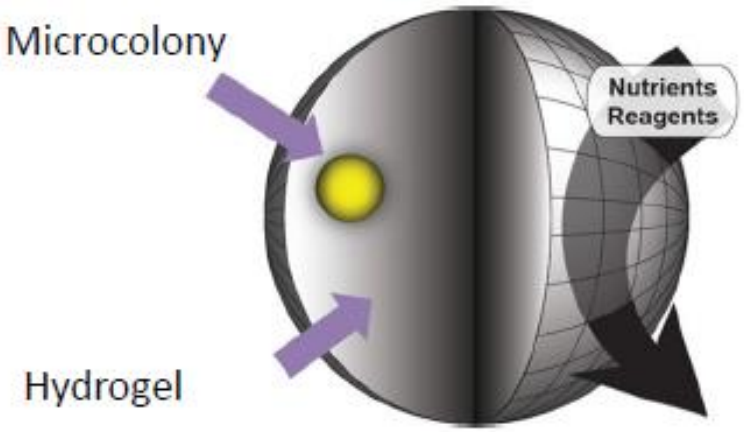
https://www.ginkgobioworks.com/2022/03/14/fgen/

NANOLITER-REACTORS (NLRs)

fgen

NLRs serve as confinement for single clones during their proliferation to microcolonies and as translucent "vial" during analysis.



Production [#/s]:	1'000 to 100'000
Size [ø µm]:	20 to 500 (highly monodisperse)
Volume [nL]:	0.005 to 100
Material:	transparent hydrogel

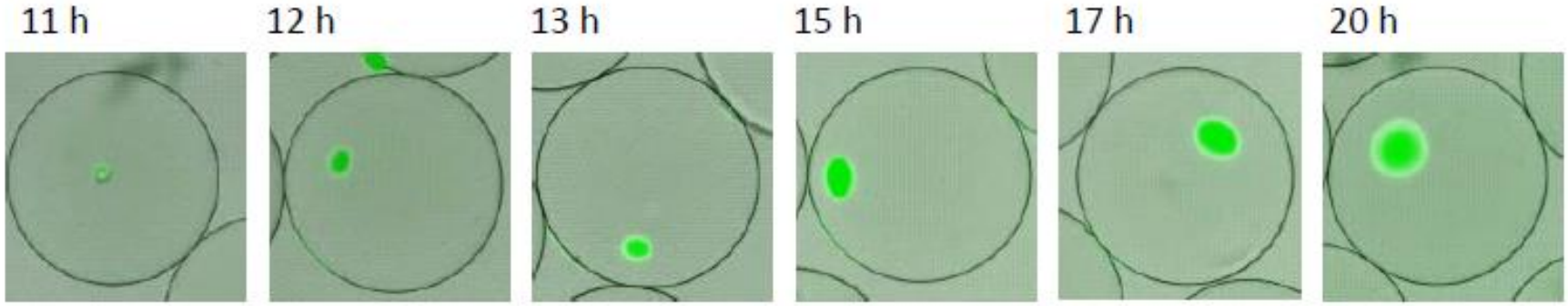
Microscopic pictures of microcolonies in NLRs

GROWTH OF MICROORGANISMS IN NLRS

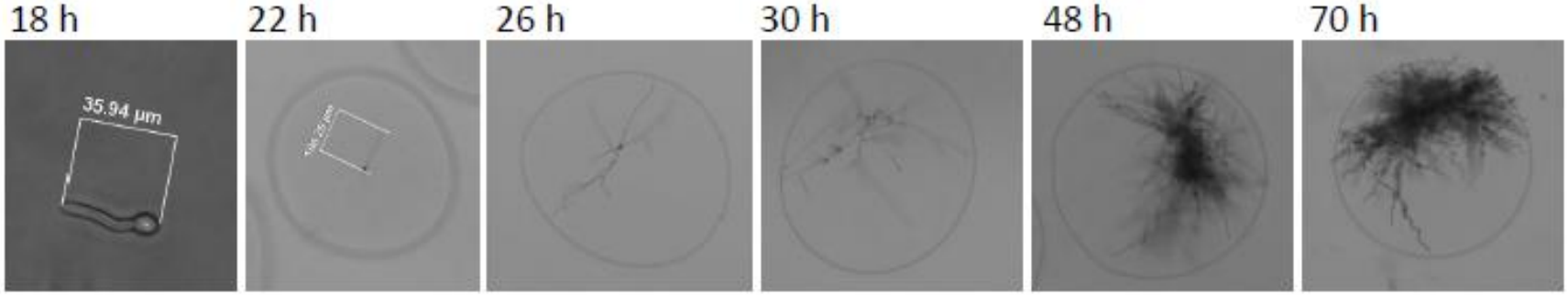
fgen

Microbes of any genera can be cultivated in NLRs.

