The P170 Protein Expression System

An Overview of Bioneer's Proprietary Protein Expression Technology

January 2010

Lactococcus lactis as production host

- Gram-positive, formerly group N *Streptococcus*
- Low GC content (35-37%)
- *L. lactis* subsp. *cremoris* MG1363
  - Model organism
  - Complete DNA sequence
- Genetic tools available
- Secretion of proteins
- Few native, secreted proteins
- Few proteases
- No endotoxins
- No inclusion bodies
- No sporulation
- Non-aerobic and easily scalable
- Host for protein production/live delivery vehicle

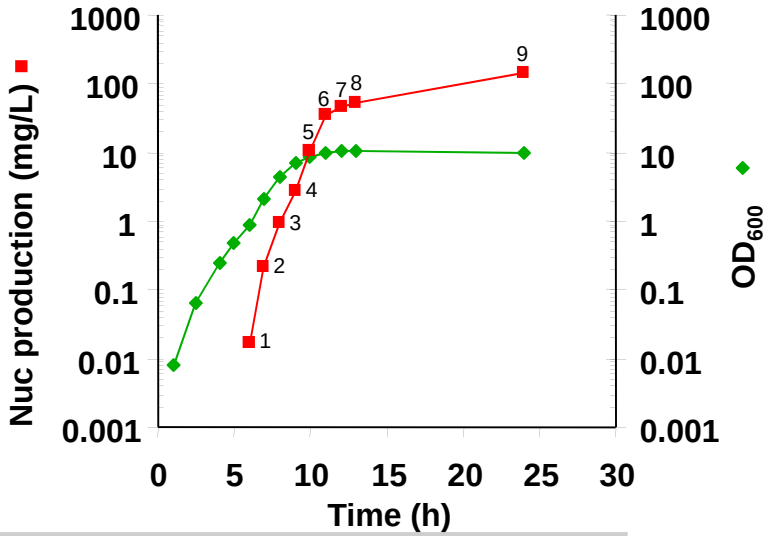
P170 Expression System

- Auto-inducible promoter (no need for addition of inducer)
- Secretion of recombinant proteins to the growth medium
- Non-aerobic and easy scalability
- Application of REED technology increases the biomass and titer of recombinant proteins more than 10 fold
- Phase II clinical trials have been undertaken using the P170 Expression System (malaria vaccine)

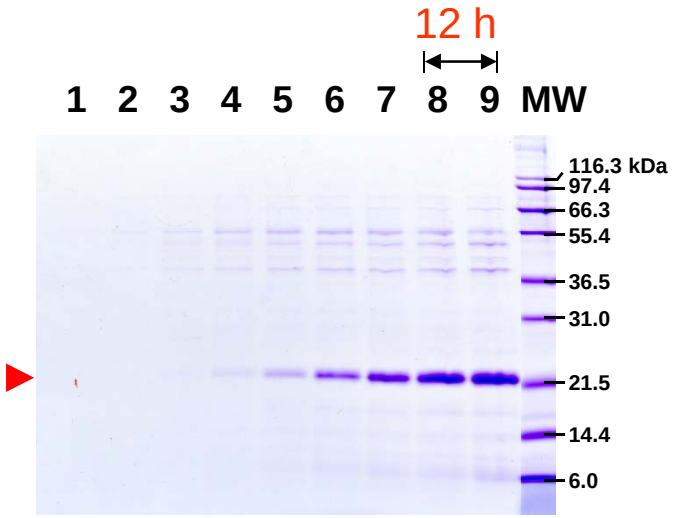
P170 fermentation technology

- Well-established batch and fed-batch technologies
- Chemically defined growth medium, free of animal protein, prions and vira
- Easy upscaling (no mass-transfer of oxygen)
- Utilize a novel, in-licensed membrane system (REED) to help increase OD600 to well over 150

Expression during pH controlled batch fermentation



L. lactis expressing the *S. aureus* nuclease was grown in pH controlled fermentor. Accumulation of lactic acid during growth induces the P170 promoter leading to production and secretion of the nuclease. Samples were taken for measurement of optical density and nuclease activity (secreted to growth medium).

