Development of a novel culture-independent method for comprehensive detection and identification of beer-spoilage microorganisms

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   ～screening a membrane ×pressure cycling technology～
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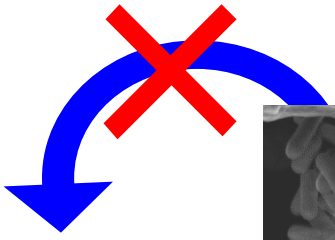
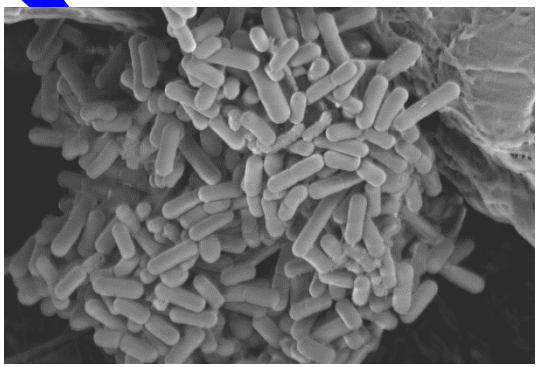
Features of Japanese beer

Unpasteurized beer has dominated the Japanese beer market.

Beer is a microbiologically stable beverage.





Beer-spoilage microorganisms

Stringent microbial quality control required.

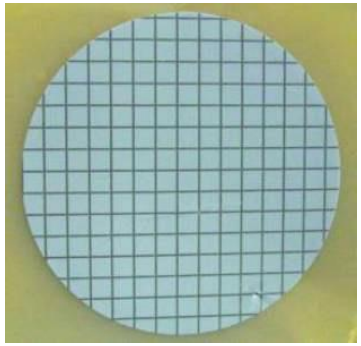
Beer-spoilage microorganisms

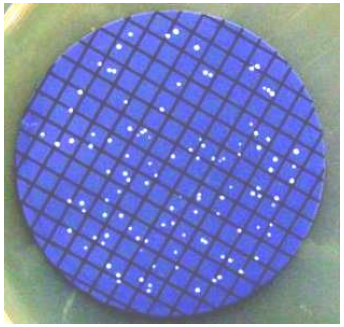
Lactobacillus	***Pediococcus***	***Pectinatus***
L. brevis	*Ped. damnosus*	*P. frisingensis*
L. lindneri	*Ped. claussenii*	*P. crevisiiphilus*
L. paracollinoides	*Ped. inopinatus*	*P. haikarae*
L. backi	other Pediococcus	Other Pectinatus
L. coryniformis	**Wild yeasts**	***Megasphaera***
L. paucivorans	*S. cerevisiae*	*M. cerevisiae*
L. casei /paracasei	*D. anomalla*	*M. paucivorans*
L. plantarum	*D. buruxellensis*	*M. sueciensis*
other lacobacilli	*B. custersianus*	

Currently new species are also emerging!

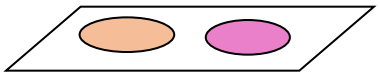
Conventional microbiological QC test

Beer filtration → **Culture** → **Identification**





- Microscopic observations
- Gram staining
- Catalase activities
- PCR tests e.t.c…

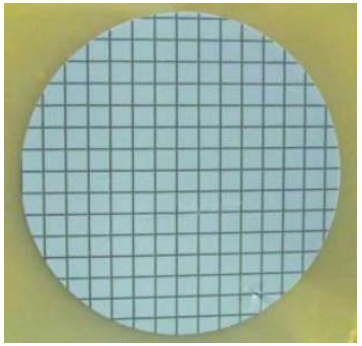


Rate-limited process

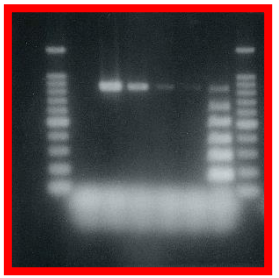
In some urgent cases, more rapid methods may be needed!

Would it be possible to detect without culture?

Beer filtration



Extract DNA directly



Limitations of culture-independent method

	species	membrane	filtration volume	detection limit
Tsuchiya, Y. (1992)	*Lactobacillus brevis*	PVDF	250	30 cells/250 mL
Tsuchiya, Y. (1993)	*L.brevis*	Polycarbonate	250	1-9 cells/250 mL
DiMichele, L. J. (1993)	*Lactobacillus*	Polycarbonate	50	20 cells/mL
Satokari, R. (1997)	*Pectinatus*	Polycarbonate	100	20 cells/mL
Yasui, T. (1997)	*L.lindneri*	PVDF	100	63 cfu/100 mL

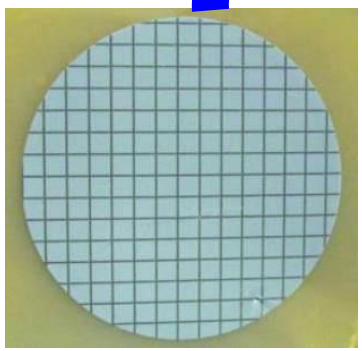
A comprehensive direct method with higher detection limits has been pursued for the past 20 years.

Purpose and strategy of this study

Purpose

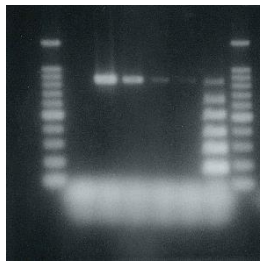
Development of a comprehensive highly sensitive culture-independent detection method

Beer filtration



Increase filtration vol.

