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Phenotypic Characterization and RAPD-PCR Profiling of *Acetobacter* sp. Isolated from Spirit Vinegar Production

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Summary

For the isolation of acetic acid bacteria from the »seed vinegar« culture, laboratory vinegar production was initiated with a culture for spirit vinegar production. The second and the third cycles of the process were followed. Samples for the isolation of acetic acid bacteria from the bioprocess liquid were taken four times during each cycle. A total of forty-seven strains were isolated. They were phenotyped as **Acetobacter** *sp. Forty-one of them were identified as* **A. europaeus.** *Differentiation among 13 strains of* **A. europaeus** *and four other* **Acetobacter** *strains not identified as* **A. europaeus** *was done by random amplification of polymorphic DNA (RAPD) analysis. Seven different RAPD profiles were identified. From this it was assumed that in spirit vinegar genotypically different strains of acetic acid bacteria were present.*

Keywords: vinegar, *Acetobacter* sp., RAPD analysis

Introduction

Vinegar is a product of microbial oxidation of ethanol containing substrate to acetic acid. The process is certainly as old as wine. First industrial vinegar production dates back to 14th century. In spite of its long tradition, commercial vinegar production is still initiated with an undefined inoculum (»seed vinegar«), taken from a previous batch. The commonest technological process for the production of spirit vinegar is submersion technology performed in acetators. The process is semicontinuous, with the starting concentration of acetic acid 70–100 g/100 L and ethanol volume fraction about 5% in each cycle, for the production of vinegar with more than 10 g acetic acid per 100 mL. When ethanol fraction in the fermentation liquid drops down to 0.05–0.3%, a part of the bioprocess liquid is replaced with a new ethanol substrate (*1*).

The reason why the industry still does not use a pure defined starter culture can be explained by the difficulties concerning isolation, cultivation and especially preservation of microorganisms producing high percentage vinegar. Great progress in the isolation strategy of *Acetobacter* strains for high acetic acid production has been achieved with the double-layer agar of Entani *et al.* (*2*). They found that acetic acid in the cultivation medium was essential for the growth of strains from industrial vinegar bioreactors. This new medium allowed them to isolate *Acetobacter polyoxogenes* from Japanese vinegar bioreactors (*2*). Using the modified Entani double-layer agar medium Sievers *et al.* (*3*) isolated *Acetobacter* species from German and Swiss industrial bioreactors and generators using submersion technology. The isolated strains have been identified as a new species – *Acetobacter europaeus*. The main phenotypic characteristic of the species is an absolute requirement of acetic acid for growth (*3*). However, Sievers and Teuber (*4*) have later reported that the isolation of acetic acid bacteria from high percentage vinegar is also possible with a simplified modification of Entani double-layer agar medium into a single-layer agar.

For the strain characterization of cultures producing high percentage vinegar, Sievers *et al.* (*3*) used plasmid analysis. From the isolated colonies they obtained an identical plasmid profile as from the bioreactors liquid. From these results they assumed that a major part of the microflora of an acetator was composed of one strain (*3,4*).

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Several papers have been published about random amplification of polymorphic DNA (RAPD) technique, as a convenient one for the strain differentiation of bacterial species, e.g. *Listeria monocytogenes* (*5*), *Campylobacter jejuni* (*6*), *Mycobacterium malmoense* (*7*), *Legionella pneumophila* (*8*), *Staphylococcus haemolyticus* (*9*), *Aeromonas salmonicida* subsp. *salmonocoda* (*10*), *etc.* The aim of our work was to test RAPD analysis for the genotypization of the previously phenotyped *Acetobacter* strains.

Materials and Methods

Running of the laboratory bioprocess

A laboratory bioreactor for submerged fermentation (equipped with a blade stirrer) with the working volume of 10 litres was used to activate *Acetobacter* strains. To start and run the process »seed vinegar« was taken from a bioreactor for commercial spirit vinegar production at the vinegar factory in Ljubljana. The substrate (composed of acetic acid and ethanol in the proportion 2.5 : 1) and the »seed vinegar« were mixed in a proportion 19 : 1. The process was carried out in a semicontinuous manner (temperature 30 °C, agitation 1100 rpm, yield 80%). When the volume fraction of ethanol dropped down to 0.5%, 20% of the liquid was replaced by a fresh alcoholic substrate. Ethanol fraction was measured densitometrically and the concentration of acetic acid by titration with 0.1 M NaOH. Bacterial biomass was followed by measuring the absorbance (λ = 620 nm) of the cultivation medium and it reached the value 0.15.

