Co-culture of a Cellulose-Producing Acetic Acid Bacterium and a Beer Yeast

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Abstract: The bacterium *Gluconacetobacter hansenii* NOK21 was identified as a cellulose pellicle-forming bacterium from rancid wine. The bacterium did not show growth when glucose concentration or ethanol concentration was equal to or greater than 20% or 8%, respectively. Production of the cellulose pellicle was greatest with a concentration of cellulose polymer of 5 g/l in glucose medium. The NOK21 oxidized ethanol to acetic acid like other acetic acid bacteria and did not appear to convert glucose to acetic acid. The maximum titratable acidity of 4.13% was obtained from 5% ethanol-containing medium. Under static co-culture conditions of the acetic acid bacterium and *Saccharomyces cerevisiae*, the beer yeast grew under the pellicle at the bottom of the culture vessel through anaerobic metabolism whereas the bacteria grew to form a pellicle on the surface of the culture medium through aerobic metabolism. The bacterial strain NOK21 grew and synthesized cellulose polymer and produced acetic acid using glucose as a substrate in the presence of the beer yeast. Our findings about the behavior in co-culture of the alcoholic fermentation yeast and the acetic acid bacterium provide information helpful for the manufacture and improvement of fermented food products that are sour and alcoholic.
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1. Introduction

Ethanol fermentation by beer yeasts is an anaerobic energy-yielding metabolism through which glucose is converted to pyruvic acid, and the pyruvic acid is subsequently decarboxylated and reduced to ethanol (Stryer, 1988). Meanwhile, acetic acid bacteria (AAB) oxidize ethanol to acetic acid, and the acetic acid can be further oxidized to carbon dioxide and water by *Gluconacetobacter* species (Mamlouk and Gullo, 2013). No AAB of the genus *Acetobacter, Gluconobacter,* or *Gluconacetobacter* are known to produce acetic acid using glucose as a substrate. Also, AAB forming cellulose pellicles are known, and their cellulose polymers have been extensively studied (Ross *et al*., 1991; Keshk, 2014; Valera *et al*., 2015).

Considering the microbial symbiosis, it is not surprising that AAB are found in ethanol fermentation products (Joyeux *et al*., 1984). Simultaneous progress of ethanol and acetate fermentation are undesirable in wine brewing, whereas it has been utilized in the manufacture of fermented food such as Kombucha (Nguyen *et al*., 2008), traditional vinegar (Tan, 2005; Nanda *et al*., 2001), and Nata de Coco. The presence of both acid and ethanol in fermentation medium prevent quality deterioration by restricting growth of undesirable microorganisms. The traditional Nata de Coco is a chewy, translucent, jelly-like substance produced by the mixed microbial fermentation of coconut water (Seumahu *et al*., 2007). Kombucha is a lightly effervescent fermented drink that is produced by fermentation of sweetened tea using AAB and alcoholic fermentation yeasts (Dufresne and Farnworth, 2000).

The influence of certain AAB on grape juice fermentation by alcoholic fermentation yeast was reported earlier by Drysdale and Fleet (1989). They investigated the effects of AAB on ethanol fermentation by yeasts, but they did not use pellicle forming bacteria. The pellicle formed by AAB on the surface of the fermentation medium can exert great effects on a mixed culture. Especially the pellicle promotes anaerobic conditions beneath itself by blocking and consuming atmospheric oxygen. Pellicle-forming AAB are easy to handle and maintain with alcoholic fermentation yeasts at home. Better understanding of microbial interactions between pellicle-forming AAB and alcoholic fermentation yeasts will provide better know-how for the manufacture of traditional fermented foods such as vinegar, Nata de Coco, Pulque, and Kombucha.

Gluconacetobacter hansenii NOK21 forms a thick cellulose pellicle on the surface of glucose broth (Ko *et al*., 2015). The bacterium was co-cultivated with a beer yeast *Saccharomyces cerevisiae* in glucose-containing broth in the presence and absence of co-substrate ethanol. Their growth behavior, acidity change, and ethanol production during cultivation were investigated and analyzed in this study.