Extract DNA efficiently



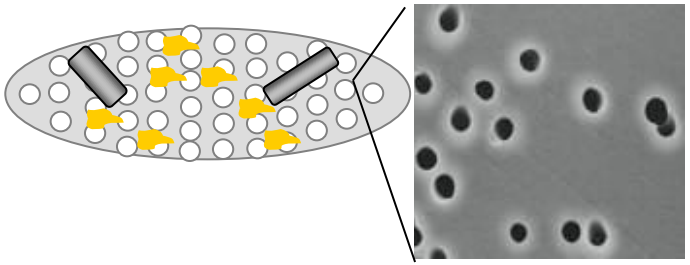
Multiplex PCR

Screening of a membrane

pros and cons of each membrane type

Polycarbonate membrane

Polycarbonate
(isopore type)



Advantages
Sensitivity is relatively high.
(approximately 10cells/membrane)

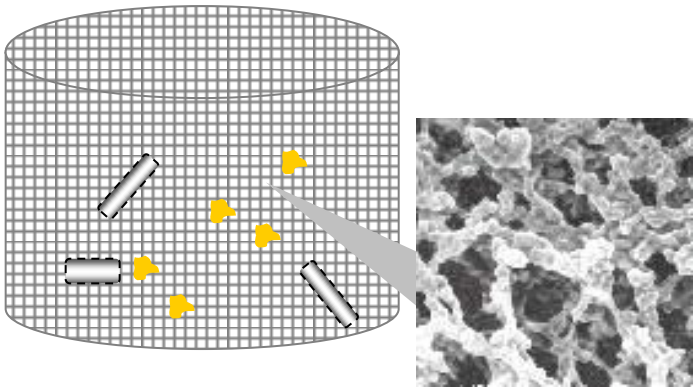
Disadvantages
Filtration volume is limited.
(up to 250ml)

Lotus root-like structure
(**Bacteria trapped on the surface**.)

A trace level of beer spoilers cannot be detected

Cellulose membrane

Mixed cellulose ester



<u>Advantages</u>
Filtration volume is larger.
(3000ml is possible)

<u>Disadvantages</u>
Sensitivity is low.
(Approx. 100 cells/membrane)

Mesh-like structure
(Bacteria trapped somewhere in the middle.)

Cellulose membrane → **Cells are buried in membrane.**
Cells and DNA must be recovered more efficiently.

Mixed cellulose ester membrane

×

Pressure cycling technology

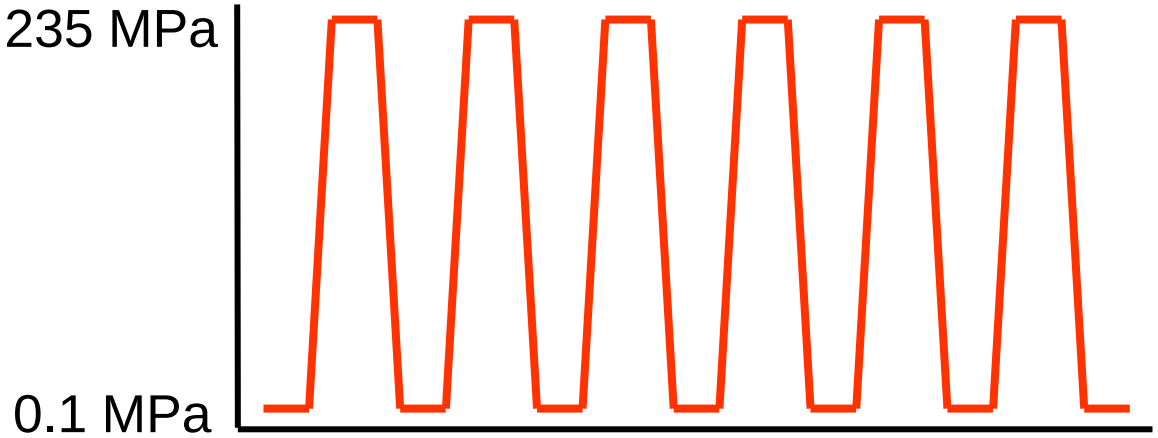
Pressure cycling technology (PCT)

Barocycler™ NEP2320

A novel approach for sample preparation method using alternating levels of hydrostatic pressure.

