Fed–batch fermentation of onion vinegar using *Acetobacter tropicalis*

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Abstract To produce onion vinegar with high efficiency, various fermentation conditions, such as varying initial ethanol concentrations and the addition of ethanol or onion juice were optimized. *Acetobacter tropicalis* KFCC 11476P consumed ethanol at a rate of 0.125–0.140 g/h when the initial ethanol concentrations were 4, 5, and 6%, and the acidity of the fermentation broth reached the maximum level when the ethanol was completely starved. In the case of fed-batch fermentation with continuous feeding, when a small amount of ethanol and onion juice was continuously supplied into the broth after 30 h of fermentation, the acidity continued to increase up to 4.5% at 45 h. All the while, the remaining ethanol content of the fermentation broth was 1.13–1.69%. The maximum acidity of onion vinegar in the pilot–scale fermenter reached 4.6% at 48 h of which fermentation speed was five times faster than the general standard.

Keywords: onion, vinegar, fermentation, acetic acid bacteria, fed–batch

Introduction

Vinegar is a product of the aerobic fermentation of various vegetables or fruits and is produced when alcohol is oxidized by acetic acid bacteria (1). The sour taste of vinegar helps digestion by stimulating digestive organs and accelerates the secretion of digestive juices. In addition, vinegar contains many organic acids that are involved in the TCA cycle and have positive health effects, like recovery from fatigue, alkalization of blood, and so on. Vinegar has therapeutic activities including weight loss, the prevention of arteriosclerosis, the secretion of adrenocortical hormones, the acceleration of calcium absorption, and the reduction of blood pressure (1–3). The manufacturing methods of vinegar are semi-continuous fermentation or continuous fermentation, and the acidity is known to be 5–7%. In Maselli and Horwath's study (4), the final acidity of vinegar was increased to be above 18% by improving acetic acid bacteria. Acetic acid bacteria were collected by filtering the fermented culture of first-stage fermentation and were inoculated into the culture broth of second-stage fermentation, and thereby the viable cell number of acetic acid bacteria was fortified (4). Lee *et al.* (5) had general semi-continuous fermentation in the first stage, and this product was transferred to the second stage and the final acidity of the vinegar was above 17%. Commercial vinegar is produced by a fast fermentation processes. There have been insufficient studies on the rapid fermentation of vinegar that contains the

beneficial compounds of its raw materials and reaches more than 4% acidity. In the case of brewed vinegar, the period of acetic acid fermentation is about 6–8 days, and this period has to be shortened for commercial uses.

The most common raw materials used for vinegar production are highly acidic fruits, such as apples or grapes, but we used onions in this study. The onion (*Allium cepa* L.), which belongs to the Liliaceae family and has a high content of organosulfur, has antioxidant effects on lipids, and these effects come from flavonoids, aromatic amines, phenols, and others. Recent studies have revealed other health beneficial effects, including the prevention of cardiovascular diseases, the lowering of blood glucose levels, anti-oxidative effects, the neutralization of the toxicity of lead, the lowering of serum lipid levels, repression of the peroxidation of liver lipids, and the inhibition of DNA damage (6–12). The aim of this study was to enhance the availability of onion, which has a short shelf-life, and develop an onion vinegar that contains the health benefits of both onions and vinegar. Additionally, a fed-batch culture was studied to counteract the inhibition of acetic acid bacteria's growth by ethanol and increased acidity.

Materials and Methods

Acetic acid bacteria and growth rate *Acetobacter tropicalis* KFCC

11476P was isolated from fallen peaches of the Janghowon area, Korea and *Saccharomyces cerevisiae* KCCM 7913 was taken from the Korean Federation of Culture Collections (Seoul, Korea). Growth was determined by checking the optical density of the culture broth at 660 nm using a spectrophotometer (Du 530; Beckman Coulter, Inc., Fullerton, CA, USA) in a triangular flask, and growth was also determined by the dry weight of microbial cells (g/L) in the fermenter. Vinegar was produced by yeast and acetic acid bacteria in the two-stage incubation, by which the yeast cells were discarded by centrifugation at 19,200×g after alcohol fermentation and *A. tropicalis* KFCC 11476P was inoculated in the resulting supernatant.

Measurement of pH and titratable acidity The pH of the culture broth was measured using a pH meter (pH/ion/cond. F-55; Horiba Instruments Inc., Irvine, CA, USA). Then, 10 mL of culture broth was titrated with 0.1 N NaOH using phenolphthalein as an indicator, and the volume of NaOH used was measured. The volume of 0.1 N NaOH (NaOH/kg equivalent) was determined as the titratable acidity (%) for neutralizing acetic acid.