2. Material and Methods

Strains and culture

G. hansenii NOK21 (Ko *et al*., 2015) isolated from rancid wine and a Pilsner-style yeast of *Saccharomyces cerevisiae* (Erbslöh Geisenheim AG, Germany) were cultivated in this study. As media for strain storage and subculture, SH medium (glucose, 20 g/ℓ; yeast extract, 5 g/ℓ; bactopeptone, 5 g/ℓ; Na_2HPO_4, 2.7 g/ℓ; citric acid, 1.15 g/ℓ; agar, 15 g/ℓ) (Hestrin and Schramm, 1954) and potato dextrose agar (PDA) (Becton, Dickson and Company, USA) were used for the bacteria and for the yeast, respectively. The glucose concentration in SH medium was modified as needed for cultivation, and the modification was stated properly in the text. In addition, ethanol-containing medium was prepared by adding 95% ethanol to SH medium after autoclaving.

Erlenmeyer flasks were used as culture vessels, to which 100 ml or 200 ml of the broth medium was added for pure culture or for co-culture, respectively. The broth medium was inoculated with the bacterium by transferring a loopful of cells from the colony on the solid medium to the broth, whereas inoculation of the broth with the beer yeast was performed by adding cell suspension as follows: The yeast culture grown previously till exponential growth phase in potato dextrose broth was diluted with sterile 0.9% saline solution to adjust cell concentration to $1.0X10^7$ cfu/㎖, and then 1 ㎖ of the cell suspension was added to each broth medium for inoculation. The microbes were incubated at 28℃ under static condition, and incubation time-dependent changes were examined.

Yeast cell count

Serial dilution method was used for the viable cell count of yeast. The culture sample was serially diluted with sterile distilled water, and then aliquots of 200 ㎕ each were plated by spreading on PDA and incubated at 28℃ for colony count. Counting chamber (Counting cell, Graticules Ltd., United Kingdom) was also used for direct cell number counting under an optical microscope (Olympus, Japan) as a supplementary measure.

Cellulose pellicle determination

Cells of *G. hansenii* NOK21 grow to form a cellulose pellicle on the top of broth medium. Pellicle determination is an indirect measure of the bacterial growth. The cellulose pellicle on the culture surface was recovered with forceps and soaked in 0.5 M NaOH at 90℃ for 1 hour. The pellicle was subsequently washed with distilled water repeatedly by soaking and decanting until the pH of the drained water reached neutral. The cleaned pellicle was dried at 105℃ and its weight was measured gravitationally with an electronic scale.

Titratable acidity measurement

The culture sample was filtered with a disposable syringe filter (Φ 0.45 ㎛, Advantec MFS, Inc., Japan), and 20 ㎖ each of the resulting filtrate was taken with a pipette into a 250 mL Erlenmeyer flask. Then a couple of drops of phenolphthalein solution were added. The filtrate was titrated with 0.1 N sodium hydroxide (NaOH) solution till the first color change (Lee *et al*., 2003). Titratable acidity was calculated as follows: Titratable acidity (%) = (V·A/S) x 100, where V, volume of 0.1 N-NaOH solution consumed (㎖); A, meq of acetic acid to 0.1 N-NaOH (0.006 g); S, volume of culture sample titrated (20 ㎖).

Ethanol determination

Ethanol content of the culture sample was determined by distillation followed by density determination by hydrometry (International Union of Pure and Applied Chemistry, 1968). This method was based on removing and collecting all of the alcohol contained in an exact volume (100 ㎖) of culture sample by distillation. The collected distillate was then diluted back to the exact volume with distilled water. The alcohol content was then determined by measuring its density using a special alcohol hydrometer calibrated at 15°C.

Statistics

Every experiment was replicated three times and their mean values were obtained and used.

3. Results and Discussion

When cells of *S. cerevisiae* were cultivated in modified SH broth without added ethanol, yeast cell concentrations were maintained at around 10^8 cfu/㎖ in glucose concentrations of up to 20%. Within the first 5 days of cultivation, cell numbers reached the maximum concentration and stayed at this level for another 10 days (Fig. 1). Yeast grows rapidly when dissolved oxygen is present in a medium, but the medium rapidly becomes and remains anaerobic upon cell propagation. Later, under anaerobic conditions, the yeast focuses on converting glucose to ethanol rather that increasing the number of yeast cells. At high glucose concentrations of 15% and 20%, cell numbers were several times lower than those at lower glucose concentrations (Fig. 1). High concentrations of glucose and ethanol seemed to inhibit yeast cell growth. It has been shown that osmotic pressure and ethanol had effects on yeast viability (Pratt *et al*., 2003).