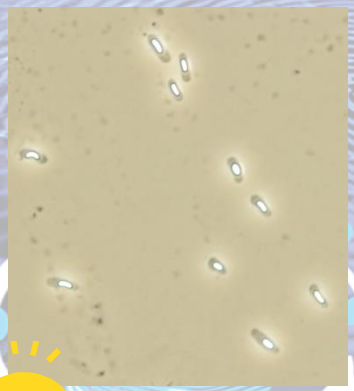
0.1 MPa ➡ 235 MPa : less than 3sec

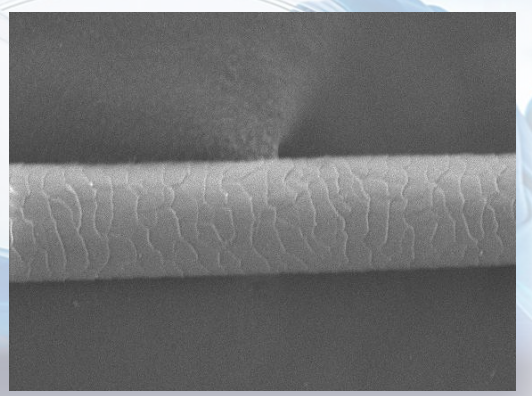
235 MPa ➡ 0.1 MPa : less than 1sec

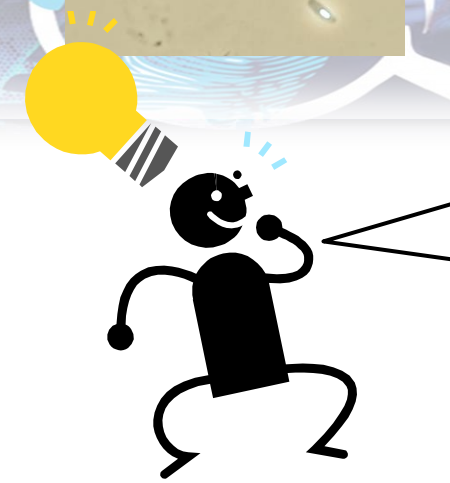


Performance of Pressure Cycling Technology

Significant improvements in DNA yield from challenging biological and forensic samples using Pressure Cycling Technology.

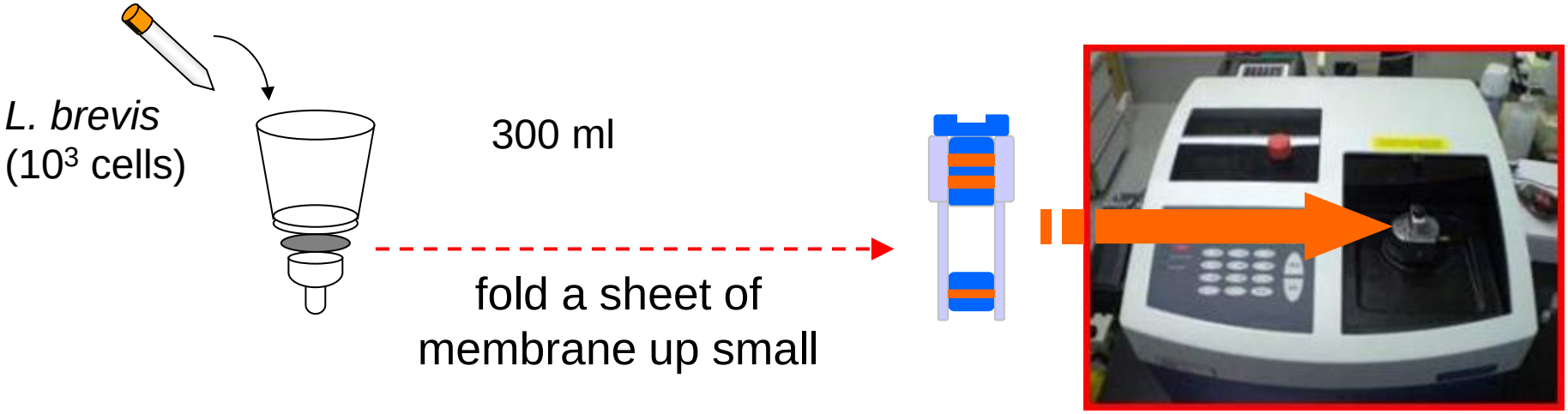






It might be possible to extract DNA from cells buried in membrane without disruption!

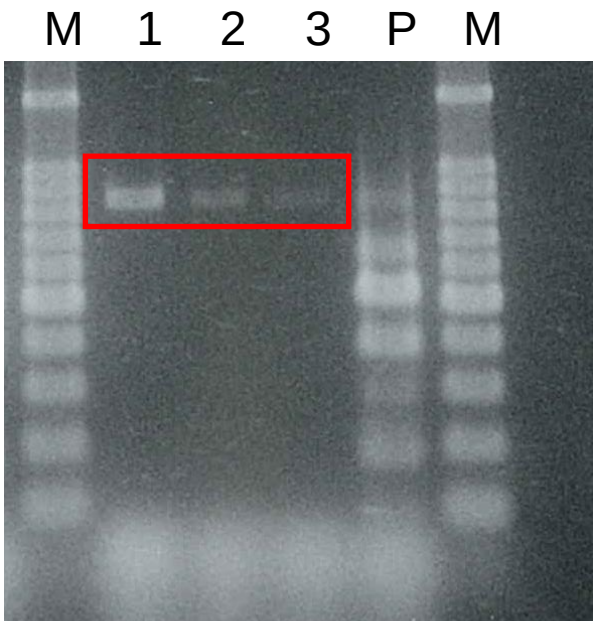
The performance of PCT for DNA extraction



Three extraction methods were compared

1. PCT with enzymatic treatment
2. Only enzymatic treatment
3. Only PCT

PCT was used once in this protocol.

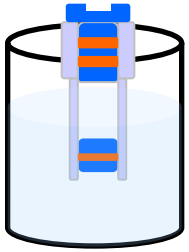


Schematic flow of DNA extraction

Pressure cycling technology

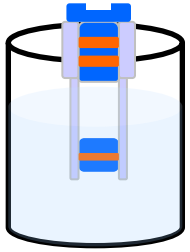
1

Incubation1
(37℃,30min)



2

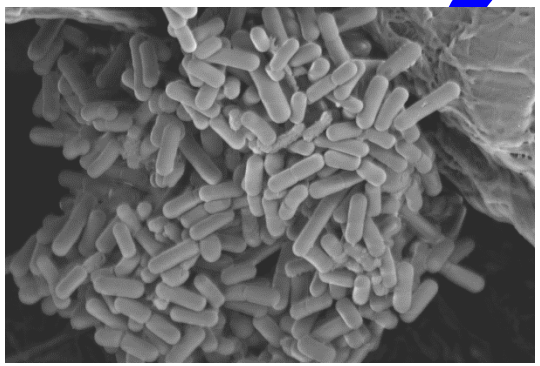
Incubation2
(50℃,60min)