Production of onion vinegar using a fermenter The fermentation of onion vinegar was performed at 30°C in a fermenter at 300 rpm and 1.5 vvm. In a fed-batch culture, 200 mL of substrate was continuously supplied for 24 h using a peristaltic pump P-1 (Pharmacia Co., Uppsala, Sweden). The working volume was 120 L for the pilot-scale fermenter (KoBioTech Co., Ltd., Incheon, Korea). To prepare the onion extract, the onion bulbs were sliced into several pieces and extracted in equal volume of water for 20 min at 110°C. A seed culture was inoculated at a concentration of 5% (v/v) after the extract was filtered using felt and underwent sterilization. The resulting culture was incubated at 300 rpm and 1.5 vvm. Foam produced during the process was discarded using silicone resins.

Measurement of the reducing sugar The reducing sugar of the culture broth was measured by the Somogyi–Nelson method (13). One milliliter of centrifuged culture broth, mixed with 1 mL of copper reagent, was heated for 10 min in boiling water. After cooling with tap water, 1 mL of arsenomolybdate reagent and 3 mL of distilled water were added and then the optical density was measured at 520 nm with a spectrophotometer (Beckman Coulter, Inc.). The reducing sugar content was determined using a glucose standard curve, and the values were measured in triplicate.

Measurement of the fermentation yield The fermentation yield was converted into percentages using the initial acidity, initial alcohol concentration, and final acidity.

Fermentation yield = $\frac{[\text{Final acidity (\%, w/v)} - \text{Initial acidity (\%, w/v)}]}{\text{Initial alcohol concentration (\%, v/v)} \times 1.304} \times 100$

Fig. 1. Profiles of ethanol concentration, acidity, and cell dry weight during the single stage fed-batch culture of onion juice with *Acetobacter tropicalis* 11476P at an initial ethanol concentration of 4% by pure ethanol addition. Seven-step feeding: 1, 2% ethanol and 0.1% yeast extract; 2, 1% ethanol and 0.1% yeast extract; 3, 1% ethanol and 0.1% yeast extract; 4, 1% ethanol and 0.05% yeast extract; 5, 1% ethanol and 0.075% yeast extract; 6, 1% ethanol and 0.15% yeast extract; and 7, 1% ethanol and 0.2% yeast extract (arrow indicates the start of the step feeding of nutrients).

Results and Discussion

Fed-batch fermentation of *A. tropicalis* 11476P in a fermenter *A. tropicalis* 11476P was inoculated in 2 L of onion extract containing 4% (v/v) pure ethanol at 300 rpm and 1.5 vvm (Fig. 1). The ethanol was starved after 36 h, even though the residual glucose was 4.18%, of which the acidity was 3.11% and the fermentation yield was 59.62%, which is slightly lower than that of fermenter incubation using yeast and *A. tropicalis* 11476P. The onion extract containing 2% of ethanol and 0.1% of yeast extract was fermented after 3 days (arrow 1 of Fig. 1), during which glucose was not added to the onion extract because the reducing sugar was at a concentration of 4.18%. Consequently, the acidity reached 4.1% after 5 days of fermentation, and 1% of pure ethanol and 0.1% of yeast extract were added to the fermentation broth on days 5 and 6 of fermentation (arrow 2, 3 of Fig. 1). After 1% of pure ethanol and 0.05% of yeast extract were added after 7 days of fermentation, the acidity reached 5.6% on day 8 of fermentation (arrow 4, 5 of Fig. 1). The dry cell weight increased from 4.06 to 5.59 g/L at 36 h and 8 days of fermentation, respectively. Additionally, 1% of pure ethanol and 0.075, 0.15, and 1% of yeast extract were added to the fermentation broth after 8, 9, and 11 days of fermentation, respectively (arrow 5, 6, and 7 of Fig. 1). The acetic acid productivity, which shows the amount of acetic acid produced per specific volume and time, was 0.7 g/L·h and the specific acetic acid productivity, which shows the amount of acetic acid produced per dry cell weight, specific volume and time, was 0.67 g/g·L·h after

2 days of fermentation. Consequently, when the remaining sugar in the fermentation broth was high, acetic acid production was reduced. It was shown that the fermentation time was shortened by aeration compared with Erlenmeyer flask fermentation. In addition, ethanol and yeast extracts were added to the fermentation broth to increase acid production at the maximum initial cell weight. Lee *et al.* (5) reported that vinegar of 16% acidity was obtained only by adding ethanol during two-stage fermentation, while the *A. tropicalis* 11476P used in this study did not show an increase in acidity only by adding ethanol. In other studies, when onion vinegar was made by two-stage fermentation, the acidity of the vinegar was 5.2% when the concentration of initial alcohol was 5% and the fermentation time was 9 days (14). When the concentration of glucose was 15%, the acidity was 4.89% after a fermentation time of 10 days (15). Kwak (16) found that the initial concentrations of acetic acid and ethanol were 3 and 5% (v/v), respectively, and the total acidity was 5.1% when European black nightshade (*Solanum nigrum*) vinegar was optimized. In this study, the final acidity of vinegar produced by fed-batch fermentation for 14 days was 5.82%. Consequently, the results of this study were more effective than those of other studies for fermentation time and acetic acid production.