E. coli



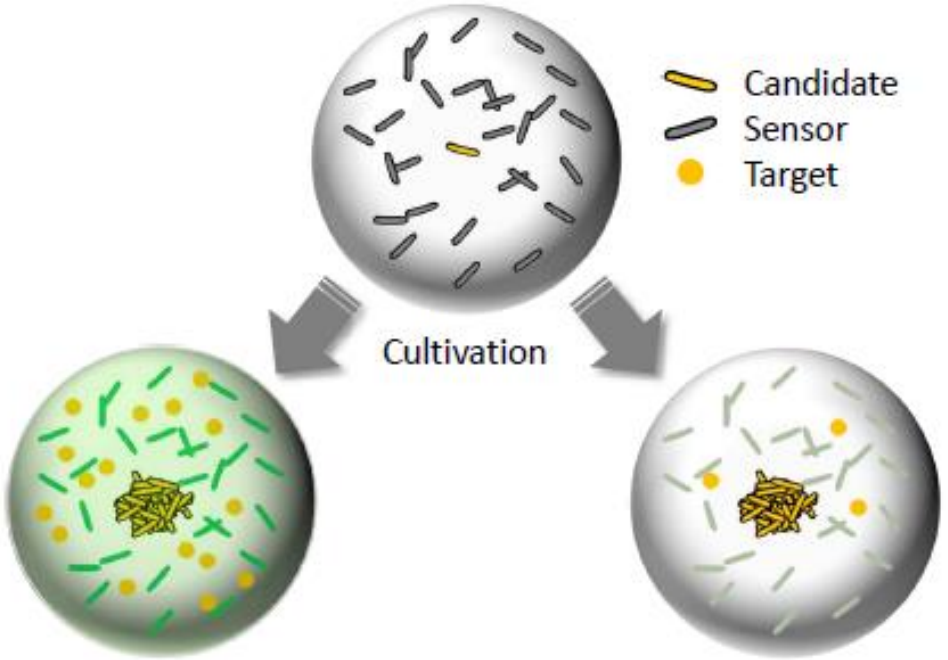
Filamentous fungus



Assay Example: Bioassays

fgen

Co-encapsulation of candidate and sensor cells that react on extracellular target



Reaction of sensor cells to target produced by positive or negative feedback

Examples

	Target	Sensor	Feedback
Positive feedback	Vitamin/AA	Auxotroph strain	Growth
	Growth factor	Cell line	Proliferation
Negative feedback	Antibiotic	Pathogen model	No growth
	Cytostatic drug	Cancer cell line	No proliferation

