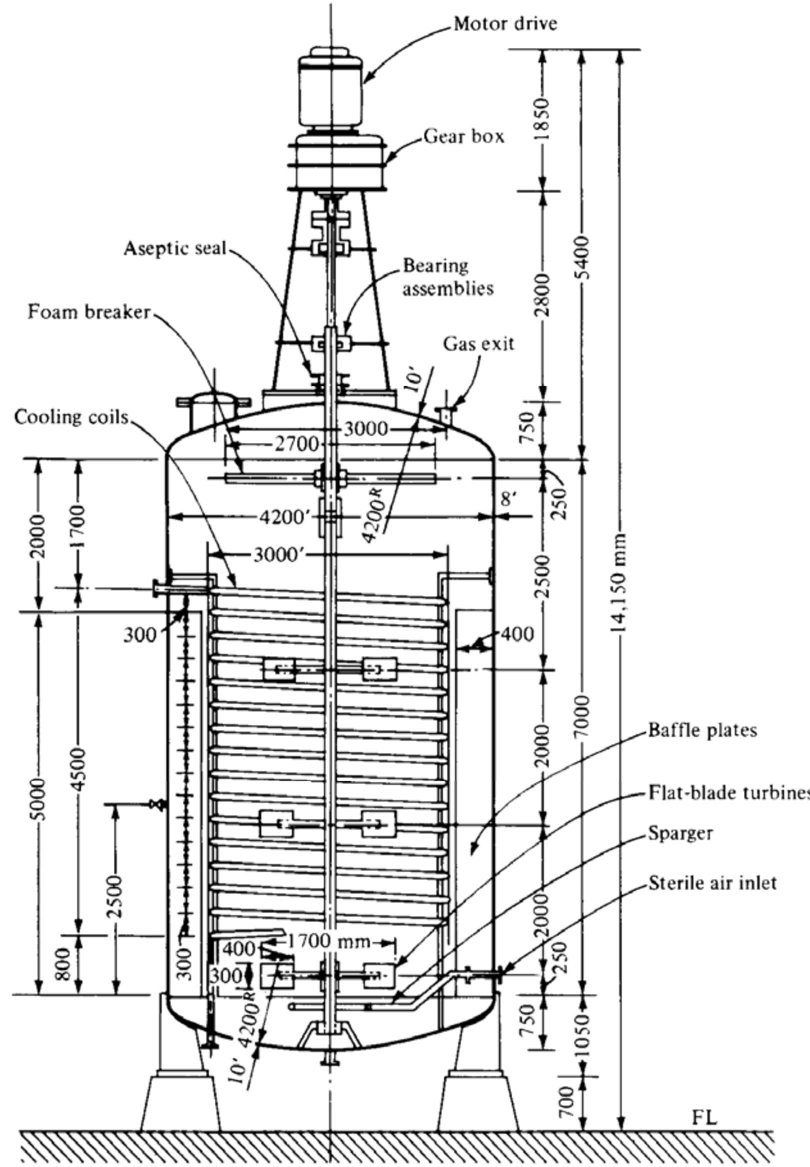
Microbial Bioengineering for Chemical Biosynthesis

Microorganisms relevant for
the production of lactic acid

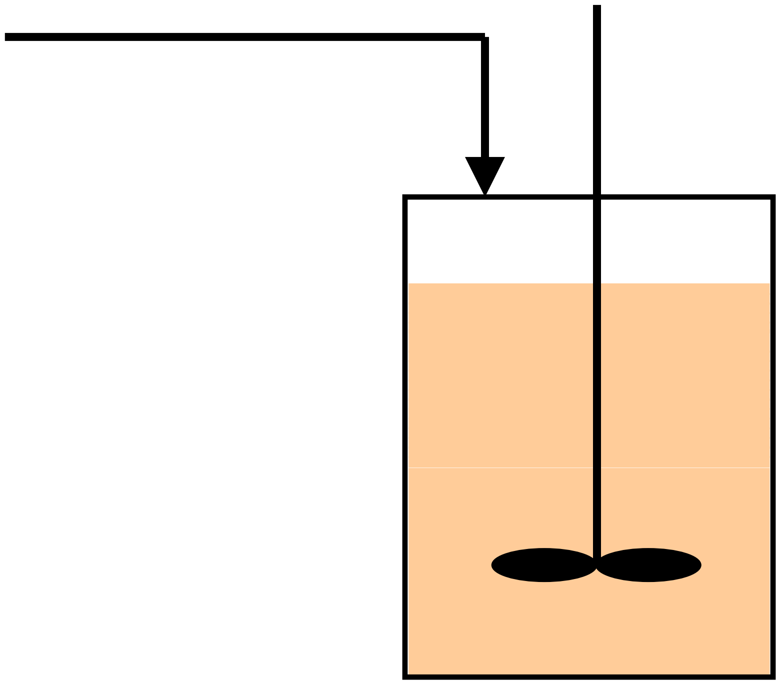
General characteristics of microorganisms used for biochemical production

1) Readily culturable and stable
2) Nonpathogenic and free of toxins
3) Flexible growth requirements
4) Fast growing
5) Ability to secrete product
6) Generate product in high yield
7) Protect itself against contaminants
8) Insensitivity to product accumulation



Motor drive
Gear box
Aseptic seal
Bearing assemblies
Foam breaker
Gas exit
Cooling coils
Baffle plates
Flat-blade turbines
Sparger
Sterile air inlet
FL
14,150 mm
1850
2800
5400
750
3000
2700
250
8'
4200'
4200R
10'
3000'
2000
1700
300
400
2500
7000
5000
4500
2000
2500
800
1700 mm
400
300
300
250
750
1050
700

Fed - Batch vs. Batch



Fed - Batch

Advantages of Fed-Batch Processes

The concentration of each nutrient exposed to the microorganism can be controlled:

1) Osmotic stress for microbial cells is reduced.
2) Substrate inhibition is avoided.
3) Cell growth can be limited by any nutrient desired.
4) Nutrient limitation prevents oxygen demand from exceeding capacity of bioreactor.

Parameters to Assess Fermentation

Yield:	How much of the desired product is formed for the amount of substrate consumed. (g product/g substrate)
Productivity	How fast is desired product formed. (g product /L h) (g product /mg cells h)

Lactic Acid

- 6000BC - cheese-making
- 1500 - sauerkraut, yogurt
- 1881 - first commercial lactic acid production
- 1895 - first successful commercial lactic acid production by Boehringer using *Lactobacillus delbrueckii* (45-48°C)

Lactic Acid

- economics of lactic acid production is driven by:
  1) ability to make lactide dimer
  2) poly(lactic acid) properties (fibers, injection molding, etc.)
  3) ability to control ratio of L- and D- lactic acid (L-form yields crystalline polymer, D- form yields amorphous polymer)
- 140,000 ton/year plant in Blair, Nebraska

Lactic Acid Bacteria (“LABs”)

- Phylogenetically related organisms which naturally accumulate significant quantity of lactic acid
- Generally, biosynthetic capacity is “underdeveloped”
- Gram-positive, non-sporulating, non-motile, aerobic to facultatively anaerobic, lack cytochromes
- Used as starter cultures in dairy products (yogurt, buttermilk, cottage cheese,…)
- Used in processing/preservation of diverse foods (cured hams, pickles, sauerkraut, beer)

Lactic Acid Bacteria

- Opportunistic pathogens (endocarditis, bacteremia, septicemia,…)
- Probiotic (use of viable microbes in foods)
  - increase immune response toward Rotavirus
  - increased number of antibody-secreting cells,
  - prevent viral intestinal infections by preventing pathogens from becoming established
- What causes antimicrobial activity?
  - pH (lactic acid is moderately strong acid, with $pK_A = 3.8$)
  - H_2O_2 (resistance: *L. lactis* 125 μg/mL, *S. aureus* 4 μg/mL)
  - inhibitory small molecules (e.g., diacetyl)
  - bacteriocins (e.g., nisin)