A Pilsner-style yeast of *S. cerevisiae* is an alcoholic fermentation yeast. Within the first 5 days of its cultivation in modified SH medium, ethanol concentration increased rapidly; thereafter no remarkable change was observed. Ethanol concentration increased in proportion to the rise in

glucose concentration (Fig. 2); however, in glucose concentrations of 15% and 20%, ethanol fermentation appeared to be retarded because the ethanol concentration increased slowly even after the first 5 days of cultivation. A previous study suggested that relatively high concentrations of ethanol might slow fermentation because of its toxicity to the beer yeast (Stanley *et al*., 2010).

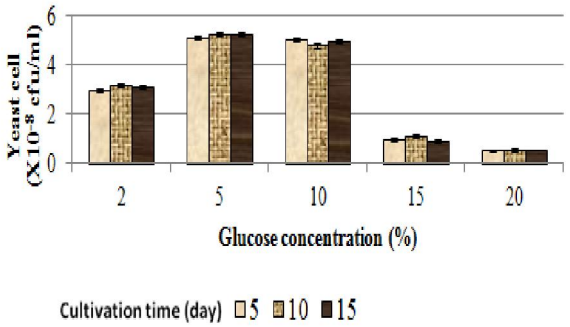

Figure 1. Changes of yeast population during cultivation of *S. cerevisiae* in modified SH broth without ethanol.

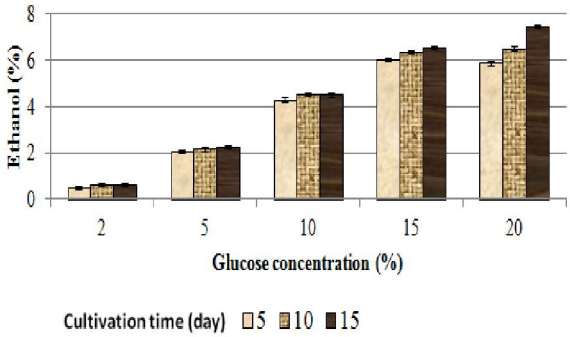

Figure 2. Changes of ethanol concentration during cultivation of *S. cerevisiae* in modified SH broth without ethanol.

The bacterium *G. hansenii* NOK21 was previously shown to synthesize a cellulose pellicle in SH medium (Ko *et al*., 2015). Pellicle formation by the bacteria reflects its corresponding growth. Cells of NOK21 made a thick pellicle on the surface of the modified SH broth in 10 days, except that no bacterial growth was observed in the broth containing 20% glucose (Fig. 3). The cause of the bacterial growth inhibition is suspected to be because of high osmotic pressure generated by glucose in the medium (Cheftel, 1995; Gullo *et al*., 2006). The highest concentration of cellulose polymer was 5 g/ℓ, which is very close to the data obtained by other researchers (Ko *et al*., 2000; Nguyen *et al*., 2008; Castro *et al*., 2012; Usha Rani and Anu Appaiah, 2013).

The strain NOK21 did not appear to metabolize glucose to acetic acid because no significant change was developed in total acidity when the bacterial cells were cultivated in SH broth without added ethanol (Fig. 4). Most AAB such as NOK21 do not produce acetic acid without ethanol as a substrate. Other organic acids produced through oxidative glucose metabolism by the bacteria were very likely responsible for the basal figures of around 0.01% of titratable acidity (Fig. 4).

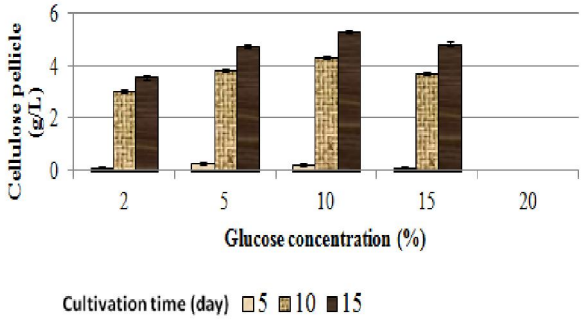

Figure 3. Changes of cellulose pellicle formation during cultivation of *G. hansenii* NOK21 in modified SH broth without ethanol.