Assay Example: Functionalized NLRs

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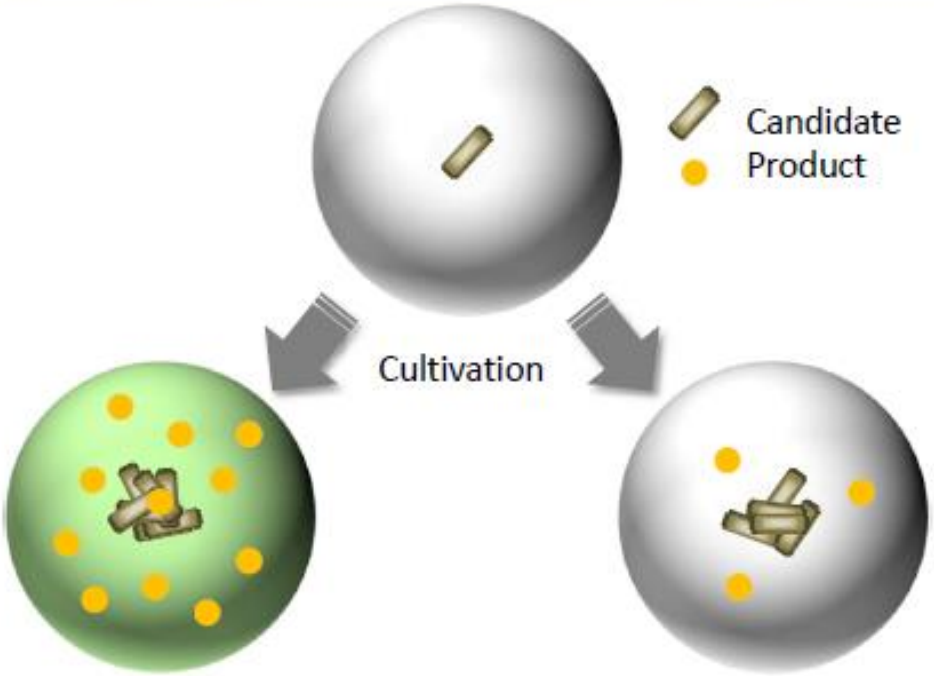
Encapsulation of candidate cells in functionalized NLR.



Secreted products reacts with the functional group and generates a signal.

Examples

Target	Functionalization
Protease	Conjugated target protein
Amylase	Fluorescently labeled starch
Antibody	Protein A/G nanoparticles
Acid or base	pH indicator

Application Example: Amylase Screening

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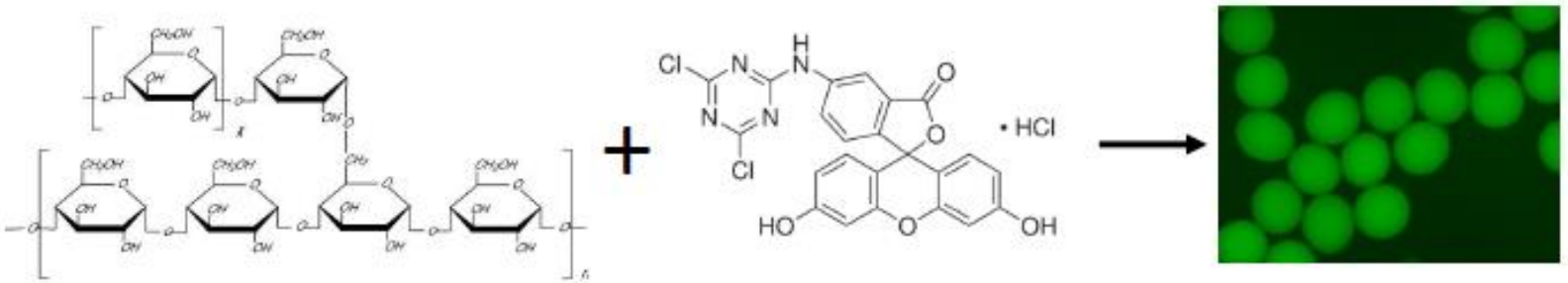
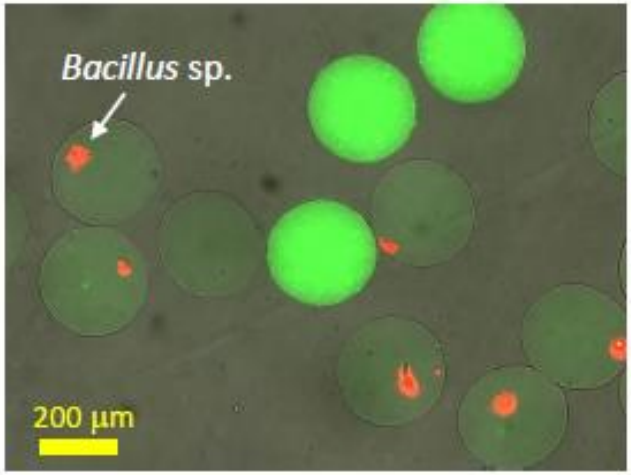
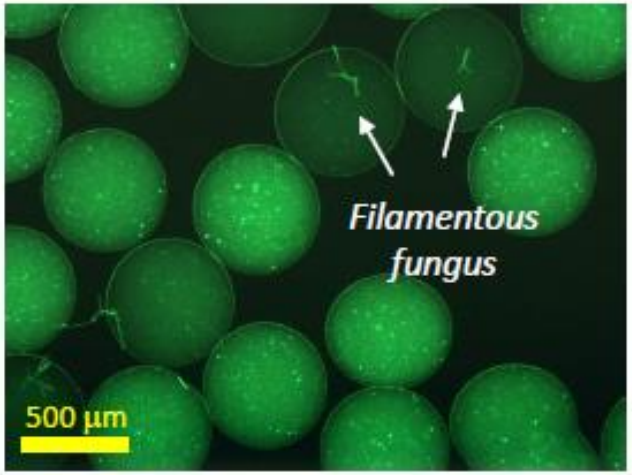
Functionalized NLRs assay based on the co-encapsulation of fluorescent labeled starch.





