

Oral Session III

Bioprocess and Bioengineering with Acetic Acid Bacteria

III-1: The Development of a System for Rice Vinegar Fermentation using Acetators

T. Aritomi, M. Izawa, K. Asada, H. Morimoto

Production Technology Center, Mizkan Sanmi Co., Ltd., Handa 475-8585, Japan.
E-mail: taritomi@mizkan.co.jp

One of our products, *Komesu* (rice flavored distilled vinegar), has a comparatively high rice extract content. This extract mainly consists of glucose, and the glucose concentration of *Komesu* is about 10%. At high extract and low ethanol concentration in submerged continuous fermentation, unfavorable oxidation and flavors are more easily produced, for example, glucose to gluconic acid. Therefore, submerged fermentation methods for high extract vinegar, which less produce less organic acids other than acetic acid, are required.

We have developed a two-step fermentation system. The first step is a seed providing process using a continuous fermentation acetator. The seed is maintained at a low extract concentration similar to spirit vinegar fermentation. This condition suppresses the generation of unfavorable oxidation and flavors. For the second main fermentation, the seed is transferred to another acetator where fed-batch fermentation is performed by feeding high concentration extract. The gradual increase of extract can shorten the period of high concentration and improve the flavor.

Gluconacetobacter polyoxogenes, which can create high acidity, has been chosen for the rice vinegar fermentation because it generates more preferable flavor and low-level gluconic acid than other acetic acid bacteria whose growth rate is higher under the high extract condition.

However, *G. polyoxogenes* is very sensitive to the lack of oxygen, which occurs in the seed transferring process. To enable a non-aeration time of less than 20 seconds, the seed is transferred to the main-fermentation acetator at the high flow rate, and the special main-fermentation acetator has been equipped for starting the aeration at the low fluid volume.

According to these features, the lag phase and death of seed at the beginning of the main fermentation can almost be disregarded.

The flow rate of the entering high extract feed stream is gradually increased because high acidity acetic acid bacteria is more sensitive to the osmotic pressure stress compared with other acetic acid bacteria.

In addition, we have made it possible to stop the fed-batch fermentation automatically by the execution of an online ethanol measurement with an NIR sensor for labor saving.

This two-step fermentation method using the acetic acid bacteria maintained under the low extract environment can be used to produce different high extract vinegars not just *Komesu*. To produce different vinegars efficiently, our system can also share the seed in the first acetator with several acetators with different kinds of entering feeds such as fruit juices. This means that our system is very suitable for high quality, high-mix low-volume production.

III-2: Population Dynamics and Kinetic Parameter Analysis of Acetic Acid Bacteria during Industrial Type Fermentation Trials

Dr. Frank Emde

Present address: H. Frings GmbH & Co. KG, Germany, Jonas-Cahn-Str. 9, D-53115 Bonn, Germany

E-mail: emde@frings.com

Acetic acid bacteria populations used for industrial vinegar fermentation at high acidities are sensitive to a number of influences like temperature, oxygen transfer, nutrient availability, substrate and product concentrations and others. Fluctuations of process performance will be the result if these process conditions change.

Uninterrupted semi-continuous processes are the aim of the industrial fermentation strategy. State of the art in the vinegar industry are repeated batch and repeated fed batch processes for submersible fermentations. In these, the population has to be maintained in a defined window of concentrations of the inhibiting substrate (ethanol) and the inhibiting product (acetic acid) to provide process stability. The cyclic change of high and low acetic acid and ethanol contents within a fermentation cycle allows the 'in process' selection of these bacteria which maintain their acid resistance and are able to grow. However, for high percentage acetic acid fermentations it is absolutely necessary not to change these conditions for a long period of time to lower acidities. Otherwise, different strains of the bacteria population or mutants, adapted to the new process conditions, will overgrow the former predominant population. It is well known in the industry that it is very difficult to reactivate the population to the previous high concentration of acetic acid.

A series of laboratory experiments has been performed to investigate the described phenomena in small scale. The response of the population to the change of process parameters was analysed by aid of kinetic studies, determination of enzyme activities and a number of microbiological methods. It was tried to answer the question whether a shift in the process conditions supports the growth of individual strains of a complex mixed culture or induces the change of the bacteria itself. The overall kinetic 'constants' and the metabolic variables have been determined for fermentations of various raw materials of agricultural origin. It will be shown how the investigation of the dynamic response to a change of process conditions can be used to optimize the industrial process performance and stability.