Lactic Acid Bacteria

	Lactic Acid Isomer	CO_2 from glucose	CO_2 from gluconate	morphology
homofermenters				
Streptococcus	L+	-	-	cocci/chains
Pediococcus	DL, L+	-	-	cocci/tetrads
Lactococcus	L+	-	-	cocci/chains
Lactobacillus				
subgroup *Streptobacteria*	DL, D-, L+	-	+	bacilli/pairs
subgroup *Thermobacteria*	DL, D-, L+	-	-	bacilli/pairs
heterofermenters				
Lactobacillus				
subgroup *Betabacteria*	DL	+	+	pairs/chains
Leuconostoc	D-	+	-/+	pairs/chains

Specific Examples of LABs

- *Streptococcus thermophilus*
  - require complex nitrogen
  - yogurt (1998 - 1.4 bil. lbs/yr), Mozzarella cheese (1998 - 2.2 bil. lbs/yr)
- *Lactococcus lactis*
  - major bacteriocin producer
  - cheddar cheese, sauerkraut
  - adept at exchanging plasmid DNA via conjugation - rapid strain adaptation and response to bacteriophage exposure, “genome plasticity”
- *Lactobacillus delbrueckii* (subgroup Thermobacteria)
  - principal choice for industrial lactic acid fermentations
- *Leuconostoc mesenteroides*
  - found on crop plants, sauerkraut
  - forms dextran and levan
  - forms viscous slimes - sugar beets and sugar cane production

see http://www.jgi.doe.gov/JGI_microbial/html/index.html

Lactic Acid Bacteria

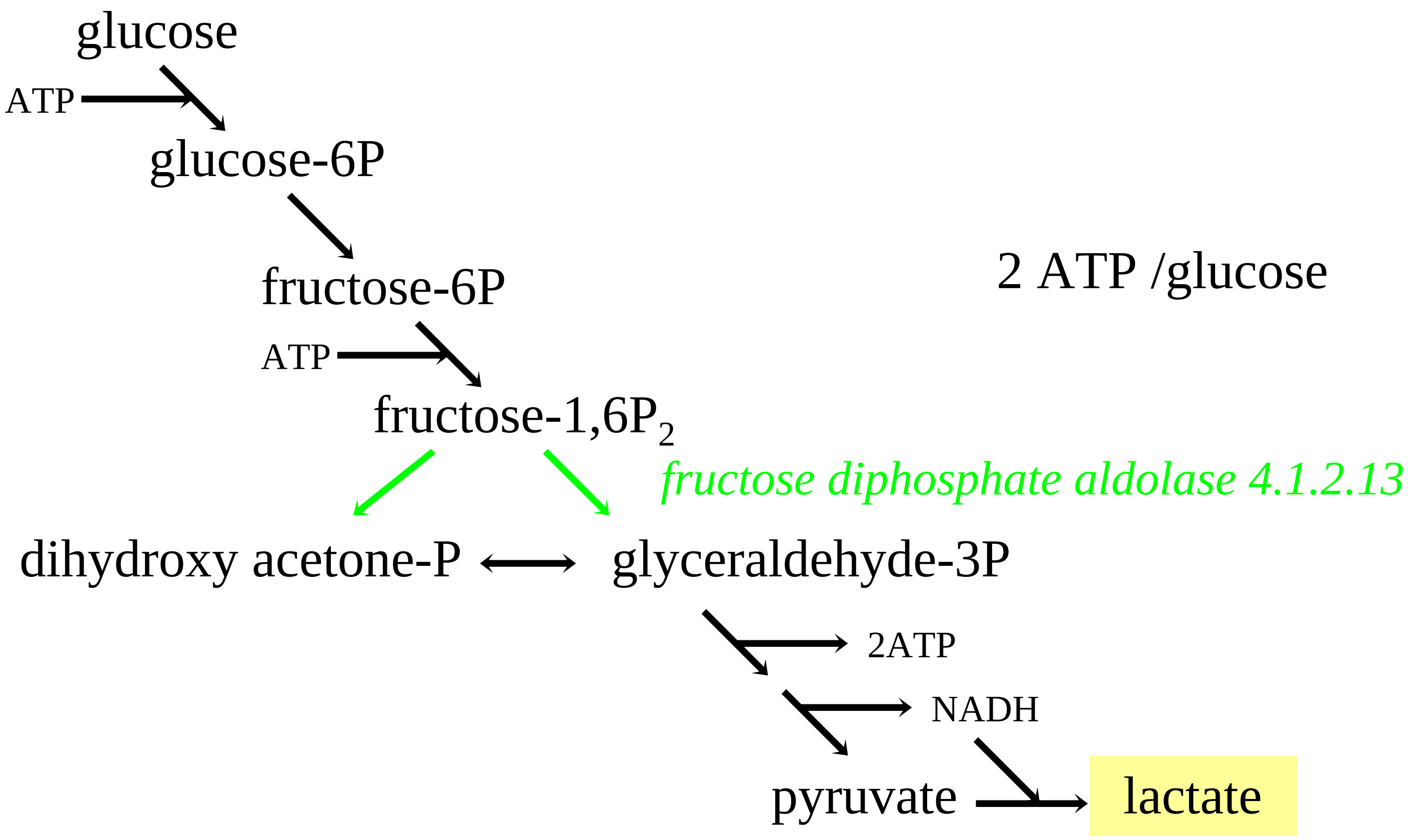
Homofermenters

- Produce (theor. max) 2 moles lactate per mole hexose
- Possess aldolase
- Those homofermenters that can consume pentoses do so heterofermentatively
- generate more ATP, but more acid - less flexibility

