http://dx.doi.org/10.5012/bkcs.2013.34.12.3877

Monitoring of Mixed Culture of *Saccharomyces cerevisiae* and *Acetobacter aceti* Using Gravitation Field-flow Fractionation and Gas Chromatography

Do-Yeon Kim, Sun Tae Kim, Hanul Kim,† In Soo Lee,† and Seungho Lee*

*Department of Chemistry, Hannam University, Daejeon 305-811, Korea. *E-mail: slee@hannam.kr*
†Department of Biological Science, Hannam University, Daejeon 305-811, Korea
Received August 27, 2013, Accepted September 17, 2013

Key Words : Gravitational field-flow fractionation (GrFFF), Gas Chromatography (GC), Mixed culture, *Saccharomyces cerevisiae* (*S. cerevisiae*), *Acetobacter aceti* (*A. aceti*)

Saccaromyces cerevisia (*S. cerevisiae*) is a species of yeast, which is widely used in wine-making and bread-baking. *Acetobacter aceti* (*A. aceti*) is used for production of vinegar.[1] Traditionally, vinegar was produced in a mixed culture of yeast and *A. aceti*.[2] The fermentation of *S. cerevisiae* yields ethanol by an anaerobic process and *A. aceti* converts ethanol to acetic acid by an aerobic process.[3]

Field-flow fractionation (FFF) is a separation technique for macromolecules, and can be useful in various fields including biotechnology.[4-6] FFF is also well suited to analytical-scale separation and characterization of nano to micron-sized particles.[7,8] FFF has been successfully applied to characterization and separation of colloids with biological interest such as yeast.[9,10]

Gravitational field-flow fractionation (GrFFF) is a member of FFF, which employs perpendicular application of Earth's gravitational field to the channel flow. GrFFF provides size-based separation with larger particles eluting earlier than smaller ones.[11,12] GrFFF has been applied for characterization of yeast cells[13,14] as well as to the study of viability and activity of *S. cerevisiae* strains during fermentation[15,16] GrFFF elution band area can be used as a measure of particle content (or concentration), and thus can be used for monitoring of change in population of cells during incubation.

Gas Chromatography (GC) has been employed for detection or identification of microorganisms.[17] GC can provide separation of ethanol and acetic acid, and allow simultaneous quantitative analysis of both species.

In this study, *S. cerevisiae* and *A. aceti* were mixed cultured in a glucose-based medium, and the mixed fermentation process was monitored as a function of time using GrFFF and GC.

Experimental

***A. aceti* Incubation.** The bacterium used for acetic acid fermentation was *A. aceti* KCTC1010 supplied by Korean collection for type culture (Daejeon, Korea). The medium for cultivation was YPD media (yeast extract 10 g/L, peptone 20 g/L and glucose 20 g/L, pH 7.0) and supplemented Agar (15 g/L) for plate. The bacterial inoculum for mixed culture was grown in 3 mL of YPD media in a 13 × 100 mm test tube tilted 45 degree by shaking at 160 rpm at 30 °C for 4-5 days. All components for preparing the media were obtained from Becton Dickinson Co. (Franklin Lakes, NJ, USA).

***S. cerevisiae* Incubation.** The yeast cell used for alcohol fermentation was *S. cerevisiae* KCTC7296, and the inoculum cells were incubated in GPYA media (yeast extract 5 g/L, peptone 5 g/L and glucose 40 g/L, pH 7.0). The culturing conditions were same as those used for *A. aceti* cultivation.

Fermentation. Each single colony was inoculated in proper broth media. And both *A. aceti* in YPD and *S. cerevisiae* in GPYA media incubated in a shaking incubator (Jeio Tech. Daejeon, South Korea) at 30 °C for about 4-5 days. Each 30 μL of pre-cultures were inoculated into 3 mL fresh GPYA media, and then vortexed for mixing. Mixed fermentations were performed by standing and shaking incubation mode. The viable counts were performed every day during incubation. Bacteria and yeast cells were serial diluted with YPD media, and plated YPD media and GPYA media, respectively.