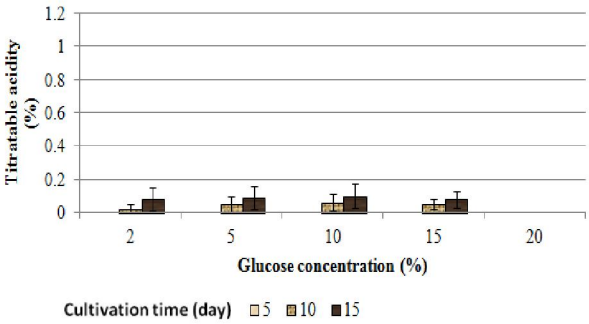

Figure 4. Changes of titratable acidity during cultivation of *G. hansenii* NOK21 in modified SH broth without ethanol.

In order to examine the effect of ethanol on the growth of NOK21, the bacterium was cultivated in SH broth containing ethanol at various concentrations. The SH broth had 2% of glucose as a basal carbon source. Cellulose pellicles were formed even at an ethanol concentration of 5% (Fig. 5); however, no pellicle developed in the broth containing 8 to 10% of ethanol, where bacterial growth is believed to be inhibited.

In the absence of ethanol, no significant acidity was detected in spite of the bacterial growth (Fig. 4); however, acidity increased in the medium containing ethanol, probably because of acetic acid production through bacterial oxidation of ethanol. The highest

acidity of 4.13% was obtained in the medium containing 5% ethanol concentration after 15 days of static culture (Fig 6). AAB are known to produce 4 to 6% of acidity under static culture conditions (Nanda *et al*., 2001).

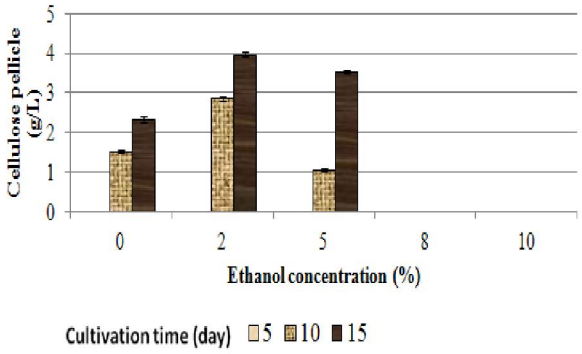

Figure 5. Changes of cellulose pellicle formation during cultivation of *G. hansenii* NOK21 in modified SH broth containing ethanol.

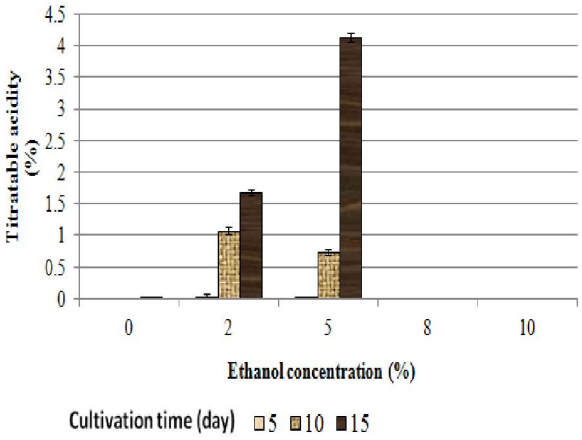

Figure 6. Changes of titratable acidity during cultivation of *G. hansenii* NOK21 in modified SH broth containing ethanol.

Coexistence of alcoholic fermentation yeasts and AAB is often observed in the natural fermentation process of carbohydrates. Co-culture of the beer yeast and the bacterium NOK21 in glucose medium was expected to produce ethanol from glucose through anaerobic ethanol fermentation by the yeast and to produce acetic acid through aerobic oxidation of the ethanol by the bacterium.

When the beer yeast *S. cerevisiae* and the AAB *G. hansenii* NOK21 were cultivated together in glucose medium, the yeast cell number appeared to be around 10^7 cfu/㎖ and lower glucose concentration had a tendency to give a slightly higher cell number (Fig. 7), which might result from ethanol toxicity to the yeast at higher glucose concentrations.

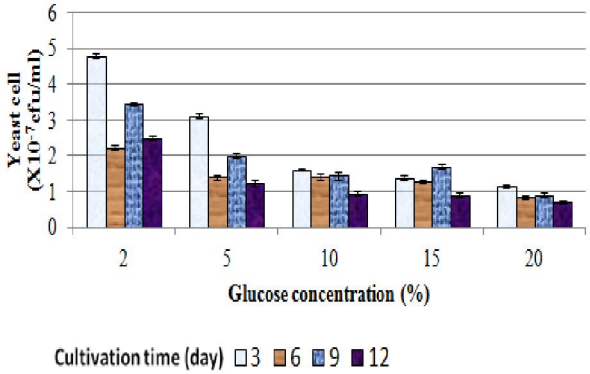

Figure 7. Changes of yeast population during co-culture of *G. hansenii* NOK21 and *S. cerevisiae* in modified SH broth without ethanol.