3

Purification

Evaluation of sensitivity



Beer spoilage microorganisms

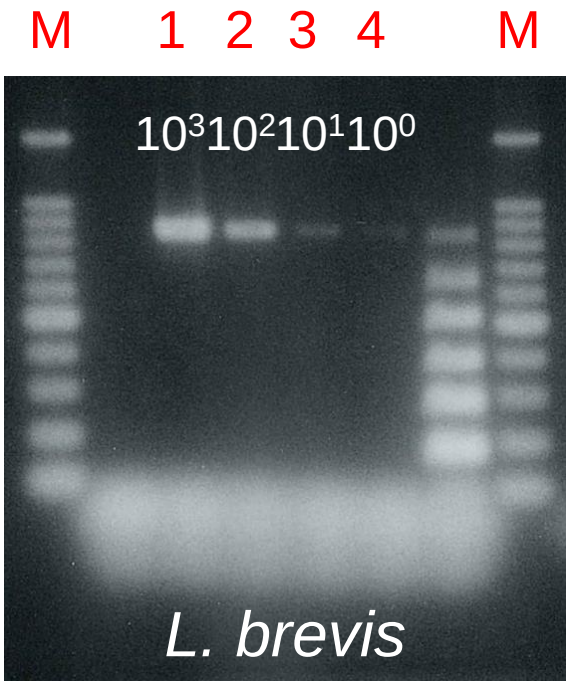
L. brevis
L. lindneri
L. paracollinoides
Wild yeasts

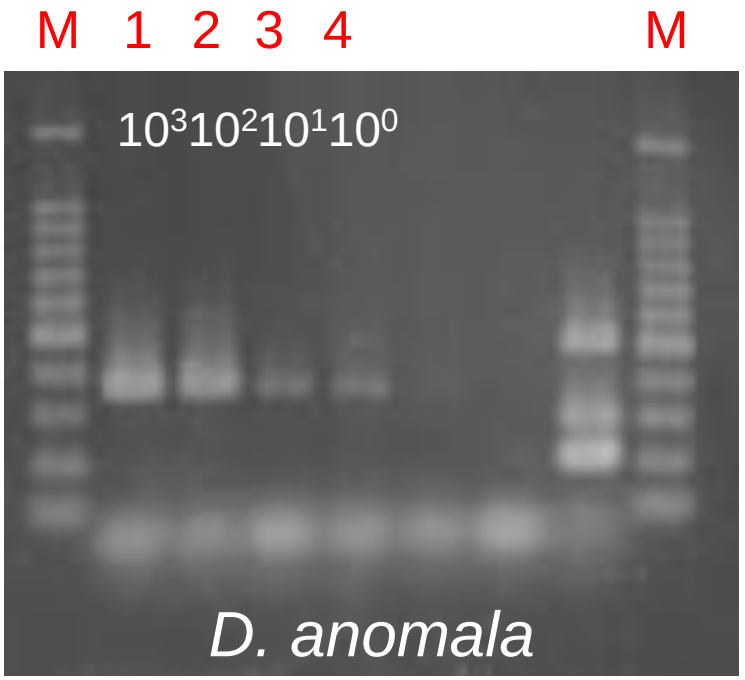
300 mL

DNA extraction with pressure cycling technology

Evaluation of sensitivity for beer spoiler

The results of *L. brevis* and *D. anomala* are shown here as an example.





As for three Lactobacillus species and four wild yeast species, the sensitivity was shown to lie between 10^0 and 10^1/300 ml of beer.

Mini summary 1

- The pressure cycling technology has enabled effective DNA extraction from the cells trapped within a cellulose membrane filter matrix.

- The detection limits of major beer-spoilage lactic acid bacteria, *L. brevis*, *L. lindneri*, *L. paracollinoides*, wild yeast (the genera *Saccharomyces* and *Dekkera*/*Brettanomyces*) species, were found to be as low as 10^0 cells/membrane (300ml beer).

Other lactic acid bacteria and strictly anaerobic bacteria are also reported as beer spoilers…

Lactobacillus	***Pediococcus***	***Pectinatus***
L. brevis	*Ped. damnosus*	*P. frisingensis*
L. lindneri	*Ped. claussenii*	*P. cresiliphilus*
L. paracollinoides	*Ped. inopinatus*	*P. haikarae*
L. backi	other Pediococcus	Other Pectinatus
L. coryniformis		
L. paucivorans		
L. casei/paracasei		
L. plantarum		
other lactobacilli		