Isolation and maintenance of the bacterial strains

Acetobacter strains were isolated from a laboratory bioreactor for spirit vinegar production. Daily sampling of the bacterial cultures (0.1 mL of the cultivation liquid) was done during the second and the third cycles of the bioprocess. Strains were isolated on the modified Entani agar (*4*). Up to five colonies were removed from each plate and transferred to a fresh medium for further purification. Single, well isolated colonies were kept on the modified Entani agar at 30 °C and transferred onto a fresh medium every tenth day.

Morphological and biochemical characterization

Stained films (the Gram method) of all the isolated strains were examined microscopically. Catalase and oxidase tests were performed (*11*). The ability of growth of each strain without acetic acid in the cultivation medium was tested on the GYC agar medium (*12*). An acid tolerance to 4 and 6% of acetic acid in the cultivation medium was performed for each strain on the modified Entani agar medium. The ability to overoxidize acetic acid to CO_2 and H_2O of the strains that did not have absolute requirement of acetic acid in the cultivation medium, was analysed on the Frateur and on the Carr agar media (*12*).

DNA isolation procedure

A single isolated colony of each strain was streaked onto four plates of the modified Entani agar medium. The plates were incubated for four to five days at 30 °C and 92–96% relative humidity. Biomass was harvested in 2 mL microcentrifuge tubes. DNA was isolated by a modification of the method of Marmur (*13*). After washing with 50 mM Tris (pH = 8) and 1 mM EDTA, the biomass was resuspended in 1 mL of lysozyme solution (5 mg/mL). The solution was incubated at 37 °C for 15 min. After the addition of 20 μL of proteinase K solution (20 mg/mL) and 20 μL of 10% sodium dodecyl sulphate, the mixture was incubated at 37 °C for 15 min or until the solution cleared. The solution was extracted with one volume of phenol – chloroform – isoamylalcohol (25 : 24 : 1). The organic extraction was repeated twice. Total DNA was precipitated by the addition of one volume of isopropanol and 0.1 volume of 3 M sodium acetate.

RAPD analysis

PCR was carried out in 10 μL solution containing 15–30 ng of *Acetobacter* strains DNA, 2 mM $MgCl_2$, 20 pmol of primer, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer), 200 μM each of dCTP, dGTP, dATP and dTTP (Perkin-Elmer) and 1 μL 10× PCR buffer II (Perkin-Elmer). Six different primers were tested (decamer primers with GC content 50–80% and two microsatellite primers – $(GACA)_{4x}$ and $(GTG)_{5x}$). On the basis of the number and the reproducibility of the amplified bands, three primers were selected for the differentiation of *Acetobacter* strains (Table 1). A Perkin-Elmer Gene Amp PCR System 2400 was used for amplification. The cycling programme started with initial denaturation of DNA at 94 °C for 5 min and continued with 35 cycles at 94 °C for 30 s, at 36–54 °C (depending on the annealing temperature of the primer) for 45 s and at 72 °C for 45 s. At the end, a final extension at 72 °C for 7 min was performed, followed by cooling to 4 °C. The PCR product was submitted to electrophoresis in 1.8% agarose gel containing 0.5 μg/mL ethidium-bromide and 1xTris acetate running buffer. The DNA molecular weight marker VI (Boehringer) was used as a length standard. DNA fragments were visualised by transillumination and photographed with a Polaroid camera.