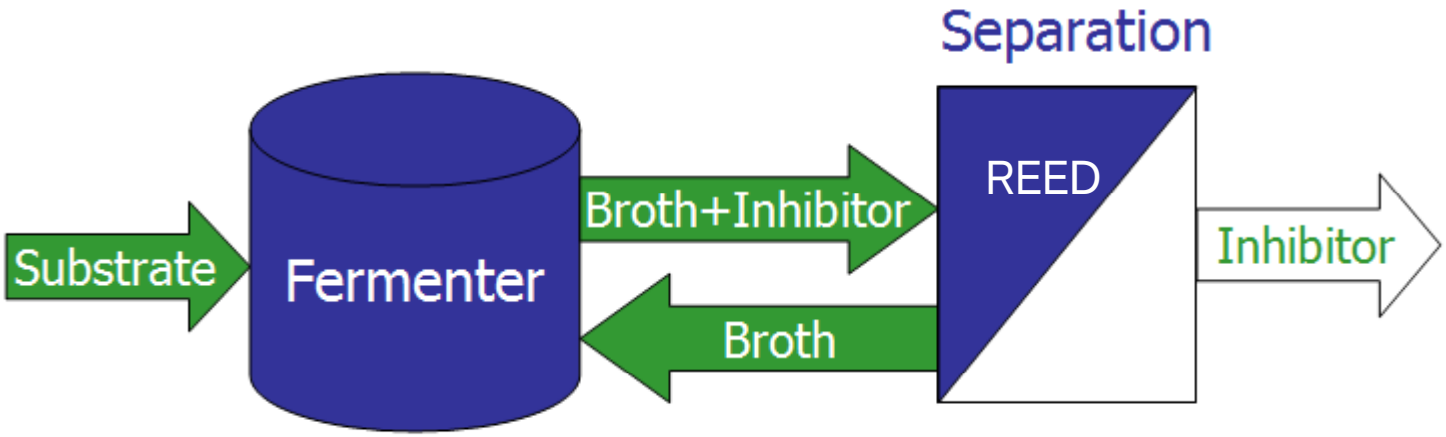


10 µl crude supernatant harvestede at the indicated time points was analyzed by SDS-PAGE and stained by coomassie blue. Estimated concentration of secreted nuclease 150mg/L.