10^{1} cells/membrane

Pectinatus $10^{1\text{-}2}$ cells/membrane

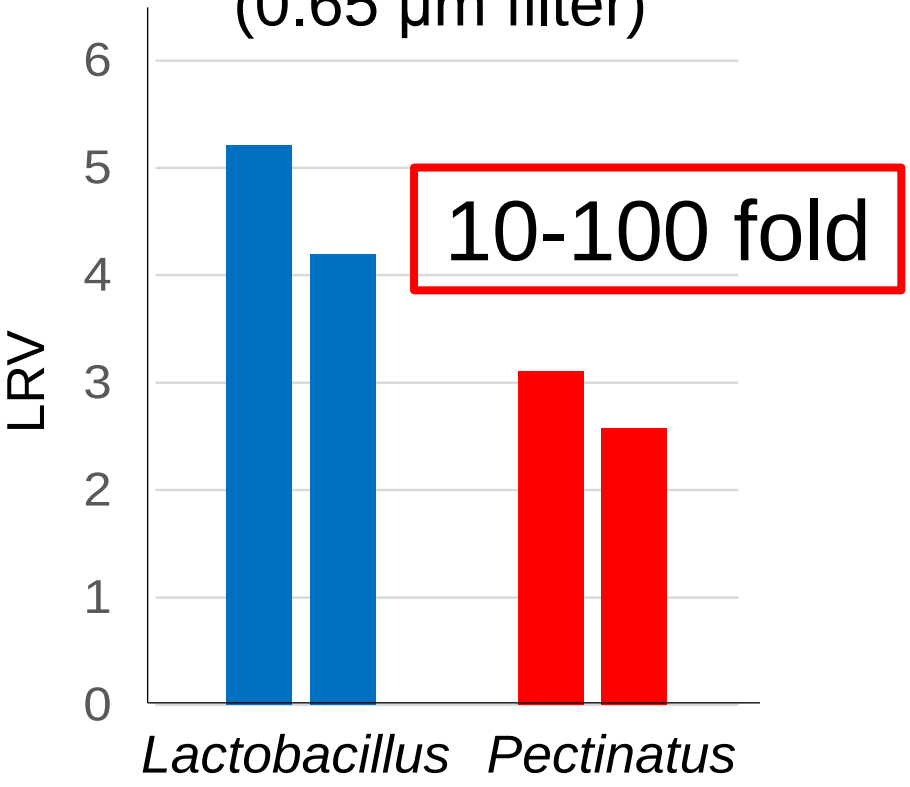
Wild yeasts	***Megasphaera***
S. cerevisiae	*M. cerevisiae*
D. anomalla	*M. paucivorans*
D. buruxellensis	*M. sueciensis*
B. custersianus	

10^{1} cells/membrane

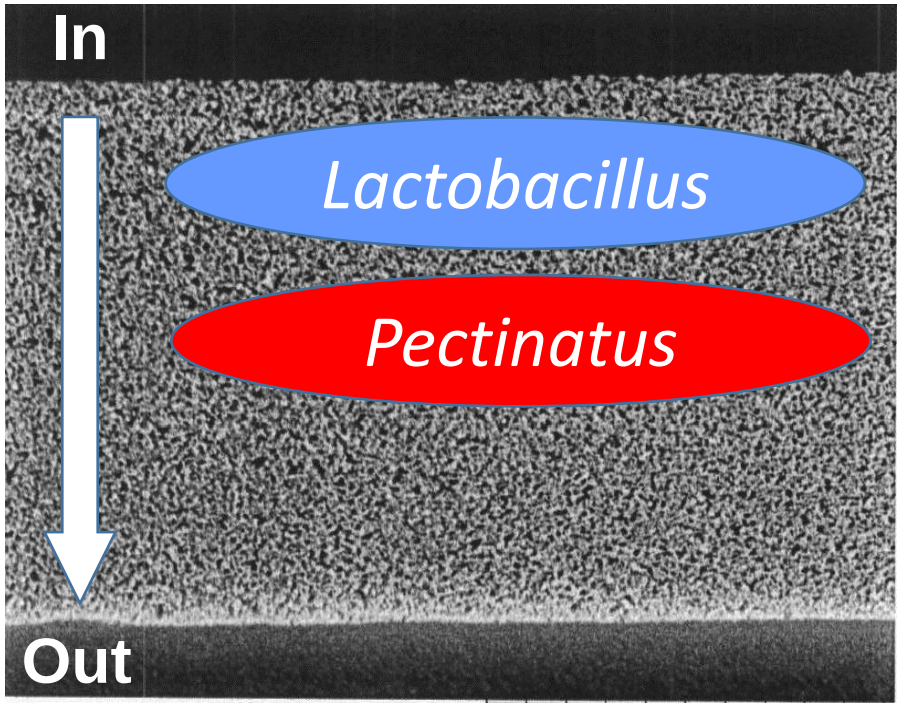
Differences between *Pectinatus* and *Lactobacillus*

Pectinatus is reported to be more likely to pass through membrane filter than *Lactobacillus.*

Log Reduction Value; LRV
(0.65 μm filter)