Bacillus sp. secreting amylase and expressing mCherry.



Filamentous fungus secreting amylase and stained with Uvitex 2B.

nature chemistry

ARTICLES

PUBLISHED ONLINE: 13 JULY 2015 | DOI: 10.1038/NCHEM.2301

Optimization of a whole-cell biocatalyst by employing genetically encoded product sensors inside nanolitre reactors

Andreas Meyer[1,2†], René Pellaux[1,2†], Sébastien Potot[3], Katja Becker[1], Hans-Peter Hohmann[3], Sven Panke[1] and Martin Held[1*]

Nature Chemistry **volume7**, pages673–678 (2015)
doi:10.1038/nchem.2301

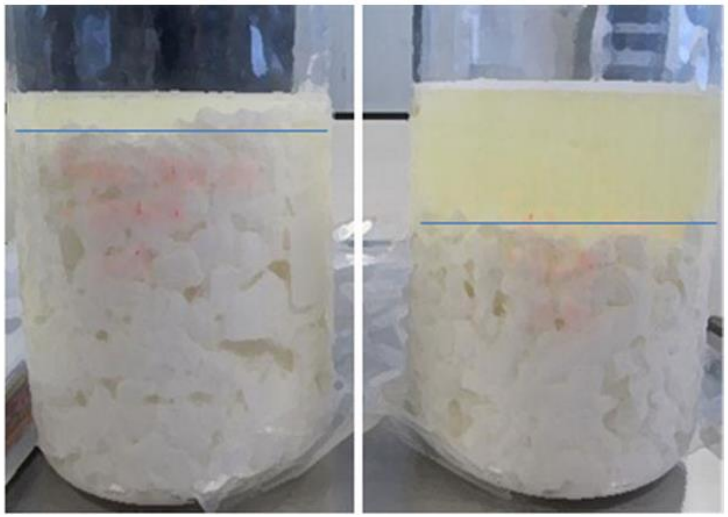
Microcompartmentalization offers a high-throughput method for screening large numbers of biocatalysts generated from genetic libraries. Here we present a microcompartmentalization protocol for benchmarking the performance of whole-cell biocatalysts. Gel capsules served as nanolitre reactors (nLRs) for the cultivation and analysis of a library of *Bacillus subtilis* biocatalysts. The *B. subtilis* cells, which were co-confined with *E. coli* sensor cells inside the nLRs, converted the starting material cellobiose into the industrial product vitamin B2. Product formation triggered a sequence of reactions in the sensor cells: (1) conversion of B2 into flavin mononucleotide (FMN), (2) binding of FMN by a RNA riboswitch and (3) self-cleavage of RNA, which resulted in (4) the synthesis of a green fluorescent protein (GFP). The intensity of GFP fluorescence was then used to isolate *B. subtilis* variants that convert cellobiose into vitamin B2 with elevated efficiency. The underlying design principles of the assay are general and enable the development of similar protocols, which ultimately will speed up the optimization of whole-cell biocatalysts.

Strain Improvement: It is all about Selection

Selection for Urease negative mutants of *S. thermophilus*

Traditionally, cottage cheese is made by fermenting milk with *L. lactis*.