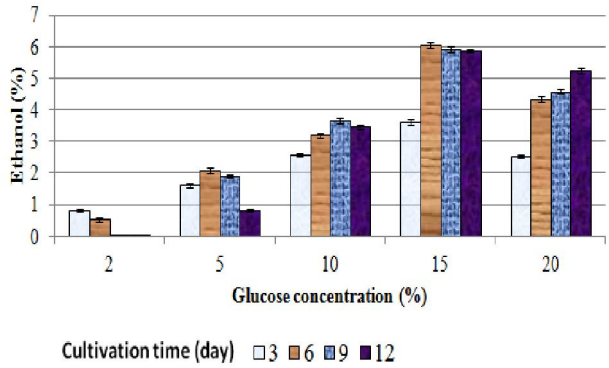

Figure 8. Changes of ethanol concentration during co-culture of *G. hansenii* NOK21 and *S. cerevisiae* in modified SH broth without ethanol.

A considerable amount of ethanol produced by the yeast was detected even in the presence of the bacterium (Fig. 8). Its concentration exceeded 5% in the medium containing 15% glucose, where the bacterium NOK21 showed neither pellicle formation (Fig. 9) nor acetic acid production (Fig. 10) because the bacterium did not grow as a result of ethanol toxicity (Fig. 5). However, in the medium containing 2~10% glucose, both the beer yeast and the bacterium NOK21 grew together, and the glucose was converted to ethanol (Fig. 8) and a cellulose pellicle (Fig. 9). Part of ethanol produced is likely to have been used as a substrate for acetic acid fermentation by the bacterium NOK21, although total acidity was quite low (Fig. 10). If the shared environment and effect of oxygen on growth are considered, it can be assumed that the yeast grew under the pellicle at the bottom of culture vessel through anaerobic metabolism whereas the bacteria grew forming pellicles on the surface of the culture medium through aerobic metabolism.

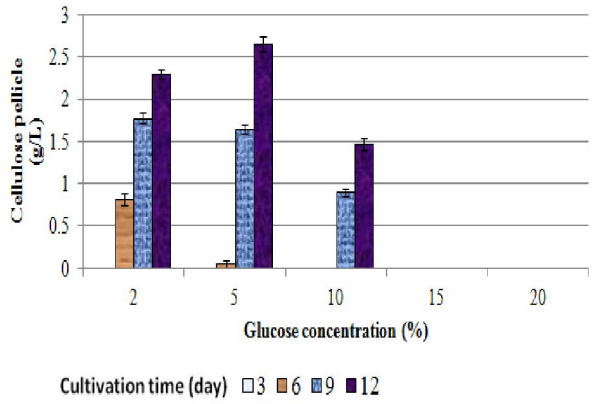

Figure 9. Changes of cellulose pellicle formation during co-culture of *G. hansenii* NOK21 and *S. cerevisiae* in modified SH broth without ethanol.

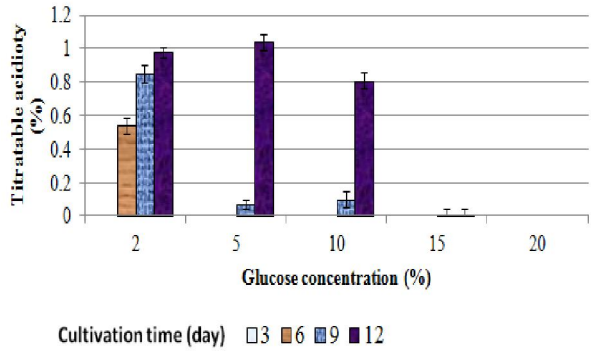

Figure 10. Changes of titratable acidity during co-culture of *G. hansenii* NOK21 and *S. cerevisiae* in modified SH broth without ethanol.

These findings about the co-culture behavior of the alcoholic fermentation yeast and the acetic acid bacterium provide information helpful for the manufacture and improvement of fermented food products such as Kombucha and Nata de Coco and other fermented food products that are sour, alcoholic, and sparkling. Control of acidity and ethanol levels are suitable measures for the prevention of food spoilage by pathogenic bacteria.

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