Lactate: Growth inhibitor and inducer of the P170 promoter

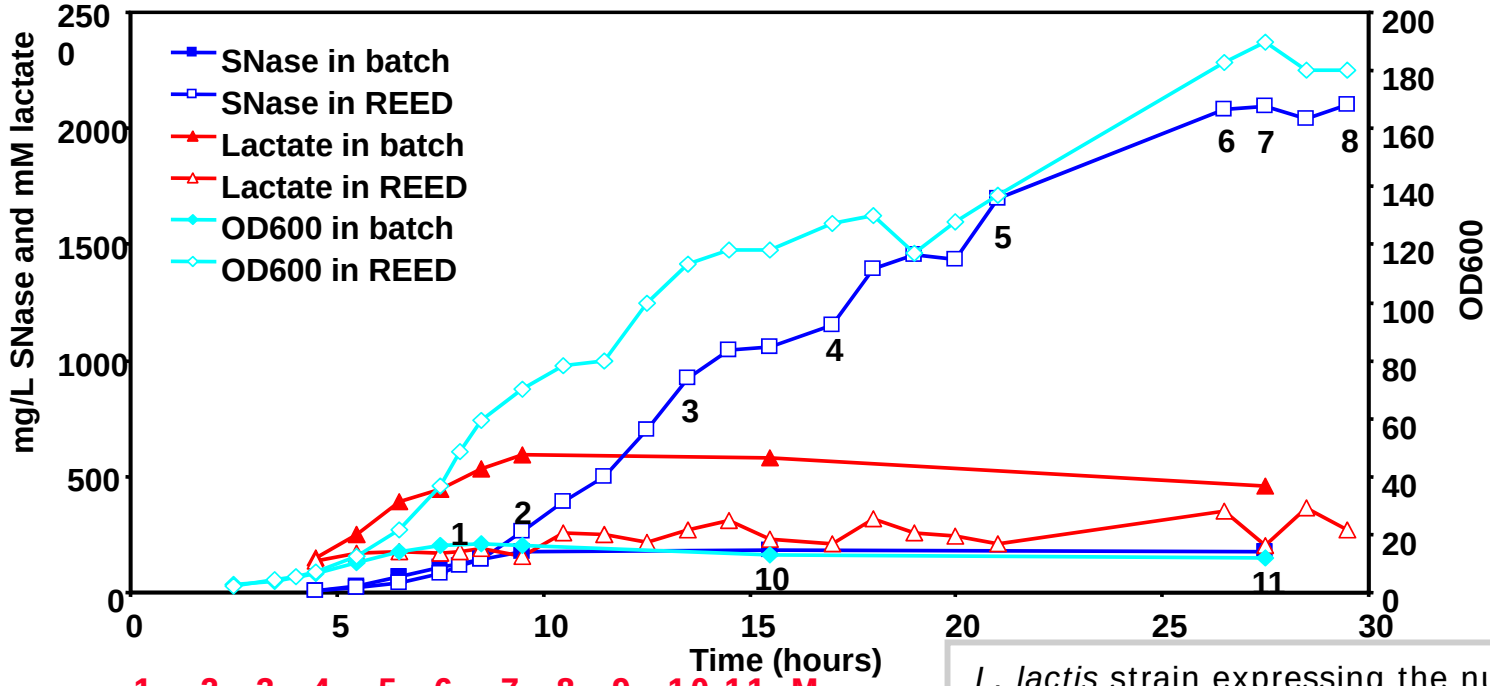
- As shown on the previous figure, growth is inhibited, which is due to accumulation of lactic acid in *L. lactis*
- As a consequence, biomass yield in *L. lactis* is 6-7 g/L cell dry weight vs 150-200 g/L (CDW) in *E. coli*
- Lactate concentration controls P170 promoter activity. The optimal level for production of recombinant proteins at pH 6.5 is 225-275 mM
- Therefore, a cell density independent control of lactate level would provide a dual advantage:
  - Extension of growth phase before P170 induction -> higher cell mass for production
  - Prolonged production phase in the P170 system
- A novel fermentation technology, REED, is able to control the lactate concentration allowing much higher biomass production

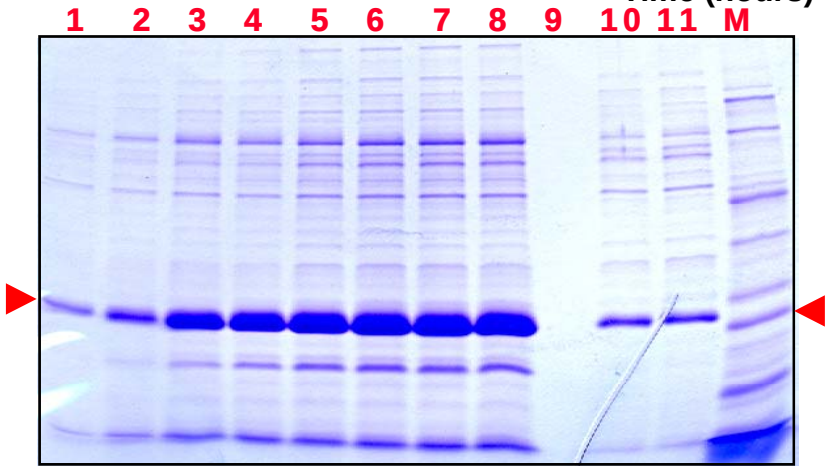
Reverse electro-enhanced dialysis (REED) for lactate control in the P170 system