Two-stage fermentation of yeast and acetic acid bacteria For the production of acetic acid, two-stage fermentation process, which means two sequential but independent fermentations with yeast to produce ethanol and with acetic acid bacteria to produce acetic acid, was applied in this study. To produce ethanol, 40 mL of *Saccharomyces cerevisiae* KCCM 7913 was inoculated into 2 L of sterilized onion extract and the supernatant was transferred to the fermenter. Then, the fermented broth was centrifuged to remove yeast cells and the supernatant was adjusted to 4% initial ethanol and was inoculated with 4% (v/v) of *A. tropicalis* 11476P, which was incubated for 4 days at 300 rpm and 1.5 vvm (Fig. 2). The lag phase of *A. tropicalis* 11476P took 18 h, thereafter the total acidity started to increase and the residual ethanol concentration was 3.02% (v/v).

The ethanol was completely consumed within 28 h, when the total acidity, the fermentation yield, and the dry cell weight were 3%, 57%, and 3.48 g/L, respectively. After 30 h of fermentation when the total acidity was 3.02%, 2% (v/v) of ethanol and 5% (v/v) of corn steep liquor (CSL), which is commercially more reasonable than yeast

Fig. 2. Profiles of ethanol concentration, acidity, and cell dry weight during the fed-batch culture of *Acetobacter tropicalis* 11476P at an initial ethanol concentration of 4% by ethanol production of *Saccharomyces cerevisiae* KCCM 7913. Four-step feeding: 1, 2% ethanol and 0.5% corn steep liquor (CSL); 2, 2% ethanol; 3, 2% ethanol; and 4, 3% ethanol and 0.3% CSL (arrow indicates the start of the step feeding of nutrients).

extract, were added (arrow 1 of Fig. 2). When 2% (v/v) of ethanol was added thereafter (second addition, arrow 2 of Fig. 2), the total acidity reached 4.8% after a fermentation time of 114 h. Then, when 2% (v/v) of ethanol was added thereafter (third addition, arrow 3 of Fig. 2), the total acidity was 5.4% after 160 h of fermentation. At that time, ethanol and CSL were added at 3 and 0.3% (v/v), respectively (fourth addition, arrow 4 of Fig. 2), and the acidity and residual ethanol content were 6.1 and 0% (v/v) at the fermentation time of 232 h, respectively. CSL is used as a nutrient replacement of yeast extract for yeast-based fermentation for ethanol production (17,18). However, the addition of CSL produced a more brown color when compared to the addition of yeast extract.

Kinetic parameters of yeast and acetic acid bacteria batch fermentation for onion vinegar

The kinetic parameter evaluation was compared between the use of *S. cerevisiae* KCCM 7913 and *A. tropicalis* 11476P, and *A. tropicalis* 11476P and ethanol, respectively, for onion vinegar production (Table 1). The parameter values were calculated after 18 h of the microorganism growth lag phase, from

Table 1. Parameters obtained from batch fermentation under aerated conditions

Parameters	Initial ethanol concentration (%)			
	4% (yeast+acetic acid bacteria)	4% (acetic acid bacteria)	5% (acetic acid bacteria)	6% (acetic acid bacteria)
μ (h^{-1}) ¹⁾	0.341	0.344	0.191	0.114
Y_p/e ²⁾	57	60	59	62
P ($\text{gh}^{-1}\text{L}^{-1}$) ³⁾	2.05	2.02	2.38	3.23
Π (h^{-1}) ⁴⁾	0.69	0.70	1.82	5.42

¹⁾Specific growth rate

²⁾Fermentation yield=[(final acidity (% w/v)–initial acidity (% w/v))/initial ethanol concentration (% v/v)×1.304]×100

³⁾Productivity: final acidity/ fermentation time

⁴⁾Specific product formation rate=[(final acidity (% w/v)–initial acidity (% w/v))/[final cell dry weight–initial cell dry weight]

Fig. 3. Profiles of ethanol concentration, acidity, and cell dry weight during the fed-batch culture of *Acetobacter tropicalis* 11476P at initial ethanol concentration of 4% by ethanol production of *Saccharomyces cerevisiae* KCCM 7913. Three-step feeding: 1, 2% ethanol and 0.2% CSL; 2, 2% ethanol and 0.2% CSL; and 3, 1% ethanol and 0.1% CSL (arrow indicates the start of the step feeding of nutrients).