Table 1. Primers tested for RAPD analysis of *Acetobacter* strains

Primer	Sequence	RAPD patterns
50% G+C	5'-TGG TCA GTC A-3'	little amplification
60% G+C	5'-CGG TCA CTG T-3'	little amplification
70% G+C	5'-AGC GGG CGT A-3'	excellent patterns
80% G+C	5'-CGC GTG CCC A-3'	excellent patterns
$(GACA)_{4x}$	5'-GACA GACA GACA GACA-3'	little amplification
$(GTG)_{5x}$	5'-GTG GTG GTG GTG GTG-3'	excellent patterns

All the primers were supplied with Codon Genetic System

Results and Discussion

In our previous experiments we optimised the conditions for cultivation of acetic acid bacteria from spirit vinegar on agar media (*14,15*). We succeeded in cultivating these bacteria only if vinegar samples were taken directly from the running bioprocess and spread on agar media. The microorganisms spread on agar media from the bottled unfiltered spirit vinegar did not grow because of the stress factors which have not been com-

pletely elucidated. One of the reasons could be lack of oxygen, especially from the time of sampling until the start of the plating procedure. In this work we succeeded in isolating 47 strains of acetic acid bacteria from the vinegar samples which were taken from the second (23 strains) and the third (24 strains) cycles of the laboratory bioprocess of spirit vinegar production (Table 2). Our intention to isolate at least five colonies every twelfth hour of the bioprocess failed. In spite of several attempts we did not succeed in isolating bacterial colonies at the start and after twelve hours of the second cycle of the bioprocess in the laboratory (Fig. 1). At sampling point 1 we failed to cultivate more than two colonies. Sometimes, the isolated colonies even stopped growing after two or more preculturings, probably because of oxygen deficiency (4).

All the strains isolated from spirit vinegar were gram-negative small rods. They were catalase-positive and oxidase-negative. Forty-one strains out of a total of 47 strains had an absolute requirement of acetic acid in the cultivation medium (Table 2). This characteristic is a major phenotypic characteristic of *Acetobacter europaeus* (3). The rest of six strains had the ability for overoxidation of acetic acid. Owing to this phenotypic charac-

Table 2. Phenotypic characterization of 47 *Acetobacter* strains and RAPD types of 17 strains generated by PCR fingerprinting with the primers 70% G+C, 80% G+C and $(GTG)_{5x}C_2$

Strain No.	Sampling point	Absolute requirement of acetic acid in cultivation medium	Identified *Acetobacter* sp.	RAPD types generated with three different primers		
Second cycle				70% G+C	80% G+C	$(GTG)_{5x}$
SI/1	1	+	*A. europaeus*	not characterized		
SI/2	1	+	*A. europaeus*	not characterized		
SI/3	2	+	*A. europaeus*	A_1	B_1	C_1
SI/4	2	+	*A. europaeus*	A_2	B_2	C_2
SI/5	2	+	*A. europaeus*	not characterized		
SI/6	2	+	*A. europaeus*	not characterized		
SI/7	2	+	*A. europaeus*	not characterized		
SI/8	3	+	*A. europaeus*	not characterized		
SI/9	3	+	*A. europaeus*	not characterized		
SI/10	3	+	*A. europaeus*	not characterized		
SI/11	3	+	*A. europaeus*	A_3	B_3	C_1
SI/12	3	+	*A. europaeus*	A_3	B_3	C_1
SI/13	3	+	*A. europaeus*	not characterized		
SI/14	3	+	*A. europaeus*	not characterized		
SI/15	3	+	*A. europaeus*	not characterized		
SI/16	4	+	*A. europaeus*	not characterized		
SI/17	4	+	*A. europaeus*	not characterized		
SI/18	4	+	*A. europaeus*	not characterized		
SI/19	4	+	*A. europaeus*	A_3	B_3	C_1
SI/20	4	–	not identified	not characterized		
SI/21	4	–	not identified	A_4	B_4	C_3
SI/22	4	–	not identified	not characterized		
SI/23	4	–	not identified	A_4	B_4	C_3
Third cycle						
SII/1	5	–	not identified	A_5	B_5	C_4
SII/2	5	+	*A. europaeus*	not characterized		
SII/3	5	+	*A. europaeus*	A_2	B_2	C_2
SII/4	5	+	*A. europaeus*	not characterized		
SII/5	5	+	*A. europaeus*	not characterized		
SII/6	5	–	not identified	A_5	B_5	C_4
SII/7	6	+	*A. europaeus*	not characterized		
SII/8	6	+	*A. europaeus*	not characterized		
SII/9	6	+	*A. europaeus*	A_2	B_2	C_2
SII/10	6	+	*A. europaeus*	not characterized		
SII/11	6	+	*A. europaeus*	A_5	B_6	C_4
SII/12	6	+	*A. europaeus*	not characterized		
SII/13	6	+	*A. europaeus*	not characterized		
SII/14	7	+	*A. europaeus*	A_3	B_3	C_1
SII/15	7	+	*A. europaeus*	not characterized		
SII/16	7	+	*A. europaeus*	A_2	B_2	C_2
SII/17	7	+	*A. europaeus*	not characterized		
SII/18	7	+	*A. europaeus*	A_5	B_7	C_5
SII/19	7	+	*A. europaeus*	not characterized		
SII/20	8	+	*A. europaeus*	A_2	B_2	C_2
SII/21	8	+	*A. europaeus*	not characterized		
SII/22	8	+	*A. europaeus*	not characterized		
SII/23	8	+	*A. europaeus*	A_2	B_2	C_2
SII/24	8	+	*A. europaeus*	not characterized		