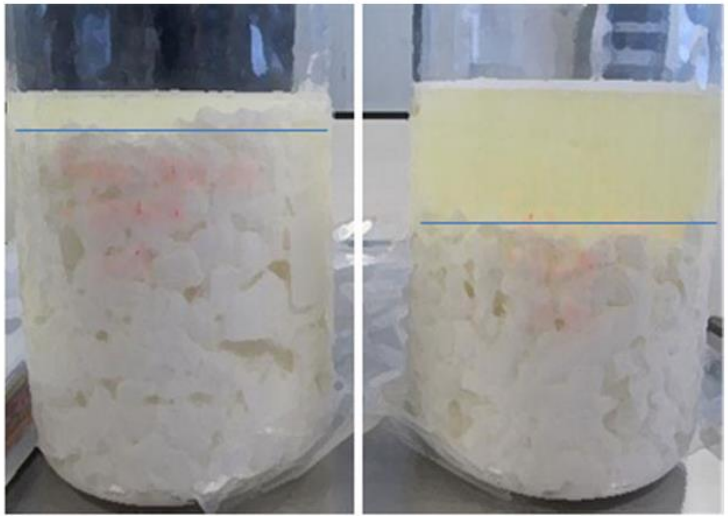
Recently, the use of S. thermophilus as a starter cultures for cottage cheese has gained popularity due to its faster acidification rate resulting in a faster production process in the dairy and a significant gain in capacity.

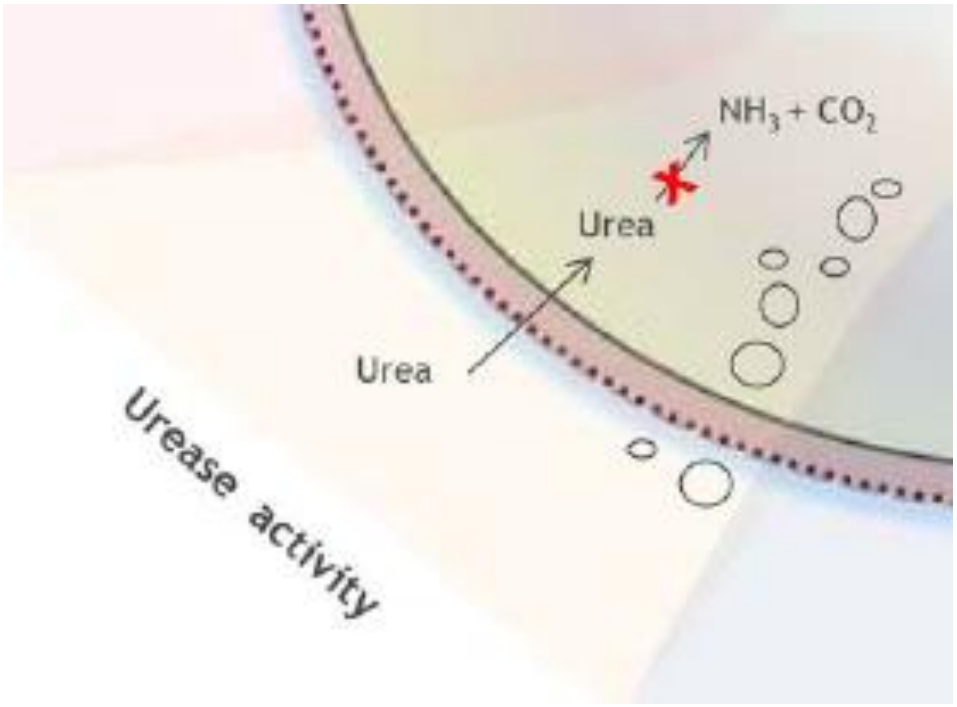


Problem: the cheese curd particles making them float which hinders whey removal and results in a loss of cheese mass.