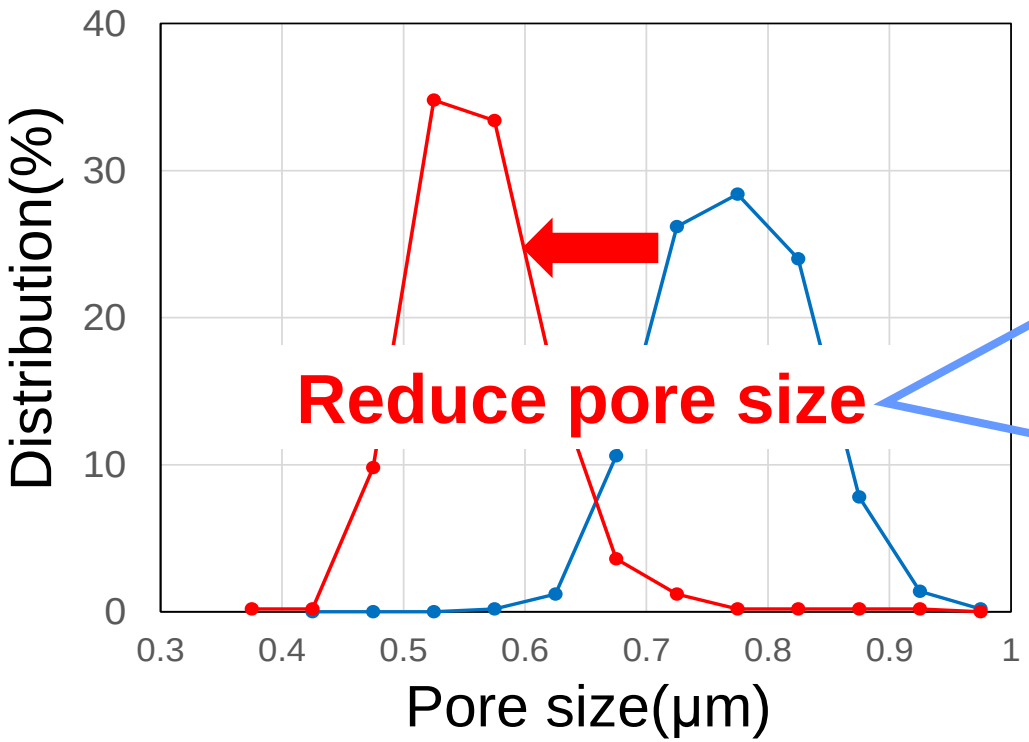


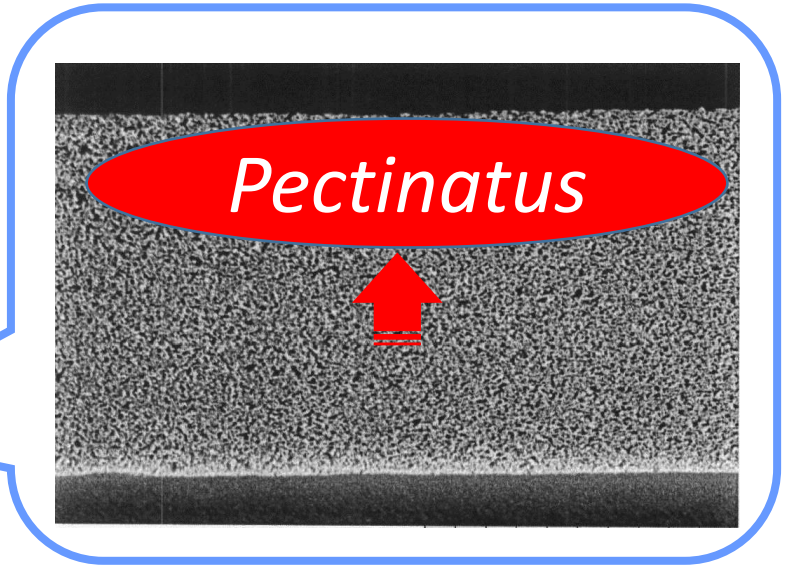
Cross-section structure
of membrane



Screening of a more optimal membrane

Optimization of pore size for cellulose membrane

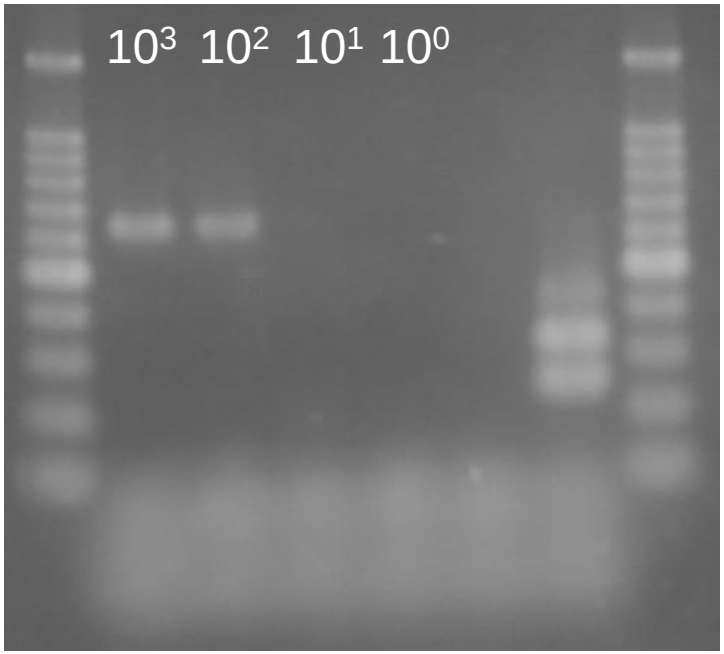


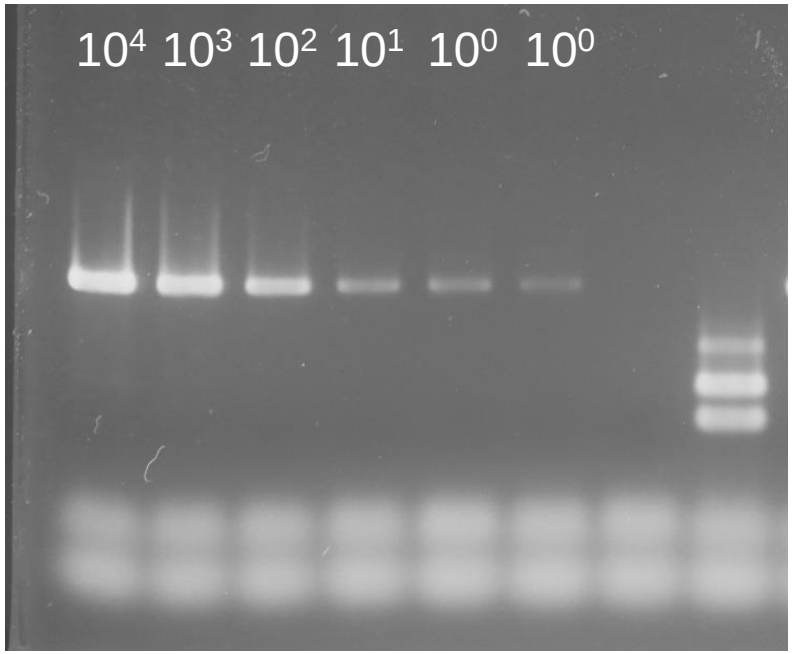


Improvement of the DNA extraction efficiency
by trapping the cells closer to the surface

The result of optimization of membrane

The results of *P. cerevisiiphilus* are shown here as an example.