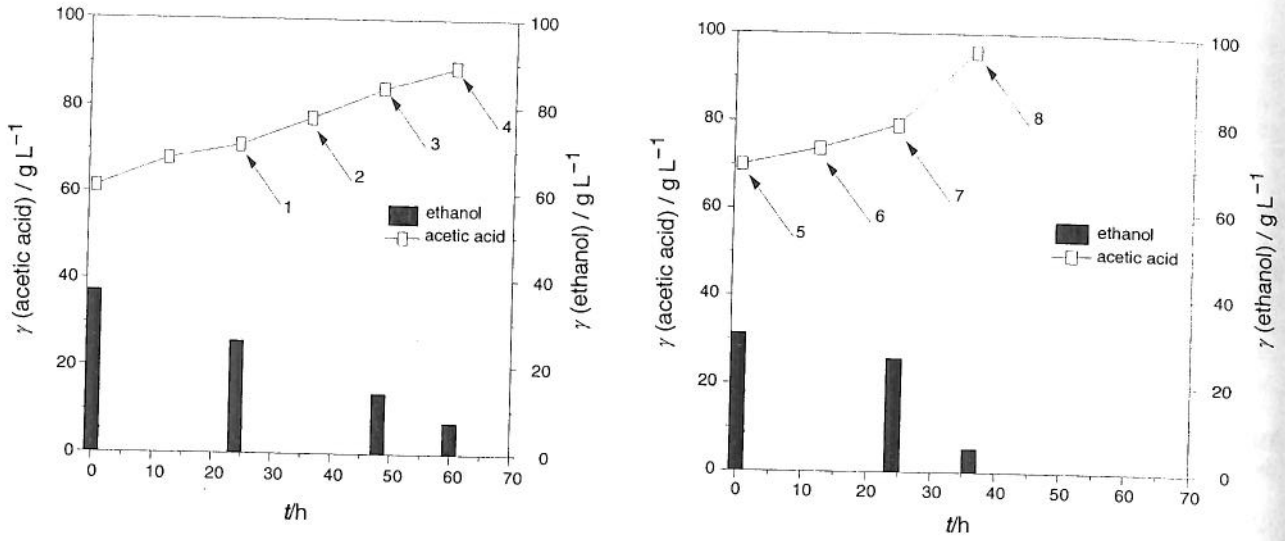

Fig. 1. Concentrations of acetic acid and ethanol during the second (left) and the third (right) cycles of the laboratory spirit vinegar production. The symbols (→) and the numbers (1–8) correspond to the sampling points of microbial cultures.

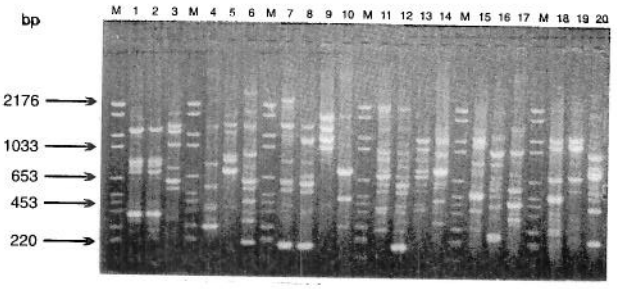

Fig. 2. RAPD profiles obtained for *Acetobacter* strains with three different primers. Lanes M, mixture of pBR328 DNA cleaved with *Bgl* I and pBR328 DNA cleaved with *Hinf* I; lanes 1–6, profiles obtained with primer 70% G+C; lanes 7–14, profiles obtained with primer 80% G+C; lanes 15–20, profiles obtained with primer $(GTG)_{5x}$. Lanes 6, 14 and 20 are the profiles of *A. europaeus*^{T} (^{T}-type strain of that species).