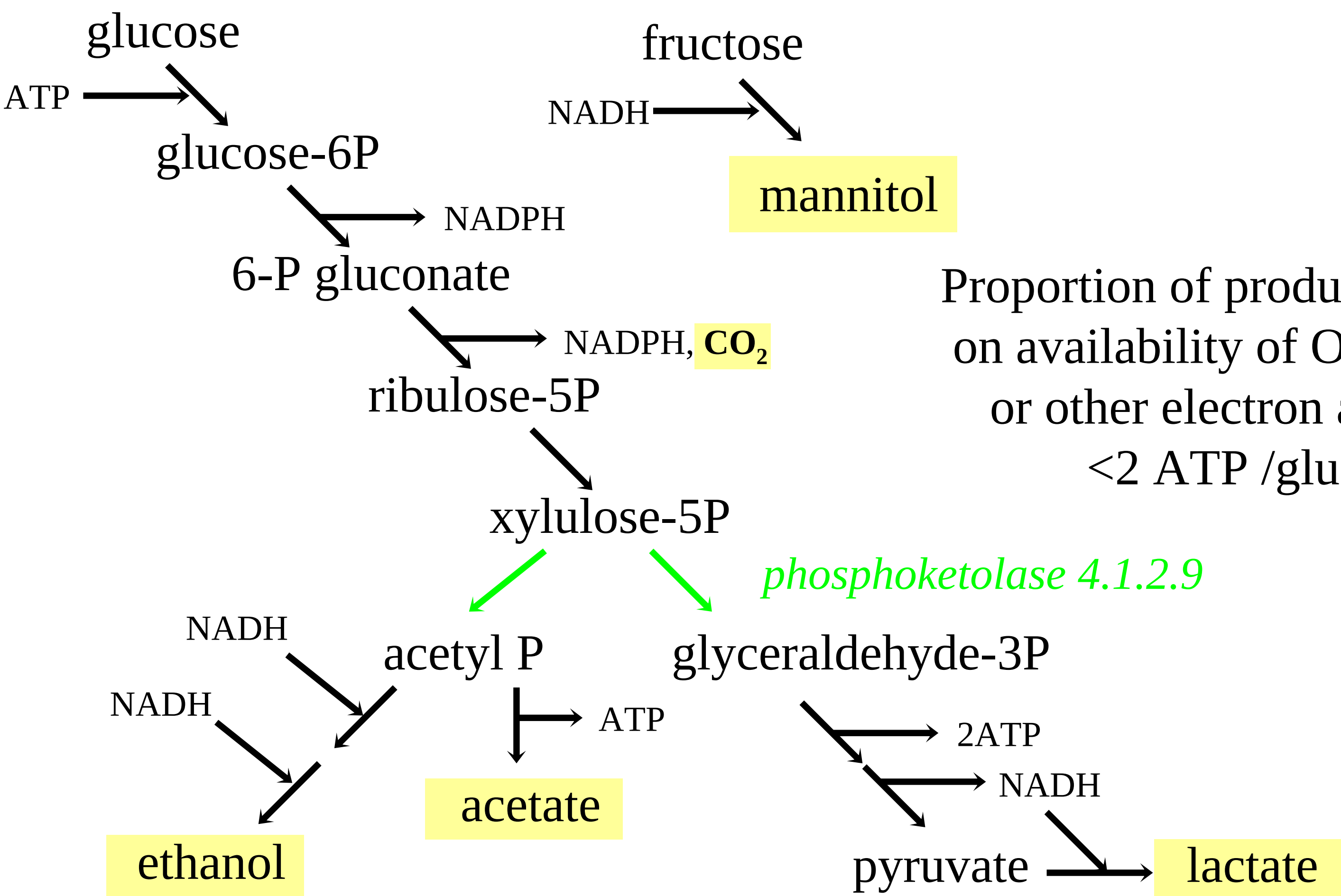
Heterofermenters

- Produce (theor. max) 1 mole lactate per mole glucose
- Produce other substances (e.g., diacetyl)
- Possess phosphoketolase (use pentose phosphate pathway)
- Generally able to ferment a wide range of pentoses
- generate less ATP, but less acid - more flexibility

Homofermentative Pathway



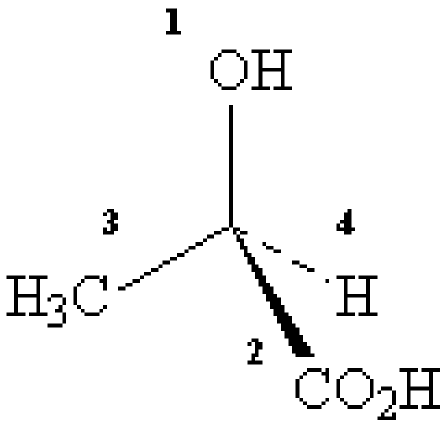
Heterofermentative Pathway

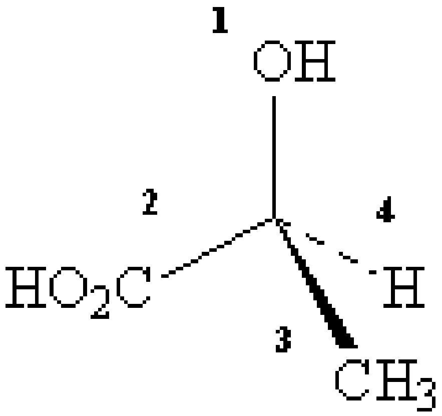


Proportion of products depends on availability of O_2, fructose, or other electron acceptors

<2 ATP /glucose

Isomers of Lactic Acid

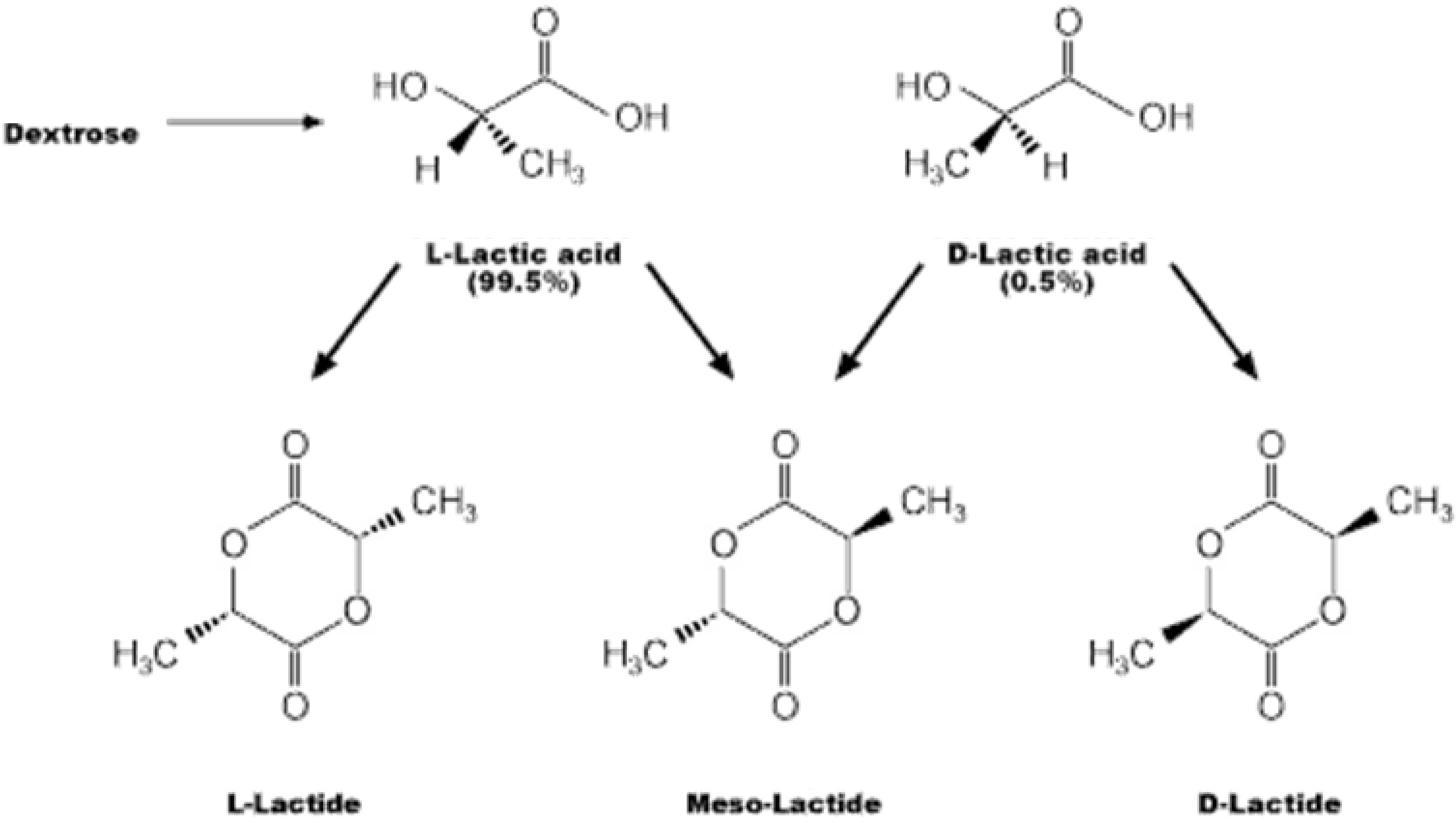




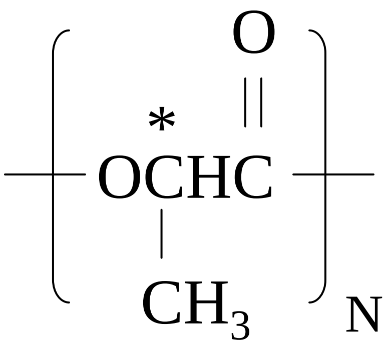
D-(-)-Lactic Acid
(dextrorotary)

L-(+)-Lactic Acid
(levorotary)

Lactide Isomers



Poly(Lactic Acid)

