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The Secret of Indigenous Vinegar Making

-Discovering new microbial communities that have been passed down for 300 years-

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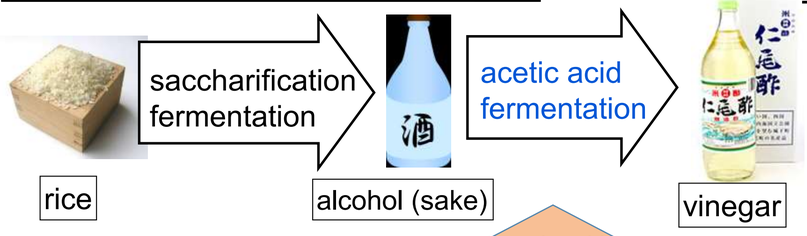
Abstract

In vinegar breweries that produce vinegar through stationary fermentation method, the bacterial biofilm formed on the acetic acid fermentation liquid surface has been carefully cultivated over generations. While it is known that acetic acid bacteria are present in this biofilm, the types of bacteria and the functions they play have yet to be determined.

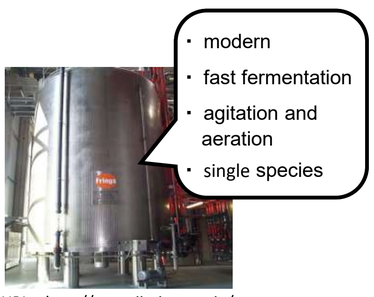
With this in mind, I conducted research to identify the types and functions of the bacteria in the acetic acid fermentation broth. First, I amplified 16SrDNA fragments contained in the fermented liquor and biofilm from samples taken from a vinegar factory *(Nakahashi Zosu*), a company founded approximately 300 years ago in Kagawa Prefecture. Then I separated the samples into separate types using DGGE, and identified them by looking at their base sequences inside the separated bands. As a result, five acetic acid bacteria types, one strain of lactic acid bacteria, and two kinds which are thought to be contaminants were identified. Because the contaminants were eliminated as the fermentation process proceeded (i.e., when the acidity rose), one might postulate that stable vinegar making is possible without strict sterilization operation procedures. Furthermore, these results suggest that the existence of various species of bacteria contributes to producing uniquely-flavored vinegar.

Introduction

1. How to make vinegar



submerged fermentation (acetator) or **standing fermentation** (Japanese cedar (*sugi*) cask)



- modern
- fast fermentation
- agitation and aeration
- single species

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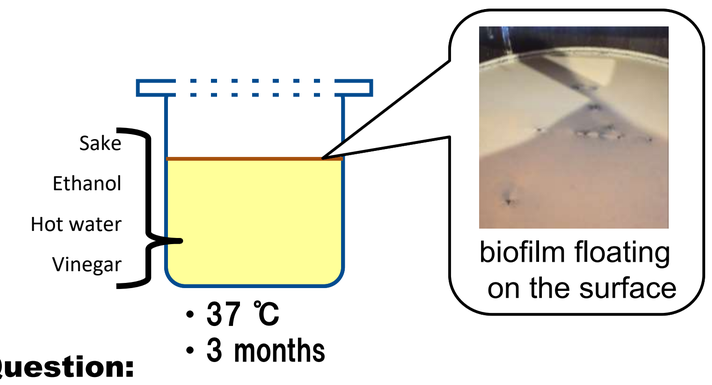
- traditional
- slow fermentation
- flavorful
- various species ?

2. What is standing fermentation?



- 37 ℃
- 3 months

Question:

- Perhaps saprophytes are mixed because the fermentation takes place in a open environment?
- What is the secret of the 'rich taste' we see in vinegar produced by standing fermentation?

Purpose

- **discovering the species of bacteria inside the cask of standing fermentation**
- **analyzing the functions of the bacteria**

Methods

3. Sampling

Nakahashi-zousu founded in Edo period

Japanese cedar (sugi) casks used over a hundred years linking up in a storehouse

sampling

- Visiting *Nakahashi-zousu* in Mitoyo city, Kagawa prefecture.
- Acetic acid bacteria has been carefully cultivated for 275 years by scooping up the biofilm from a cask that holds solution in later stage of fermentation using a basin, and planting it into a cask that holds solution in early stage of fermentation.
- Obtained biofilm and fermented liquor from the cask of

1. Early stage of fermentation (planted September 15: the third week of fermentation)
2. Middle stage of fermentation (planted September 15: the seventh week of fermentation)

(this research was supported by Japan Science and Technology Agency (JST))

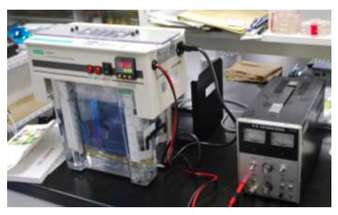
4. Sort out and identify the bacteria

PCR ⇨ DGGE ⇨ PCR ⇨ DNA sequence ⇨ BLAST® search

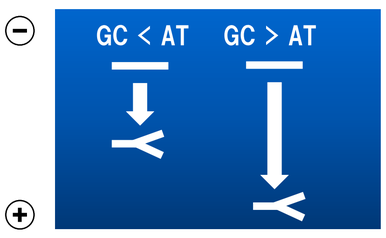
- After amplifying 16SrDNA[※1] fragment from the sample,[※2] I separated them into each type using DGGE.
- Then I amplified the separated 16SrDNA fragment, and identified them by looking at their base sequences.