teristic, which is typical of *Acetobacter* sp. and not of *Gluconobacter* sp., they were also classified as belonging to the genus *Acetobacter (16)*. All the isolates had a strong tolerance to acetic acid, 3–6% in Entani agar. We were not successful in cultivating our isolates identified as *Acetobacter europaeus* in the liquid Entani medium. This was the reason why we sampled the biomass for the DNA isolation from the surface of the agar medium.

A. europaeus was a predominant species among all the strains that we isolated from spirit vinegar. At point 4 of the second cycle, we noticed that half of the analysed isolates were not identified as *Acetobacter europaeus*. In the third cycle, *Acetobacter europaeus* strains were again predominant in spite of the controlled cultivation conditions (see Methods) during both cycles of the submerged fermentation procedure in the laboratory bioreactor.

For the RAPD analysis we tested six different primers. With three primers we got fewer than three amplified fragments, which is considered to be too low a number for acquiring information from RAPD profiles (Table 1). All the RAPD profiles were categorized by assigning different numbers to letters (Table 2). A new number was given to a profile if it differed from all the previous ones in at least one intense band. The data generated with the use of primer 80% G+C showed the highest number (seven) of different RAPD profiles of our isolated *Acetobacter* strains. With each of the other two primers, 70% G+C and $(GTG)_{5x}$, five different patterns were identified. Both analysed isolates at sampling point 4, which were not identified as *A. europaeus*, had the same RAPD profiles. The RAPD profiles of the other two isolates, which were not identified as *A. europaeus*, were similar, but differed from those mentioned previously (Fig. 2). We also noticed that the morphology of the strain colonies not identified as *A. europaeus* taken at point 4 differed from the morphology of strain colonies not identified as *A. europaeus* taken at point 5. We assumed that they were at least different strains of the same *Acetobacter* sp. From the predominant profile in the second cycle, we assumed that the strain to which it corresponded was the predominant strain. In the third cycle, the other strain predominated.

Conclusions

In this work we have found out that the Entani agar medium (*2–4*), which is reported to be suitable for the isolation and cultivation of acetic acid bacteria from vinegar production based on submerged fermentation, does not always assure successful isolation and cultivation of the bacteria. Even when we dealt with vigorous cultures on the industrial or laboratory scale the plating efficiency was sometimes very low.

Most isolates were identified as *A. europaeus*. However, RAPD analysis established the presence of different genotypes among the isolated strains.

In spite of the new technique developed for the isolation of acetic acid bacteria from high percentage vinegar, further experiments are needed to determine the op-

timal procedure for the isolation of the majority of strains from the microbial population which is responsible for acidification of ethanol to acetic acid.

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Fenotipska karakterizacija in RAPD-PCR analiza izolatov *Acetobacter* sp. iz bioprocesa proizvodnje alkoholnega kisa

Povzetek

Iz laboratorijsko vodenega bioprocesa za proizvodnjo alkoholnega kisa smo izolirali 47 sevov ocetnokislinskih bakterij. Sevi so bili izolirani iz druge in tretje šare bioprocesa. Vsi izolati so bili po fenotipski karakterizaciji uvrščeni v rod **Acetobacter**. *Enainštirideset od njih je zadostilo fenotipskim kriterijem za uvrstitev v vrsto* **A. europaeus**. *Trinajst izmed sevov identificiranih kot vrsta* **A. europaeus** *in štiri seve* **Acetobacter** *sp., ki niso zadostili kriterijem za uvrstitev v vrsto* **A. europaeus**, *smo genotipsko okarakterizirali s pomnoževanjem v verižni reakciji s polimerazo z naključno izbranimi začetnimi oligonukleotidi (analiza RAPD). Identificiranih je bilo sedem različnih profilov RAPD. Iz tega domnevamo, da so v alkoholnem kisu prisotni genotipsko različni sevi ocetnokislinskih bakterij.*