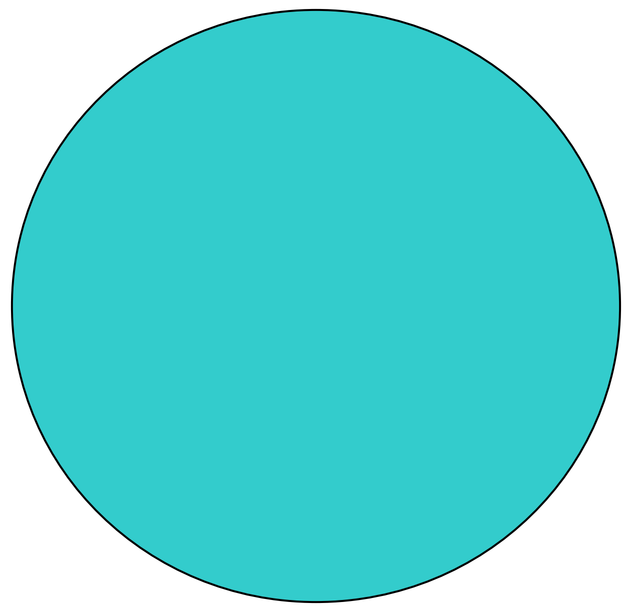


Fabrics with names: NATUREWORKS®, ECOPLA®, LACTY®, HELPON®, LACEA®

Selection/Isolation Methods

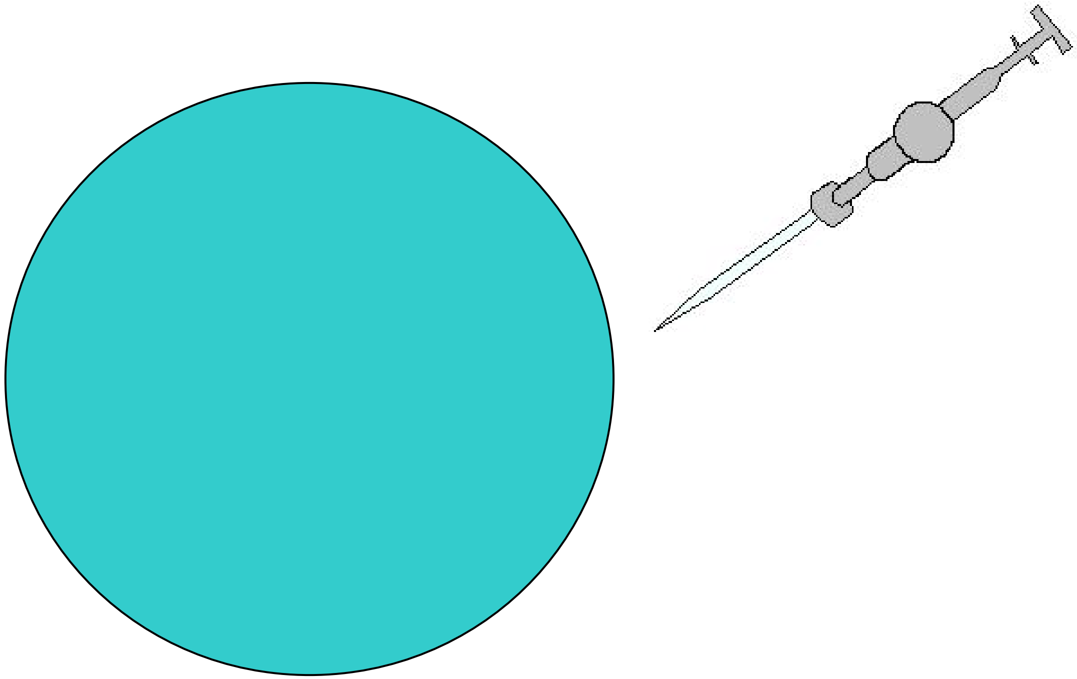
- pH of Media
  - *Lactobacillus* - pH < 4.5
  - *Pediococcus* - pH < 4.5
  - *Leuconostoc* - pH > 4.5
- Temperature
  - subgroup Thermobacteria - T > 45°C
  - subgroup Streptobacteria - T < 45°C
- Nutrient requirement
  - *Lb. plantarum* - growth on ribose
  - *Lb. coryniformis* - no growth on ribose
- Homofermentative-Heterofermentative Differential Agar
- Isomer Differential Agar

HHD Plating



1) Fructose-containing media, low buffering capacity, add bromocresol green (pK = 4.6).

HHD Plating



2) Plate with mixture of homofermentative and heterofermentative LABs.

HHD Plating

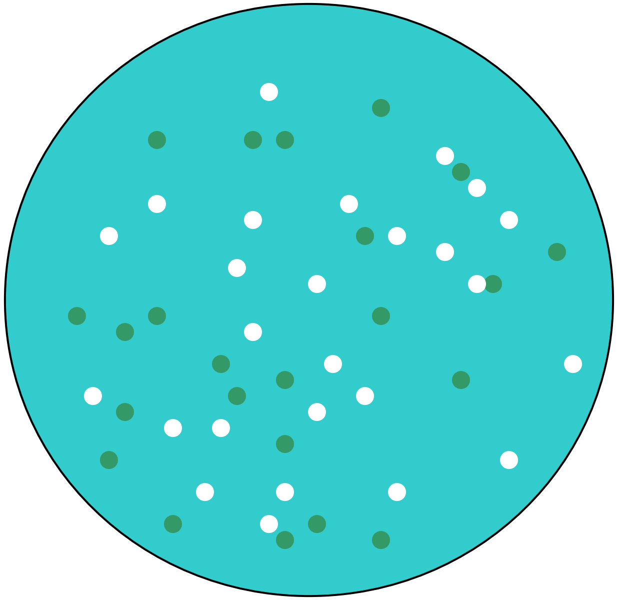
Homofermenters:

- Generate predominantly lactic acid from fructose
- significant reduction of pH
- cells ingest dye
- <u>colonies turn green</u>

Heterofermenters:

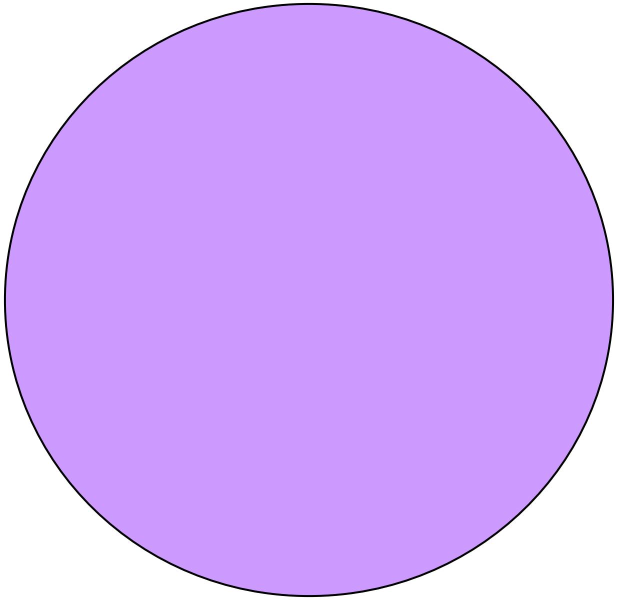
- Generate mannitol and lactic acid from fructose
- small reduction of pH
- cells to not ingest dye
- <u>colonies remain white</u>