Selection for Urease negative mutants of *S. thermophilus*

- Cottage cheese production by *S. thermophilus*
- Problem with amidohydrolase activity which converts urea into ammonia and carbon dioxide.
- Random mutagenesis and screening on plates containing a pH indicator



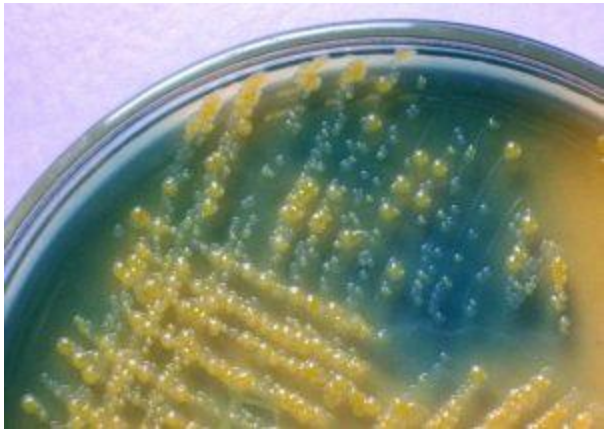


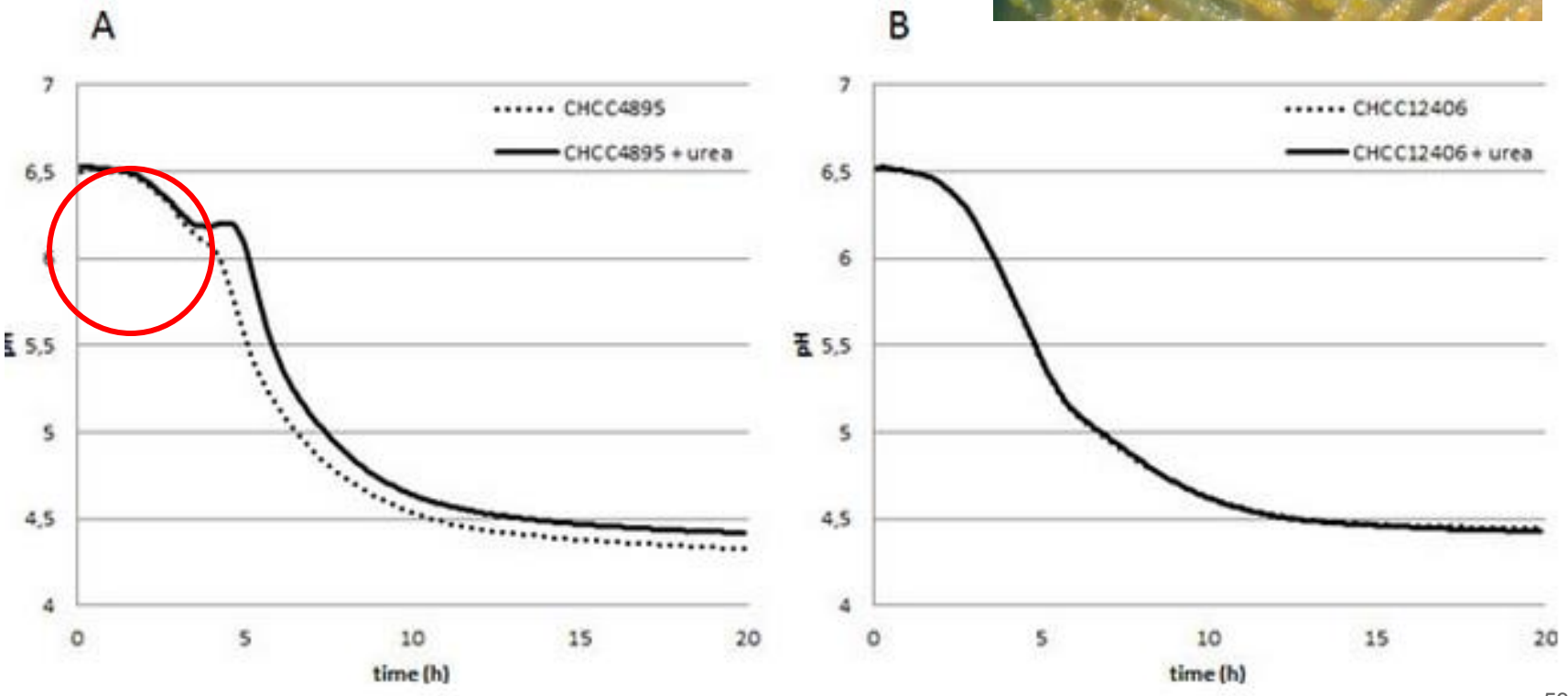
urease; EC. 3.5.1.5: urea + H2O = CO2 + 2 NH3

Selection for Urease negative mutants of *S. thermophilus*

- Screening was done on plates containing a pH indicator; mutants devoid of urease activity lack buffering capacity due to reduced ammonia production.