III-3: Biofilm formation in the mixed-culture of yeasts, lactic acid bacteria, and acetic acid bacteria isolated from Fukuyama pot vinegar brewing samples.

Soichi Furukawa¹, Kanako Yoshida¹, Natsumi Nojima¹, Eriko Suda¹, Hirokazu Ogihara¹, Makari Yamasaki², and Yasushi Morinaga

¹*Department of Food Science and Technology, College of Bioresource Sciences, Fujisawa, 252-8510, Japan*

²*Advanced Research Institute for the Sciences and Humanities, Nihon University, Tokyo 102-0073, Japan*

E-mail: furukawa@brs.nihon-u.ac.jp

Traditional Japanese fermentation of mashes to make rice-vinegar is characterized by the collaboration of lactic acid bacteria (LAB), yeasts and acetic acid bacteria (AAB), and by the fermentation in the heterogeneous liquid-solid mixture which contained steamed rice and rice Koji (culture of the koji mold, *Aspergillus oryzae*, grown on and within steamed rice). These properties of the traditional Japanese fermentation prompted us to investigate the possibility that LAB, yeasts and AAB can form a mixed-species biofilm during fermentation, and we found previously some positive combinations of LAB and yeasts in which LAB produced some extra-cellular factor(s) supporting the formation of yeast biofilm in a single culture (1). This time we focused on the biofilm formation in the mixed culture of yeast-LAB and AAB-LAB which were isolated from the samples of the Fukuyama pot vinegar, a Japanese traditional and domestic rice-vinegar. Brewing of Fukuyama pot vinegar is conducted in roughly capped large pots laid in the open-air without any refined culture-manipulation.

Ten-yeasts, 124-LABs and 26-AABs were isolated from 5th and 11th-day's brewing samples, and mixed-species biofilm formation in LAB-yeast and LAB-AAB combinations were investigated. There were totally 14 positive combinations between LAB and yeast, and the combination of the strain identified as *Lactobacillus plantarum* ML11-11 and the strain identified as *Saccharomyces cerevisiae* Y11-43 showed the most remarkable biofilm formation. Both strains were isolated from 11th-day's sample.

In contrast to our previous study (1), the biofilm formation in the mixed culture of *L. plantarum* ML11-11 and *S. cerevisiae* Y11-43 requires a direct cell-cell contact. This interaction is solely dependent on the particular property of *L. plantarum* ML11-11, i.e., some other budding yeast also formed biofilm in the mixed culture with *L. plantarum* ML11-11. Scanning electron microscopic (SEM) observation showed that the direct cell-cell contact of the both microorganisms in the extraordinarily thick (at least 100 µm) biofilm.

There were also many positive combinations between LAB and AAB. We hypothesize that mixed-cultured biofilm formation among yeast, AAB and LAB would contribute to the traditional rice-vinegar fermentation.

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III-4: A survey of some traditional acetification systems from XVIth to XXth century

Jacques F. Bourgeois

Consultant for H. Frings, D Bonn, 19 Route de Chevrier, CH- 1244 Choulex, Switzerland.

E-mail: jacques.bourgeois@bluewin.ch

A good grasp of background history is always beneficial for the deeper understanding of a subject, and forms a basis for a significant contribution to its advance. Vinegar is no exception.

A rapid historical survey from the XVIth to the XXth century serves to order and situate the principal landmarks which have contributed to the present level of scientific knowledge, and of the technological advance in vinegar production.

At the end of the XVIth century the knowledge about making vinegar had not progressed further than a range of different technics, all empirical, with uncertain production results, and no scientific knowledge to speak of. The identification of the importance of wood shavings and of air (Boerhaave), the description of the mother of vinegar, the first proposition of an elaboration of a chemical equation (Döbereiner) lead to the development of a rapid and more stable method (Schützenbach). As soon as scientific knowledge established landmarks, the resultant profit contributed to the rapid development of corresponding technology.

This progress prepared the way for the important contribution of Pasteur: the presence of "Mycoderma aceti", the importance of oxygen in the atmosphere for the oxidation of ethanol to acetic acid, the possibility of continuous production, and eventually the production of high strength vinegar. The submerged fermentation system and computerization have both contributed to these last aspects of technological advance.

From the end of the XIXth century and throughout the XXth century, the development of industrial equipment and process significantly increased the production and the quality of vinegar. Probably the most significant landmark was the development of the submerged system in 1949.

This communication attempts a rapid historical review, with corresponding references, of the fundamental advancements which have contributed to the present understanding and knowledge of the elaboration of vinegar.

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