Gravitational Field-Flow Fractionation (GrFFF). GrFFF channel was 0.02 cm thick, 2 cm wide, and 51 cm long. The channel void volume was 2.0 mL. The sample injection volume was 10 μL. A Young-Lin SP930D isocratic HPLC pump (Seoul, Korea) was used to deliver the carrier liquid. The particles in the FFF eluent were monitored by a Young-Lin M720 UV/VIS detector (Young-Lin Science, Anyang, Korea) with the wavelength fixed at 264 nm. The carrier liquid was water containing 0.1% FL-70 (Fisher Scientific, Loughborough, UK) detergent (a low-foaming; low alkalinity; and phosphate-, chromate-, silicate-free mixture of anionic and nonionic surfactants). Polystyrene (PS) latex beads of various nominal diameters (6, 8, 12, 20, 40 μm) purchased from Duke Scientific Corp. (Palo Alto, CA, USA) were used as standard particles which were dispersed at 0.1%(w/v) in the carrier liquid.

Gas Chromatography (GC). A Varian CP-3800 gas chromatograph equipped with a flame ionization detector (FID) was used for separation and quantitation of ethanol and acetic acid. GC column was a capillary (VF-5ms, Varian, Palo Alto, CA, USA), whose dimensions were 30 m × 0.25 mm × 0.25 μm. The carrier gas was He flowing at 2.0 mL/min. The injector temperature was 250 °C. Initially the column

oven temperature was 50 °C, which was maintained for 1 min, and then increased up to 240 °C at the rate of 7 °C /min. Temperature was maintained at 240 °C for 10 min. The detector temperature was 300 °C.

The mixture of *yeast* and *A. aceti* in GPYA media was added to dimethyl formamide (DMF) at the volume ratio of 2:8 to remove nutrients and byproducts of microorganisms, and then filtered through a 0.22 μm sterile filter before the injection into the GC column.. The sample injection volume was 1 μL. Calibration standards were prepared by adding known amount of ethanol and acetic acid in the GPY medium, which was diluted with DMF.

Results and Discussion

Figure 1(a) shows GrFFF fractograms of PS latex beads having various nominal diameters. The retention data shown in Figure 1(a) were used to establish the calibration curve shown in Figure 1(b), which was used to determine the size of *S. cerevisiae* and *A. aceti* during fermentation.

Figure 2 shows GrFFF fractograms of *A. aceti* (a), *S. cerevisiae* (b) and their mixed culture by standing (c) and shaking mode (d) collected at various fermentation times.

It has been reported that *A. aceti* are about 2 μm in sizes.[18]

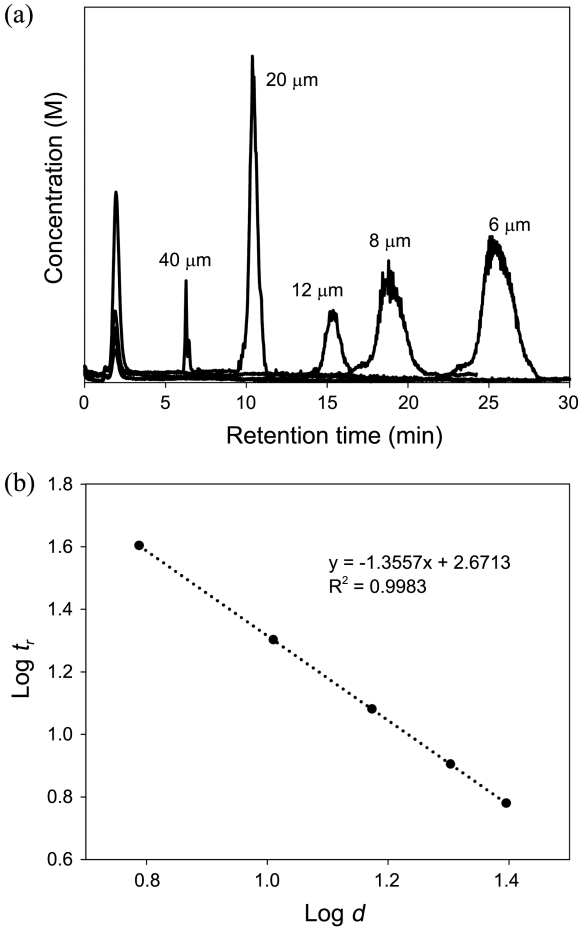

Figure 1. GrFFF fractograms of polystyrene latex beads having nominal diameters of 6, 8, 12, 20, and 40 μm (a) and a calibration curve (b).