HHD Plating



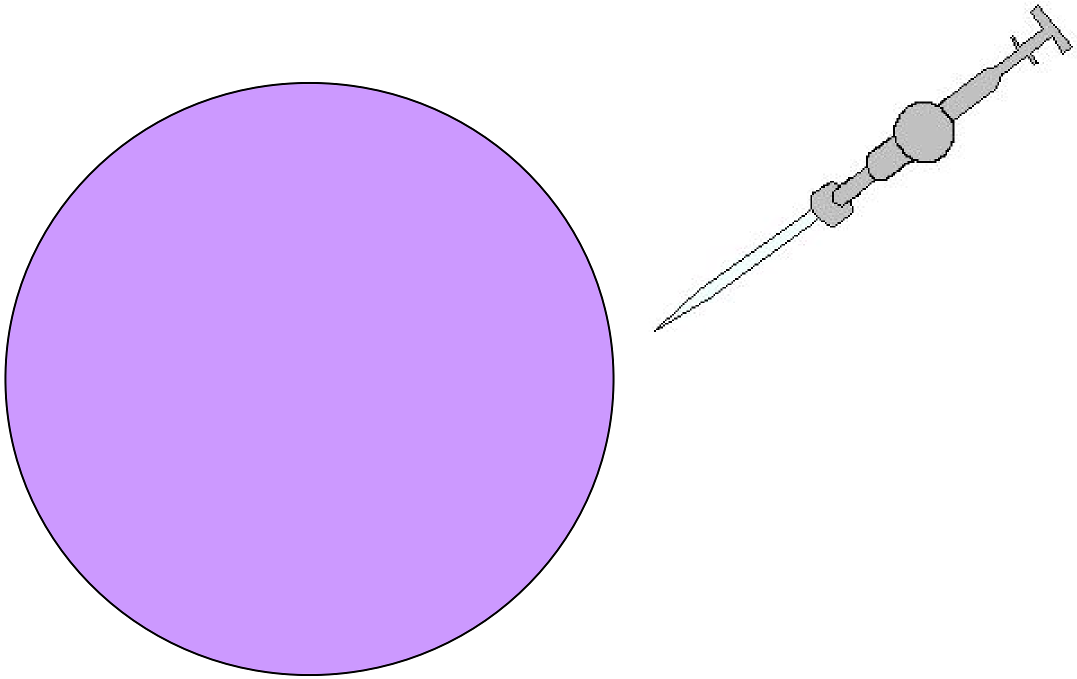
3) Permits *simultaneous enumeration* of homofermentative and heterofermentative LABs.

Isomer Differential Plating



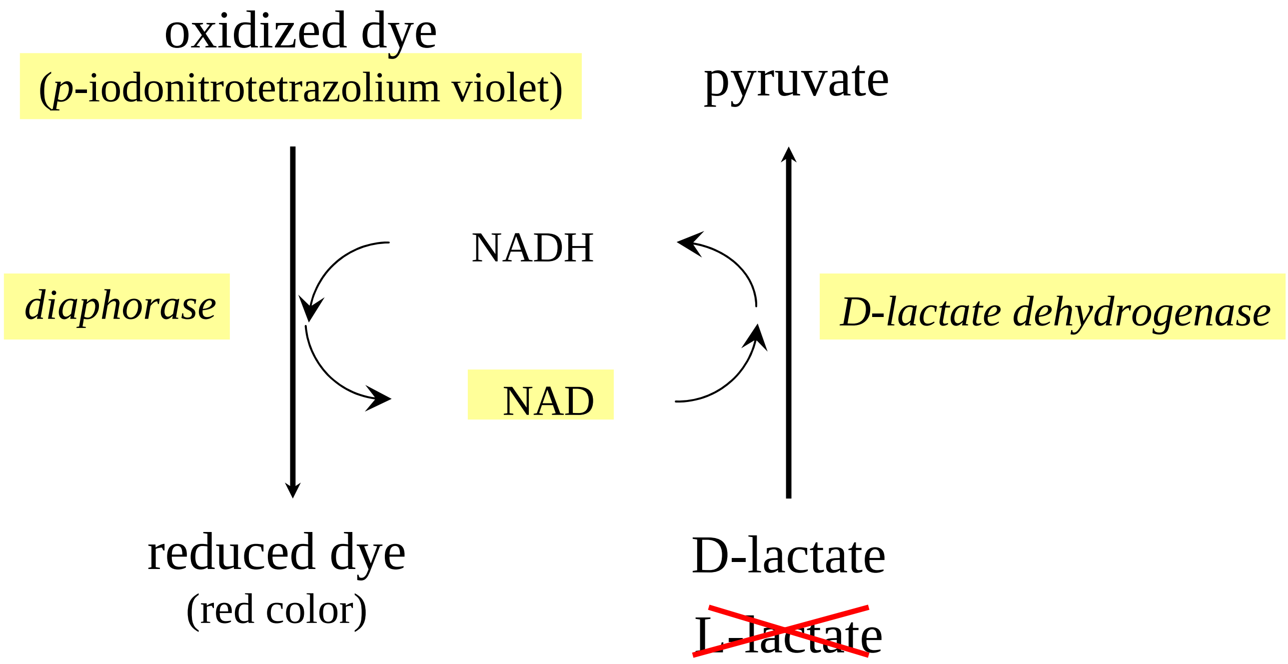
1) Prepare media with isomer-specific lactate dehydrogenase, add NAD, oxidized dye and diaphorase.

Isomer Differential Plating

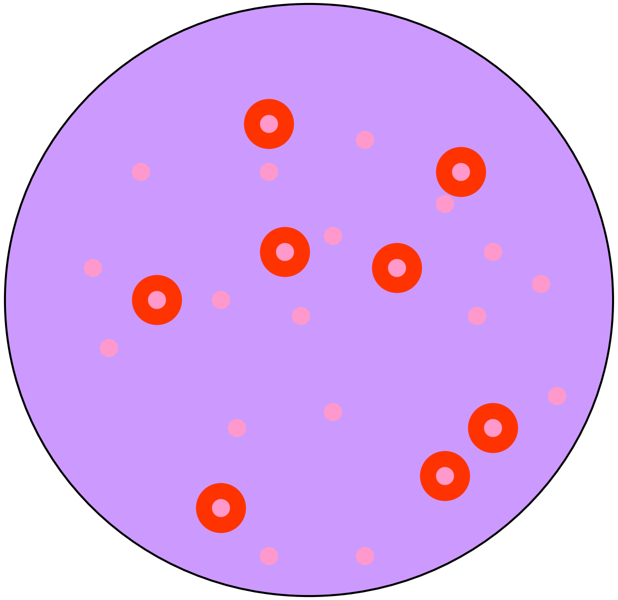


2) Plate with mixture of D-, L-, and DL- generating LABs.

Isomer Differential Plating



Isomer Differential Plating



- Colonies which generate DL-lactic acid or D-lactic acid form red halos.
- Test is based on detection of a negative character for L-lactic acid generation, and thus must be confirmed by other analyses.