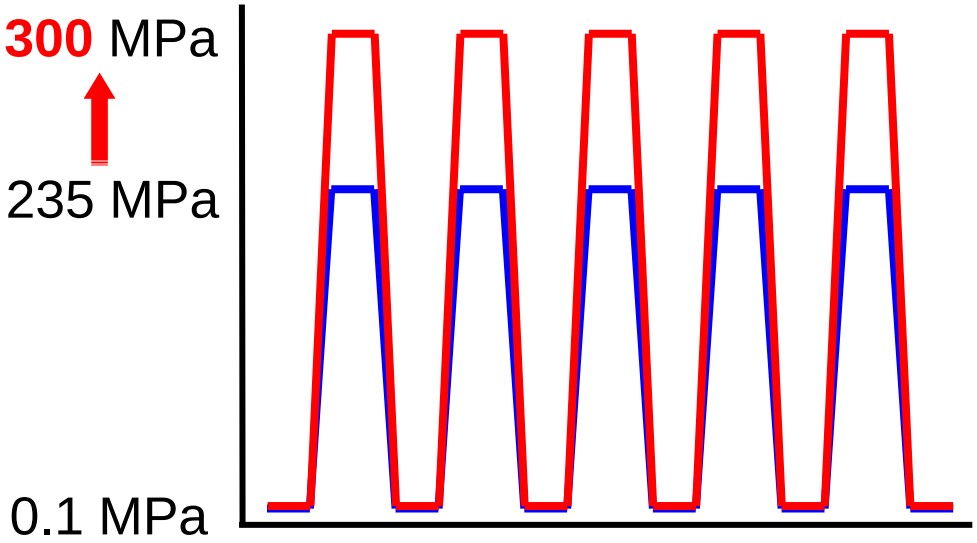




The modified approach was found to be applicable to all of the beer-spoilage *Pectinatus* species.

To further improve extraction efficiency

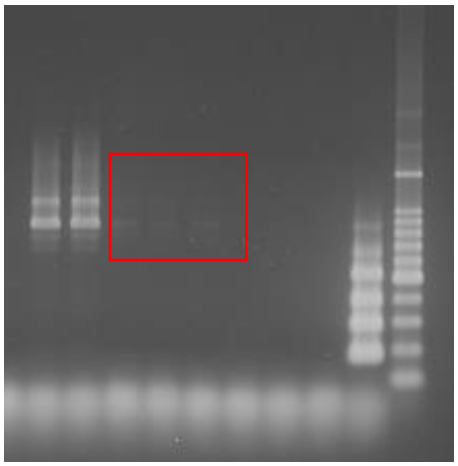
Higher pressure (300 Mpa) model was adopted



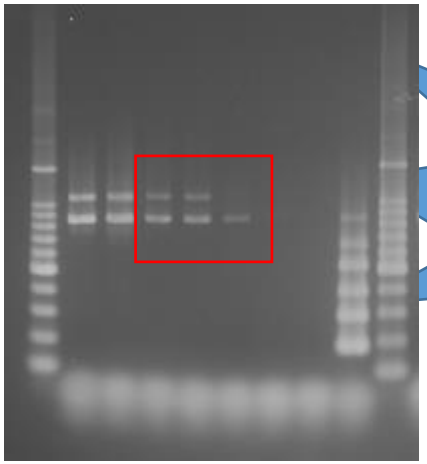
To further improve sensitivity

Vacuum concentrator

Improves the sensitivity
by evaporating DNA solution
with vacuum concentrator.