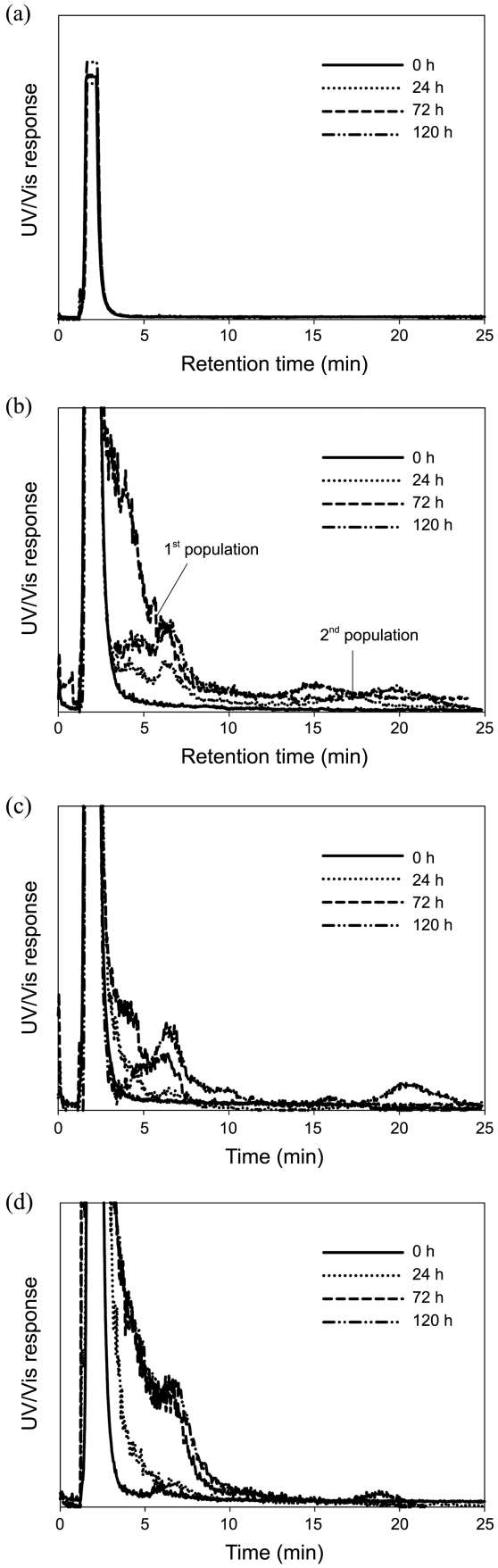

Figure 2. GrFFF fractograms of *A. aceti* (a), *S. cerevisiae* (b) and their mixed cultures by standing (c) and shaking mode (d).

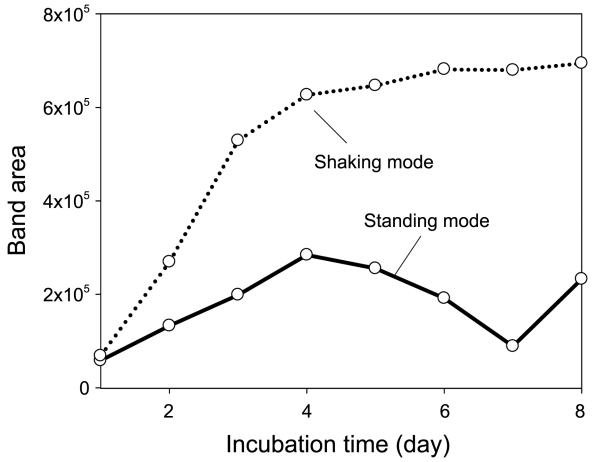

Figure 3. Variation in GrFFF band area with incubation time in mixtures cultured by two different mode.