Nisin

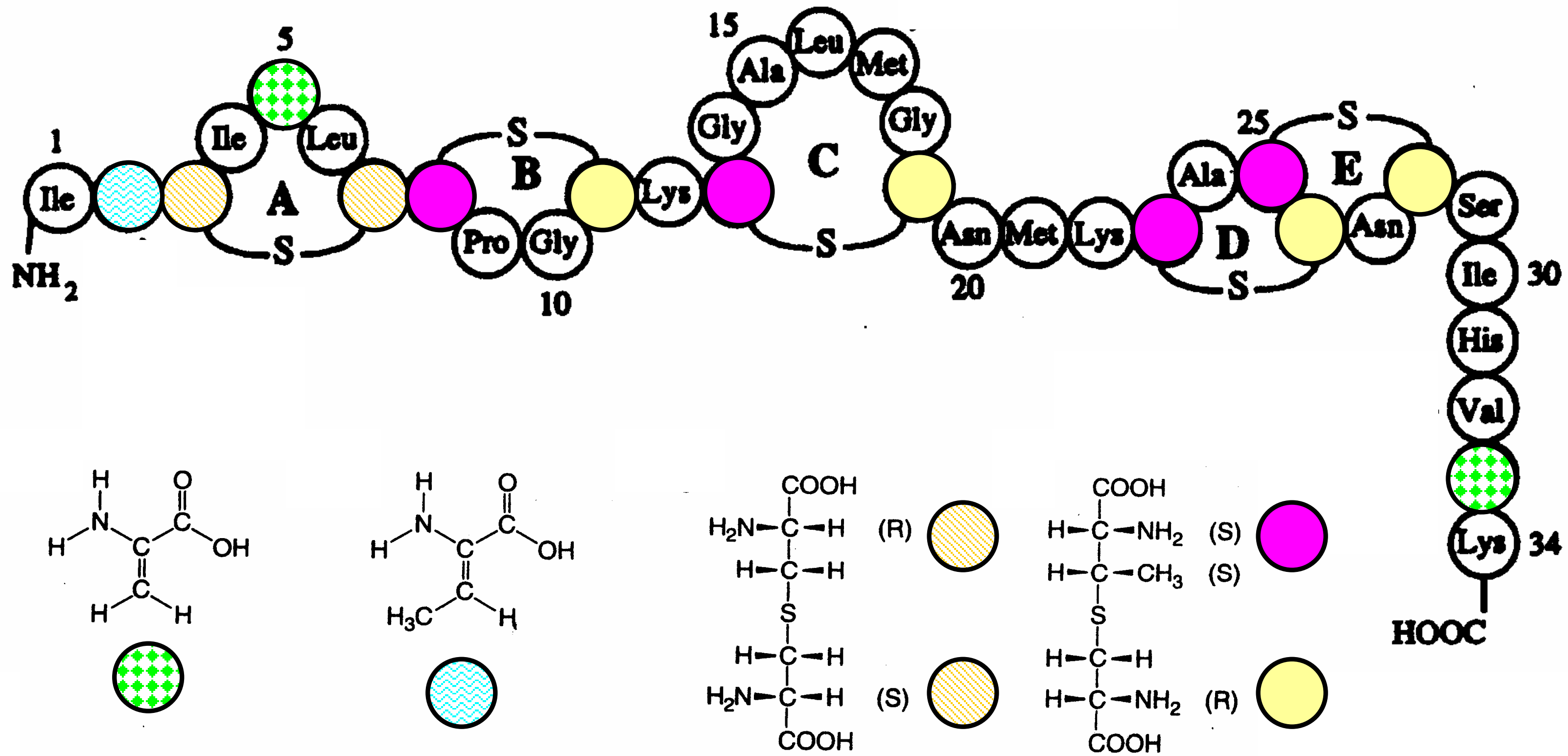
"group N inhibitory substance"
34 amino acid, positively charged peptide
13 amino acids are post-translationally modified
serine ⟶ dehydroalanine
threonine ⟶ dehydrobutyrine

1928 - Rogers and Whittier (UK) discovered that some strains of *Lactococcus* were inhibitory to other LABs
1953 - marketed in UK
1969 - FAO/WHO assessed as safe to 12.5 mg/kg
1989 - GRAS - allows use to prevent growth of *C. botulinum* in processed cheese and dairy desserts

Nisin

- Produced by *Lactococcus*
- Improves shelf-life of numerous foods
- Interacts with a docking molecule, lipid II, a membrane-bound precursor for cell wall biosynthesis to form pores. Causes “leaky cells”.
- prevents endospore germination
- effective inhibitor at nM concentrations against numerous pathogens (*Clostridium*, *Listeria*)

Nisin

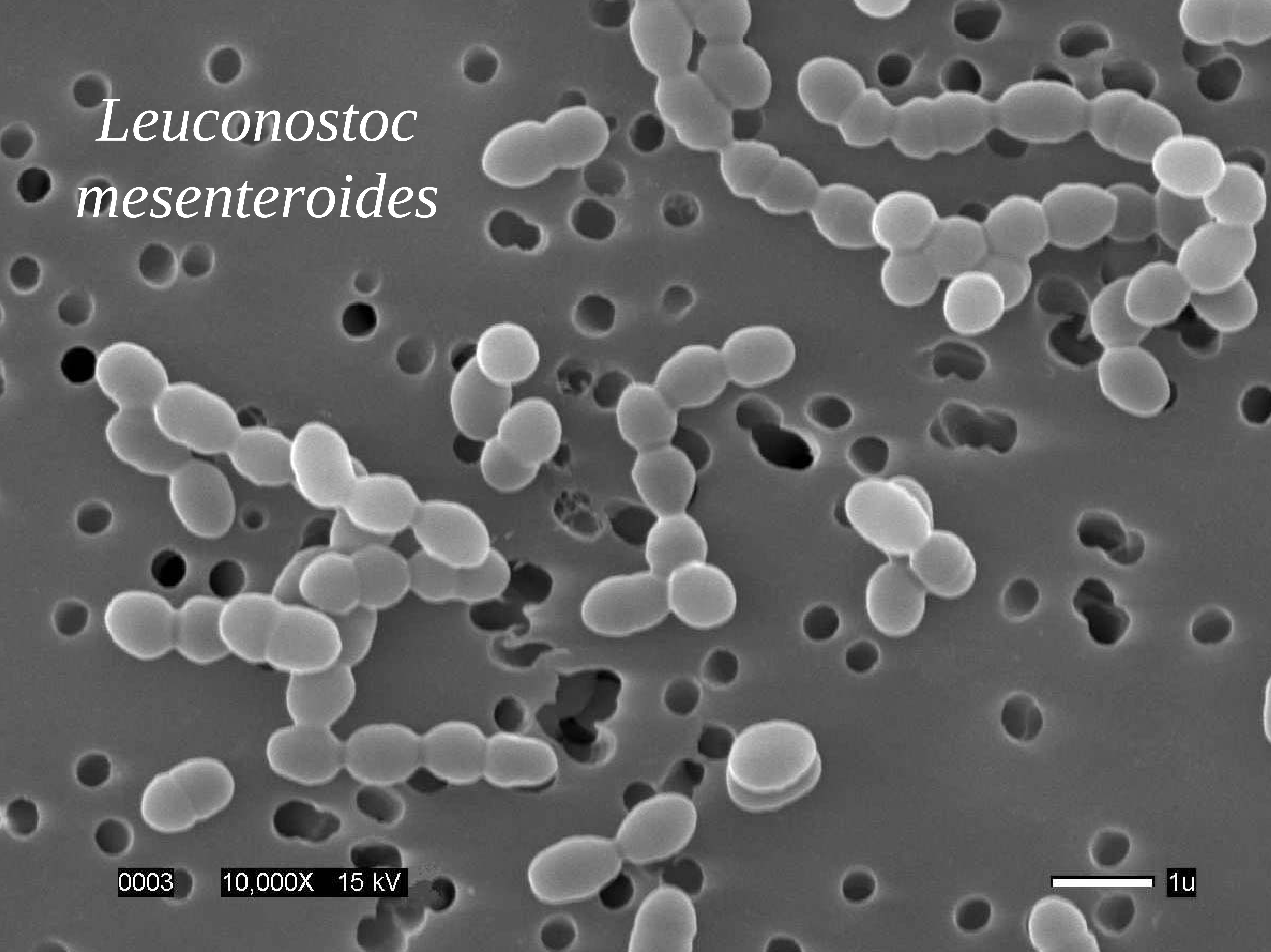


dehydroalanine dehydrobutyrine lanthionine 3-methyllanthionine

Lactobacillus delbrueckii process

- organism is homofermentative, of course!
- pH controlled (5.0 - 6.0) with $CaCO_3$ or Na_2CO_3
- complex nitrogen course (corn steep liquor)
- gentle agitation for mixing
- mildly anaerobic conditions
- vitamin/amino acid content of medium important
- dextrose (glucose) used as carbon source instead of molasses or whey because of purification costs
- yield obtained is about 0.9 g lactic acid/g glucose (90% theoretical)
- productivity of 2-3 g/Lh


Pediococcus pentosaceus


Leuconostoc
mesenteroides
0003
10,000X 15 kV
1u


Lactobacillus
delbrueckii


Lactococcus
lactis

References

Lactic Acid

O. Kandler (1983) Carbohydrate metabolism in lactic acid bacteria, *Antonie van Leeuwenhoek*, 49:209-224.

K. H. Steinkraus (1983) Lactic acid fermentation in the production of foods from vegetables, cereals and legumes, *Antonie van Leeuwenhoek*, 49:337-348.