(※1 16SrDNA: DNA which codes one of the ribosome RNA. Often used in identifying microorganism)

(※2 denaturing gradient gel electrophoresis (DGGE). Separating mixed bacterias by utilizing the fact that the content rate of GC differs depending on its specie even in same16SrDNA. (high content rate of GC = difficult to denature = long electrophoresis)



Electrophoretic apparatus



Primer Sequence

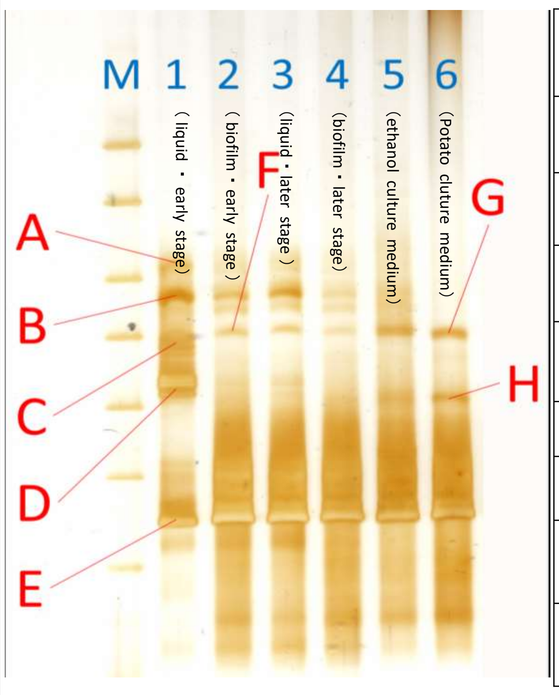
WBAC1	5′-GTCGTCAGCTCGTGTCGTGAGA-3′
WBAC2[GC]	5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCCGGGAACGTATTCACCGCG-3′

1	liquid	3 weeks	
2	biofilm	3 weeks	
3	liquid	7 weeks	
4	biofilm	7 weeks	
5	liquid	7 weeks	ethanol culture medium
6	liquid	7 weeks	potato culture medium

Results

5. The results of DGGE and database retrieval

+++ : very dense band, very many bacteria
++ : dense band, many bacteria
+ : light band, few bacteria
− : cannot confirm the band, very few bacteria or none



	species	early stage of fermentation	later stage of fermentation	features
A	*Gluconacetobacter intermedius*	+++	+	•make acetic acid by oxidizing ethanol •make cellulose
B	Uncultured *Acetobacter* sp.	+++	++	•make acetic acid by oxidizing ethanol
C	*Paenibacillus* sp.	++	−	•polysaccharide decomposition [1] •strong renitence against acetic acid [3]
D	*Lactobacillus acetotolerans*	+++	−	•lactic acid bacteria separated from rice vinegar[2] •can grow under pH3.3
E	Uncultured *Acetobacter* sp.	+++	+++	•Make acetic acid by oxidizing ethanol
F	*Saccharibacillus kuerlensis*	++	+	•cannot grow in low pH environment
G	*Acetobacter senegalensis*	potato culture medium +++		•Make acetic acid [3] •make catalase
H	*Acetobacter pasteurianus*	ethanol culture medium +++		•high acetic acid production capacity [4] •resistant to acid

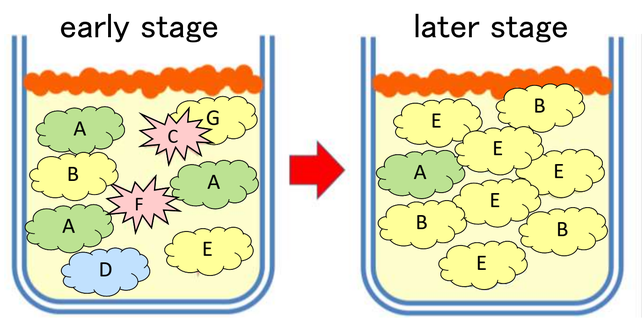
※ the coincidence was not 100% but between 70 to 97% in any case

- The result suggests that there are 8 species of bacteria
- Amplify and refine the DNA after extracting band A to H
- Search the DNA database using BLAST® after analyzing the DNA sequences

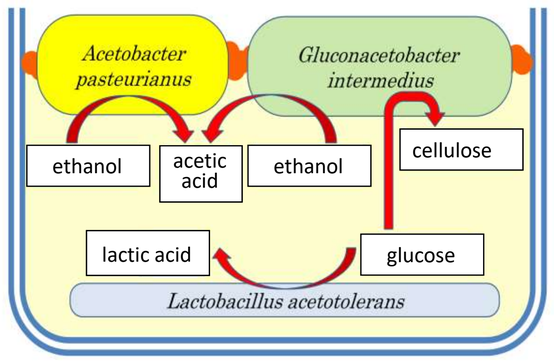
- There were 8 species of bacteria.
- There were multiple species acetic acid bacteria.
- As the fermentation proceeds the fewer the species of bacteria found.

Discussion

6. The function of bacteria in fermented liquid



- Bacteria (C•F) —which were thought to be contaminating bacteria— were reduced by the rise of acidity.
- This process has made safe vinegar production possible since ancient times, even without strict sterilization procedures.



- The various functions of bacteria may be affecting the taste of the vinegar
- Isolation and culturing of the bacteria is needed in order to understand each bacteria's function and to read longer sequences to conduct a more concrete identification.

Conclusions

Bacteria relating to standing fermentation were analyzed using DGGE and by running a sequence analysis in order to identify the species of bacteria passed down from Edo period and their functions.

I was able to identify 8 species of bacteria including bacteria other than lactic acid bacteria.

Through this research we cay assume that:

⇒ the existance of multiple species of bacteria in the fermented solution results in the rich taste of vinegar

⇒ the reproduction of saprophytes are suppressed by the rise of acidity as the fermentation proceeds

References

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3) Ndoye,B et al. (2007) Int.J. Syst.Evolutionary Microbiology, **57**, 1576–1581.
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