As shown in Figure 2(a), *A. aceti* are eluted at the void time without being retained, suggesting they are too small to be retained in GrFFF. Figure 2(b) shows that the *S. cerevisiae* is bimodal in size distribution with one population (earlier-eluting) in sizes ranging about 20-50 and another (late eluting) in sizes ranging 7-10 μm based on PS calibration (Figure 1(b)). It is likely that the earlier eluting population is consisted of aggregates.

Figure 2(c) and (d) show that the mixed cultures are also bimodal in size distributions. No distinct difference was observed between the mixtures cultured by standing and shaking mode, except that the overall band area from the shaking mode grows faster than that from the standing mode.

Figure 3 shows daily variation in the overall band area with incubation time for mixtures cultured by two different modes (standing and shaking). In the shaking mode, the band area increases rather rapidly for the first 3-4 days, after which does not change significantly. In the standing mode, the band area also increases for the first 3-4 days, this time at relatively slower rate, and then decreases and increases again without reaching any plateau. It seems the fermentation

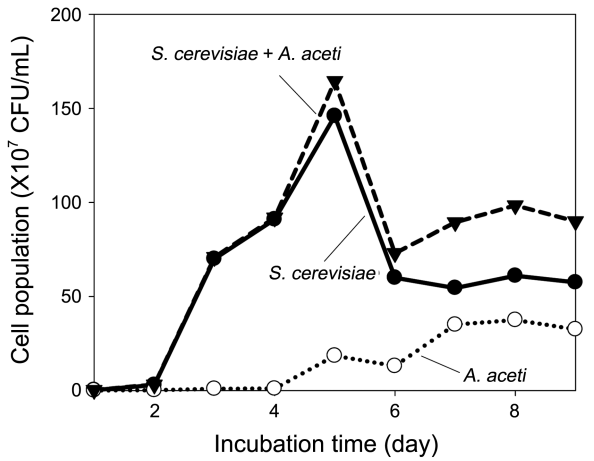

Figure 4. Variation in cell population with incubation time in shaking mode.

Table 1. Concentrations of ethanol and Acetic acid determined by GC in mixed cultures

Incubation time (day)	Concentration (M) in mixture cultured by			
	Shaking mode		Standing mode	
	Ethanol	Acetic acid	Ethanol	Acetic acid
1	0.226	0.131	0.030	0.142
2	0.526	0.057	0.176	0.142
3	0.468	0.133	0.658	0.010
4	0.309	0.177	0.665	0.010
5	0.246	0.238	0.670	0.010
6	0.139	0.390	0.617	0.010
7	0.069	0.424	0.667	0.010
8	0.009	0.220	0.653	0.010

reaches equilibrium much faster by the shaking mode than the standing mode.

Figure 4 shows daily variation in cell population obtained by viable cell counting in the mixed culture incubated by the shaking mode. The cell population of *A. aceti* gradually increases during the first 7-8 days of incubation. However the increase is relatively slow and the change in the cell population is small. The cell population of *S. cerevisiae* also gradually increases during the first 5 days (this time at much higher rate than that of *A. aceti*), and then quickly decreases in next day. It is noted that, while the *S. cerevisiae* population decreases, *A. aceti* population increases. The total cell population (*S. cerevisiae* + *A. aceti)* showed similar trend with that of *S. cerevisiae*. As mentioned earlier, *S. cerevisiae* produces ethanol anaerobically, while *A. aceti* produces acetic acid using ethanol aerobically.

To determine the ethanol and acetic acid in mixtures, calibration were plotted with various concentrations of ethanol and acetic acid standard solutions which show an excellent linearity with $R^2 = 0.9962$ for ethanol and $R^2 = 0.9953$ for acetic acid, respectively.

The ethanol and acetic acid concentration determined by GC in the mixtures are listed in Table 1. Figure 5 shows plots of data shown in Table 1.

It can be seen in Table 1 that, in the shaking mode, the ethanol concentration increases for the first couple of days reaching at 0.526 M, after which decreases continuously.