E. Breukink et al. (1998) The orientation of nisin in membranes, *Biochemistry*, 37:8153-8162.

D. Jehanno et al. (1992) Development of a method for detection of lactic acid bacteria producing exclusively the L-(+)-isomer of lactic acid, *Applied and Environmental Microbiology*, 58(12):4064-4067.

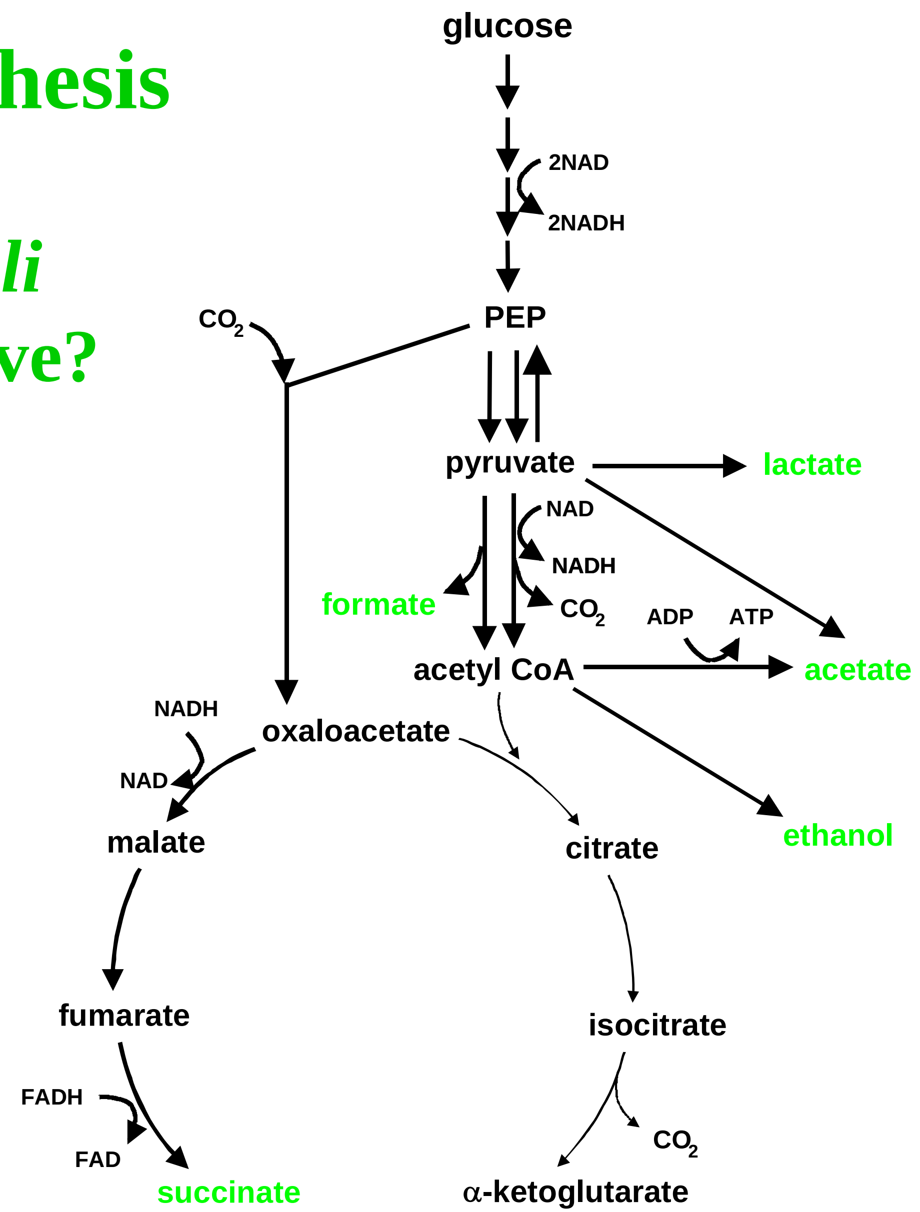
L. C. McDonald et al. (1987) A differential medium for the enumeration of homofermentative and heterofermentative lactic acid bacteria, *Applied and Environmental Microbiology*, 53(6):1382-1384.

R. Paul Ross et al. (2002) Preservation and fermentations: past, present and future. *International Journal of Food Microbiology*, 79:3-16.

Lactic Acid Biosynthesis

How do we make *E. coli* lactate `homofermentative?

Anaerobic conditions

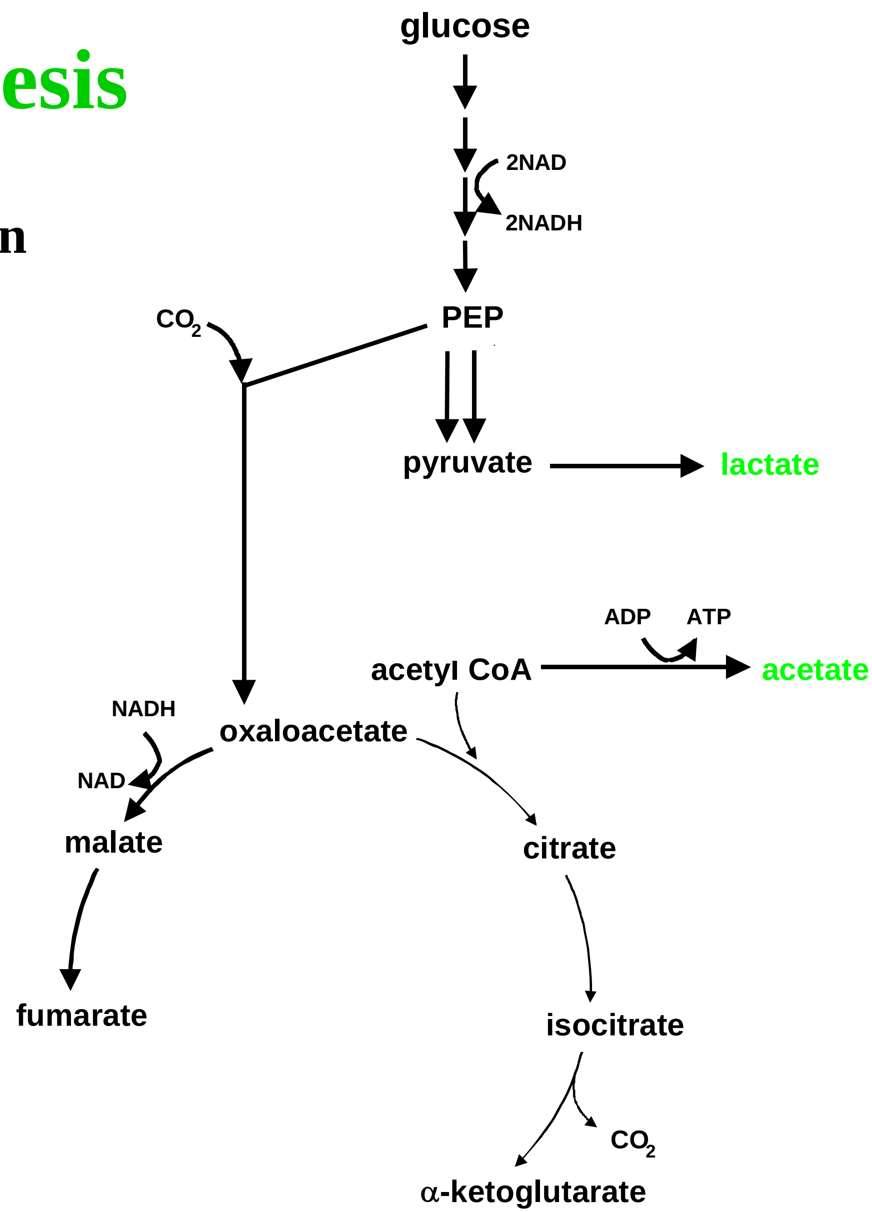


Lactic Acid Biosynthesis

Goal: Reduce By-Product Formation

Deletions

1. pyruvate formate lyase (*pfl*)
2. pyruvate dehydrogenase (*aceEF*)
3. PEP synthase (*pps*)
4. fumarate reductase (*frdABCD)*
5. pyruvate oxidase (*poxB*)