- Direct removal of inhibiting by-products such as lactate through anion membranes
- Lactate ions replaced by hydroxide ions
- REED unit provides pH regulation of the fermentor
- Membranes allow passage of negatively charged molecules < 1 KDa
- Higher biomass and yield of recombinant protein production is obtained by combining the P170 expression technology and the REED system

Nuclease production using REED process





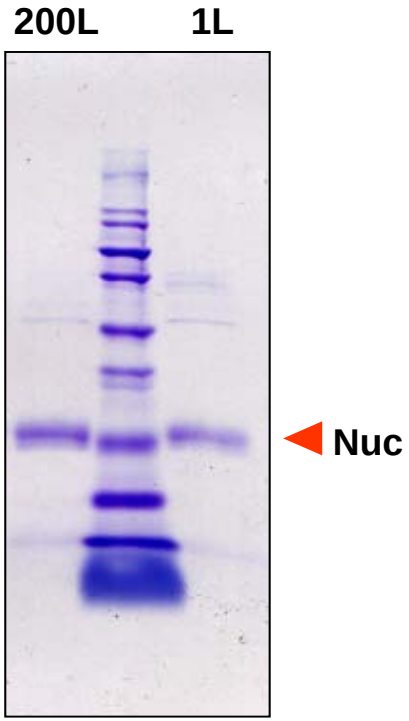
L. lactis strain expressing the nuclease was fermented in batch mode and compared to REED mode. Samples were taken at the indicated time points for determination of cell density, lactate concentration and nuclease activity.

2,5 µl crude supernatant was analyzed by SDS-PAGE using coomassie staining.

9 fold improvement was obtained in REED mode ~ 2000 mg/L

Up-scaling of fermentation volume

Up-scale experience from 1 L to 200 L (batch)



L. Lactis expressing the nuclease was grown in 1 L and 200 L fermentors. Product formation was analyzed by SDS-PAGE

Similar growth, products yields, purity and stability were demonstrated

In collaboration with a CMO the production of a pharmaceutical component was scaled up to 2000 L scale

Successfully secreted proteins

Protein	Origin	MW
Nuc	*Staph. aureus*	18,8
S	*Strep. spp*	31
MSP3-GLURP fusion	*P. falciparum* (malaria parasite)	74,6
GLURP	*P. falciparum*	56,1
X	*P. falciparum*	150
P1-5	Gram positive bacterium	24,5
MSP3	*P. falciparum*	20,1
P3-21	Gram positive bacterium	24,1
P3-17	Gram positive bacterium	21,9
L7/L12	*Brucella abortus*	13
MPT64	*M. tuberculosis*	22,4
dESAT6	*M. tuberculosis*	20,2
B1	Higher Plant	17
B2	Higher Plant	17
D	Arachniae	14
β-lactamase	*E. coli*	29
P1-7	Gram positive bacterium	22,7
β–lac globulin	Bovine	18
α–lac albumin	Bovine	14
P	Higher Plant	26
B-12 protein	Human	45
HOX	*C. crispus* (sea kelp)	62
tPA	Bovine	61
Plasminogen	Bovine	59
PAH	Human	54
PhhAB	*Pseudomonas aeruginosa*	46
Sheathlin	Porcine	43,5
P2-2	Gram positive bacterium	43,1
P3-1	Gram positive bacterium	23
CP	Gram positive bacterium	21,9

- MW 13-150 kDa, size **not** linked to yield
- Origin of proteins:
  - Gram + and Gram - bacteria
  - Plasmodium
  - Higher plants
  - Invertebrates
  - Mammals
- Production ranges up to 2 g/L using REED technology

IP and regulatory documentation for P170

Patents on P170 Expression System:

- WO94/16086: Recombinant LAB containing an inserted promoter
- WO98/10079: A LAB bacterial regulatable expression system
- WO01/10079: Method of isolating secretion signals in LAB and novel secretion signals isolated from *Lactococcus lactis*
- US20020117140: Improved method for production of heterologous gene products in LAB

Regulatory documentation:

- Report on the safety of the *Lactococcus* P170 system for the production of proteins for pharmaceutical use, prepared by an independent consulting company.
- The report supports and answers ICH* Q5B and Q5D; where it is possible, with the P170 system, to give:
  - Description of gene expression system, strain history etc.
  - Risk assessment, biohazard information

* International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

System summary

- Secretory system
- Several advantages (no endotoxins, no exogenous components for induction)
- Purity, stability and solubility of product
- Simple upscaling (1 L = 200 L)
- Non-animal source growth medium
- Optical density ~200 and 2 g/L of secreted product
- Several optimised production strains
- Exclusive licensee of innovative technologies (REED)
- Intellectual property rights
- Regulatory documentation prepared by independent consulting company

Feasibility study (I)

- Native or synthetic genes are produced by PCR or gene synthesis
- Genes are cloned into expression vectors
  - Test of different copy numbers
  - Intracellular vs extracellular
  - Antibiotic vs non-antibiotic based selection
  - Fusion tags
- Transformation of *L. lactis* strains
  - Wild type and mutant strains
- Flask experiments
  - Expression analysis by SDS-PAGE; coomassie staining and western blotting
- Fermentation experiments
  - 1 L fermentations
  - Expression analysis by SDS-PAGE
  - Plasmid stability
  - Optimization of fermentation media and process
  - 15 L fermentation

Feasibility study (II)

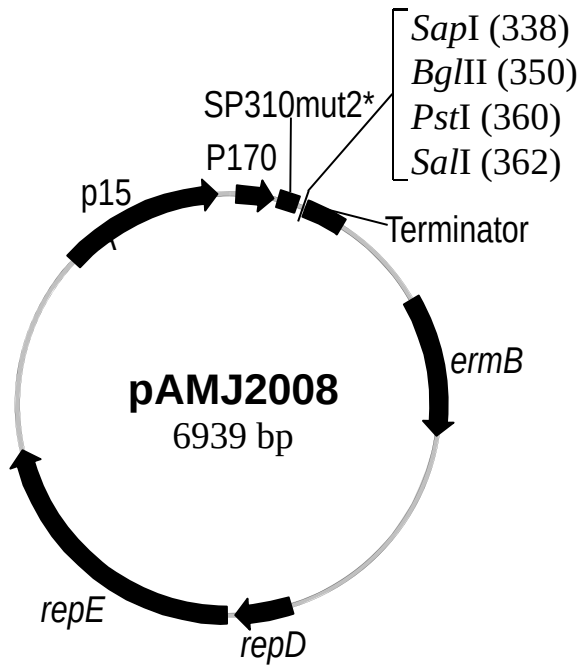
- REED fermentation
  - 1 L fermentation
  - Expression analysis by SDS-PAGE
  - Plasmid stability
  - Optimization of fermentation media and process
  - 15 L fermentation

- Purification and characterization of produced proteins
  - Ion exchange chromatography
  - Size exclusion chromatography
  - Hydrophobic interaction chromatography
  - Enzyme assays
  - Characterization by partners (MS, N-terminal sequencing etc)

Optimization of production strain

- Optimization of expression construct
  - Vector elements
  - Gene specific optimization
- Optimization of host strain
  - Mutagenesis of strain to increase the production of specific protein
- Optimization of fermentation
  - Medium composition
  - Fermentation process

Available P170 expression vectors



Vector	pCT1138A	pAMβ1	p15A	*ermB*	*tetK*	*hom-thrB*	SP310mut2	SP310mut2*
pAMJ398	•		•	•				
pAMJ397	•		•	•			•	
pAMJ328		•	•	•				
pAMJ399		•	•	•			•	
pAMJ2006	•		•	•				•
pAMJ2008		•	•	•				•
pAMJ2023	•		•		•		•	
pAMJ2024	•		•		•			•
pAMJ1222		•				•		•
pAMJ1223	•					•		•

Development of a new selection system (vectors and strains) that avoids the use of antibiotics is in progress.

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