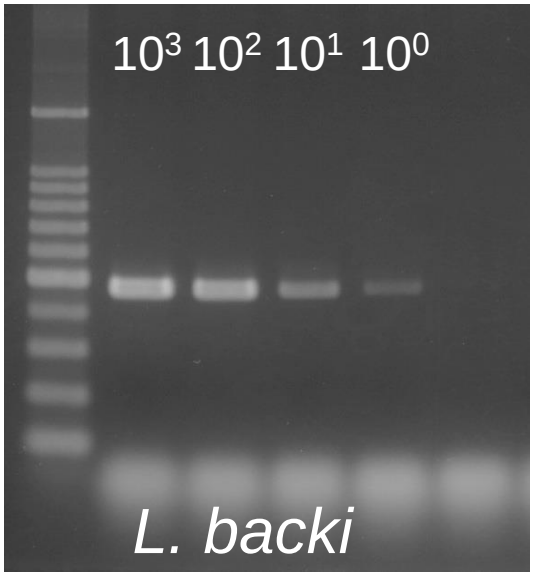


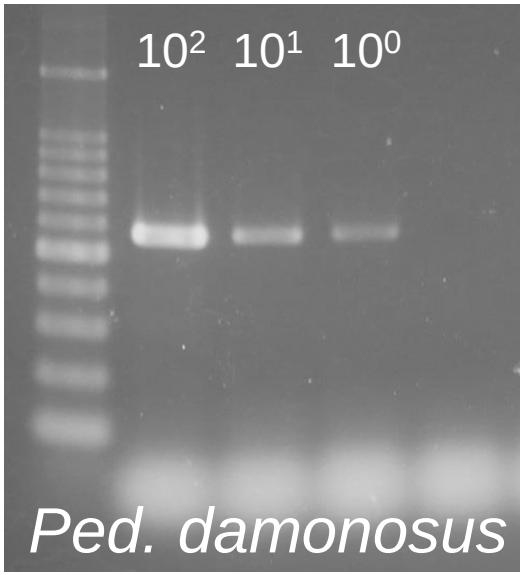
10-fold
concentrated

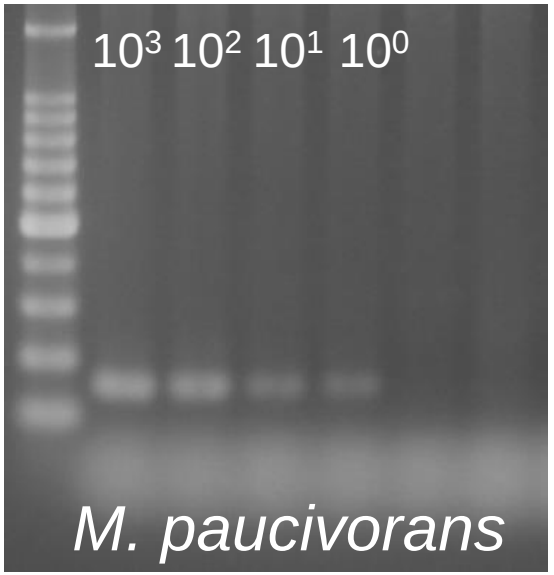


Recovers a trace
amount of DNA

Result of using a wider range of beer spoilers







The identical detection limits were accomplished.

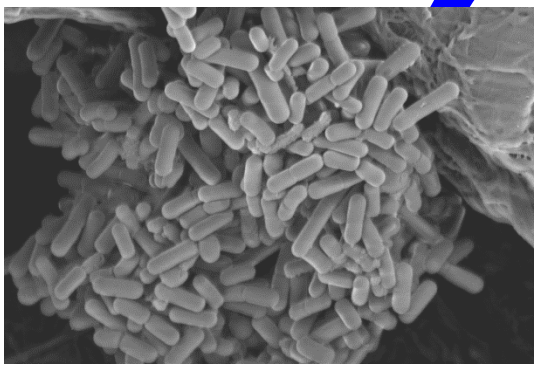
Our modified method was also shown to be applicable to other *Lactobacillus*, *Pediococcus* and *Megasphaera.*

Mini summary 2

Series of measures in combination finally allow the detection of 22 species of beer-spoilage microorganisms with the detection limits of 10^0 cells/membrane

Lactobacillus	***Pediococcus***	***Pectinatus***
L. brevis	*Ped. damnosus*	*P. frisingensis*
L. lindneri	*Ped. claussenii*	*P. crevisiiphilus*
L. paracollinoides	*Ped. inopinatus*	*P. haikarae*
L. backi	**Wild yeasts**	***Megasphaera***
L. coryniformis	*S. cerevisiae*	*M. cerevisiae*
L. paucivorans	*D. anomalla*	*M. paucivorans*
L. casei /paracasei	*D. buruxellensis*	*M. sueciensis*
L. plantarum	*B. custersianus*	

If filtration volume is increased up to 3000 mL

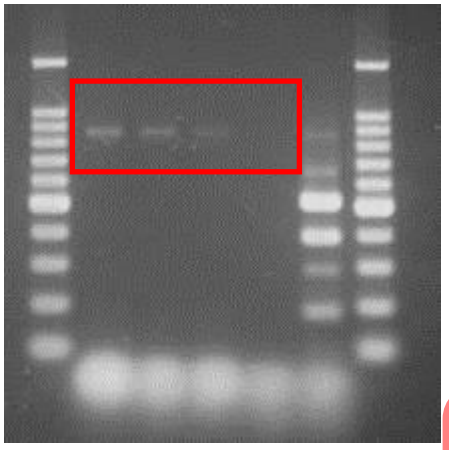


L.brevis

3000 mL

DNA extraction with pressure cycling technology

Increased filtration volume



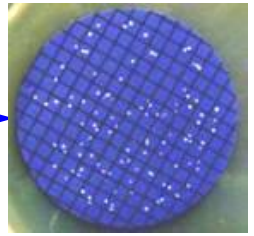
Inoculation level (cfu)

1	10^2 cells
2	10^1 cells
3	10^0 cells

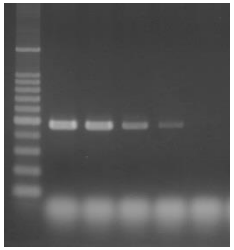
Detection limit
10^0 cell/3,000ml

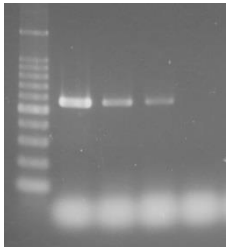
A trace amount of bacteria in larger volume of beer was detected.

3-14 days



Culture





Direct detection
(8hrs)

Summary

- The pressure cycling technology has enabled effective DNA extraction from cells trapped within a cellulose membrane filter matrix.
- Series of measures in combination finally allow the detection of 22 species of beer-spoilage microorganisms with detection limits of 10^0 cells/membrane.
- Our method is able to cope with an extremely low level of contamination (10^0 cell/3000ml-beer).

Comprehensive detection and identification of low levels of beer-spoilage microorganisms is achieved by direct PCR

Thank you for your attention