- Mutant CHCC12406 did not show the typical pH increase when grown in milk with added urea, verifying the urease negative phenotype

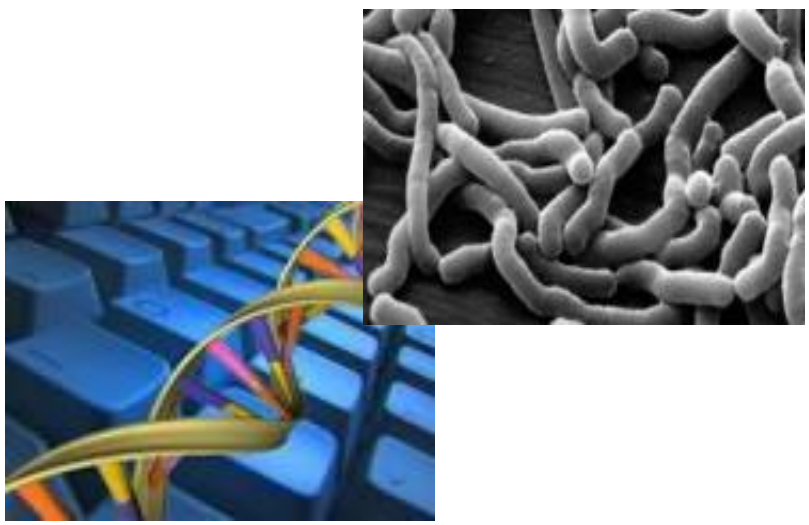


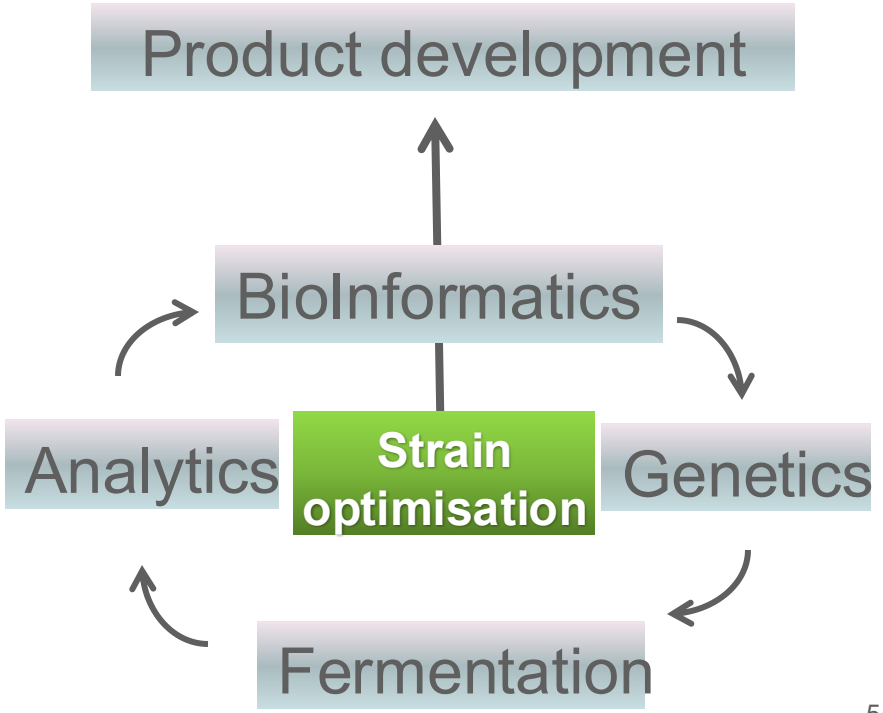


Conclusions

Development of a strain improvement platform
to enhance functional properties or increase yield

- Know your target
- Know the physiology of your strain
- Key to success is the screening and selection approach
- Be aware of potential negative effects
- GMO/nonGMO





Introduction to CRISPR