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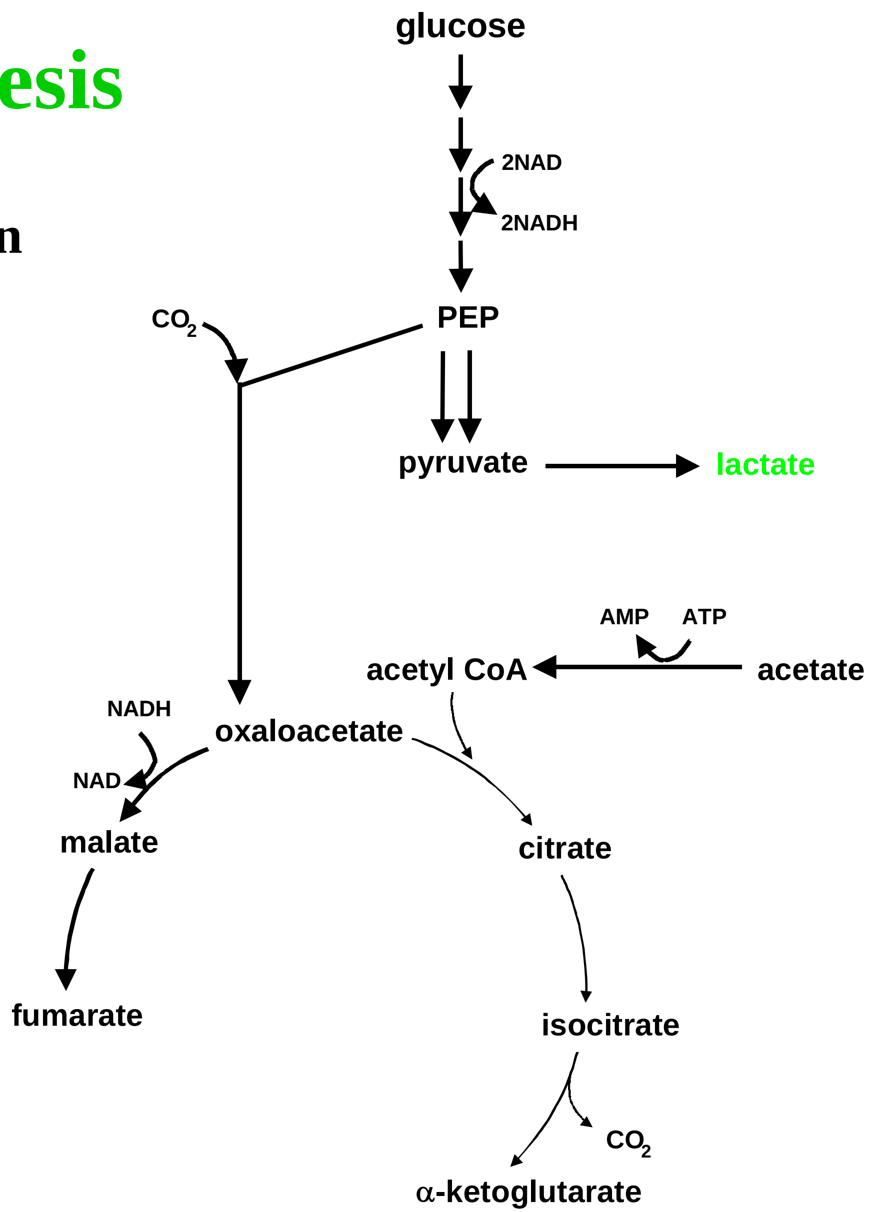
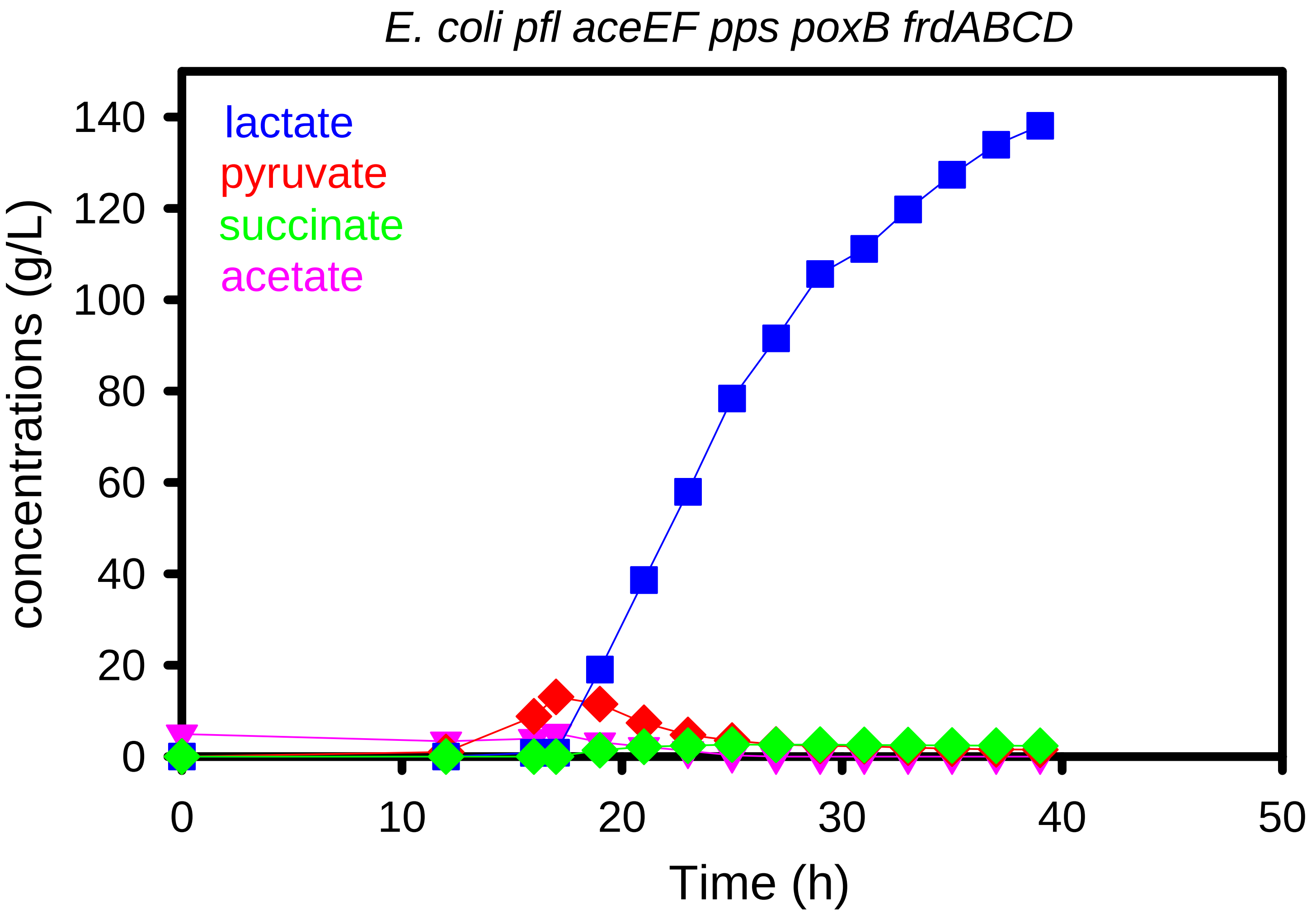


Results


E. coli pfl aceEF pps poxB frdABCD
lactate
pyruvate
succinate
acetate
concentrations (g/L)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)