The reduction in ethanol concentration is probably due to relatively faster consumption of glucose in the GPY medium. On the other hand, in the standing mode, the ethanol concentration gradually increases for the first 3 days reaching at about 0.66 M, after which does not change much.

In the shaking mode, the acetic acid concentration increases for the first 7 days reaching at 0.424 M, after which decreases, while, in the standing mode, the acetic acid concentration decreases for the first 3 days down to near zero (0.01 M). It is interesting to see that, after 3 days of incubation, there exists no significant amount of acetic acid remained in the culture. It seems the growth of *A. aceti* is ceased after 3 days in the standing mode due to oxygen depletion. Dissolved oxygen is a crucial factor for production of acetic acid in

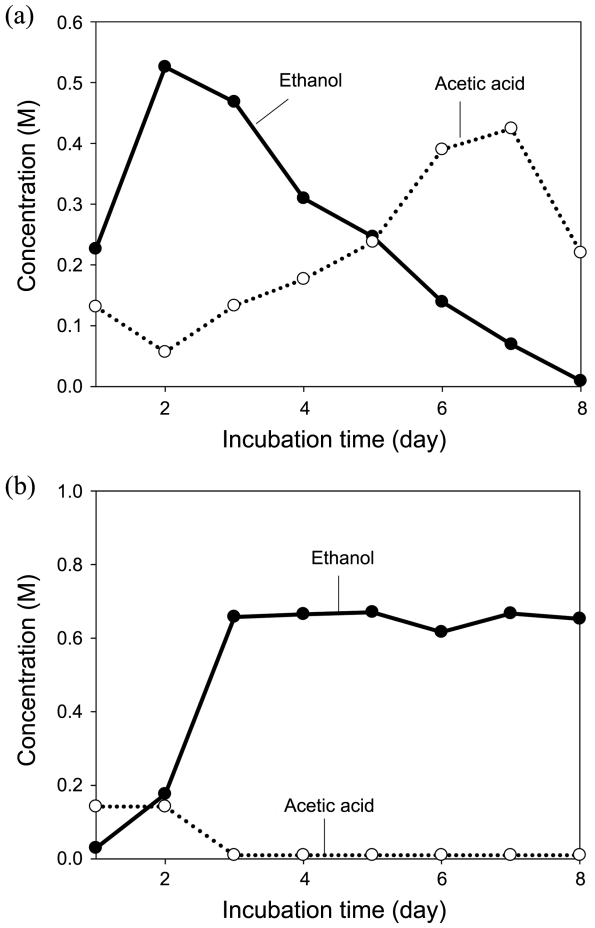

Figure 5. Variation in concentrations of ethanol and acetic acid in mixtures cultured by shaking (a) and standing mode (b).

mixed fermentation. It is noted that a certain amount of ethanol and acetic acid present in the mixture from the first day of incubation as they exist in the seed cultures.

After 3 days incubation by the standing mode, the ethanol concentration remains at higher level than in the shaking mode because the ethanol fermentation is an anaerobic process and thus ethanol is not converted to acetic acid much in the standing mode. On the other hand, the acetic acid concentration keeps increasing in the shaking mode.

Conclusion

In this study, a mixture of *S. cerevisiae* and *A. aceti* was cultured either by the shaking and the standing mode, and the culture process was monitored using GrFFF and GC. It was found that the culture cycle in the shaking mode is faster than that in the standing mode because of higher concentration of dissolved oxygen. In the shaking mode, the cell population of *A. aceti* gradually increases. The cell population of *S. cerevisiae* also gradually increases during the first few days, and then quickly decreases. While the *S. cerevisiae* population decreases, *A. aceti* population increases.

S. cerevisiae yields ethanol by an anaerobic process, so the ethanol concentration remains at higher level in the standing mode than in the shaking mode, while *A. aceti* produces acetic acid by an aerobic process, thus the acetic acid concentration is higher in the shaking mode. Results in this study suggest a combination of GrFFF and GC could be a useful tool for monitoring of various types of mixed culture processes.

Acknowledgments. This work was supported by Hannam University.

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