the time when the production of acetic acid started to increase to the time when the final acidity was reached. This showed that the specific growth rate, fermentation yield, acetic acid productivity (P), and specific product formation rate (II) had almost the same value for 4% of ethanol concentration using *S. cerevisiae* KCCM 7913 or ethanol. While the specific growth rate decreased, the acetic acid productivity and specific product formation rate increased when the concentration of ethanol was 5 and 6%. Consequently, the amount of bacterial cells decreased and the acetic acid productivity increased at a more than 4% initial ethanol concentration. Qi *et al.* (19) reported that when semi-continuous acetic acid fermentation was carried out using a 9 L pilot acetator, the acetic acid productivity was 1.80 g/L·h at the initial concentration of 3.1%. There were no big differences in the yield of this study compared with those using an acetator. Additionally, 0.125–0.140 g/h of ethanol was consumed by *A. tropicalis* 11476P at 4, 5, and 6% of the initial concentrations from the time when the ethanol was almost exhausted to the time when the final acidity was reached, and 0.024–0.070 g/h of ethanol was consumed during fed-batch fermentation after the final acidity was reached. In consequence, the additional ethanol availability of *A. tropicalis* 11476P became lower with higher acidity.

Fed-batch fermentation of high cell density for onion vinegar A cell weight of 6.28 g was obtained after *A. tropicalis* 11476P was inoculated into onion extract with no addition of ethanol and was incubated for 2 days. After that, fed-batch fermentation began with an initial ethanol concentration of 4% (Fig. 3). Consequently, the acidity consistently increased until 24 h of fermentation, while the cell weight of *A. tropicalis* 11476P did not increase and the acidity of *A. tropicalis* 11476P showed a consistent increase when ethanol and CSL were added to the fermentation broth at 24, 72, and 96 h of

Fig. 4. Profiles of ethanol concentration, acidity, and cell dry weight during the single stage fed-batch culture of *Acetobacter tropicalis* 11476P at an initial ethanol concentration 4% by ethanol production of *Saccharomyces cerevisiae* KCCM 7913. Continuous feeding (feeding rate of 100 mL/h-L); thick arrow: 30% ethanol+70% onion juice; thin arrow: 20% ethanol+80% onion juice.

fermentation (arrow 1, 2, and 3 of Fig. 3). However, Kim (20) published the results of the effect of the inoculation levels of the starter culture and acetic acid addition on the fermentation of Makgeolli vinegar, in which the amount of *A. aceti* suppressed the production of acid and retarded the increase in acidity. Park (21) showed that the final acidity was 5.2% and the residual ethanol content was lower than 1% when the initial ethanol concentration and acidity were 5 and 2%, respectively. Ito *et al.* (22) suggested that fed-batch fermentation using two fermenters was used to obtain high-acidity vinegar. In the first fermenter, acetic acid bacteria were grown to their maximum level, while a small amount of substrate was supplied in the second fermenter. A low concentration of ethanol was supplied in both the first and second fermenters. Eight percent acetic acid was obtained when the fermentation broth of the first fermenter was supplied to the second fermenter. In short, this study, in which more than 5% acetic acid was obtained with only one fermenter, is a more efficient process than the others.

Fed-batch fermentation of continuous feeding for onion vinegar

Ethanol and onion extracts were continuously supplied to the fermentation broth with an initial ethanol concentration of 4%, using a peristaltic pump for fed-batch fermentation (Fig. 4). After 28 h of fermentation, when ethanol was depleted, a substrate mixture of 30% ethanol and 70% onion extract was continuously supplied to the fermentation broth. At this time, 0.04 g of ethanol was supplied to the 2 L of fermentation working volume, and then an acidity of 4.5% was reached when the fermentation time was 45 h. After 45 h of fermentation, a mixture of 20% ethanol and 80% onion extract was supplied to the fermentation broth (0.027 g of ethanol per hour), and the acidity increased to 5%. The ethanol and onion extract concentration was modified because the ethanol availability dropped when the

Fig. 5. Profiles of ethanol concentration, acidity, and cell dry weight for the pilot scale production of onion vinegar with *Acetobacter tropicalis* 11476P at an initial ethanol concentration of 6%.

acidity exceeded 4.5%. The acetic acid productivity was 0.67 g/L·h and the specific acetic acid productivity was 0.16 g/g·L·h after 2 days of fermentation. Qi *et al.* (23) reported a final acetic acid content of more than 7% after continuous feeding fermentation when the initial ethanol concentration decreased from 6.4% to lower than 0.5%. In our work, if continuous feeding started at the time of 30 h of fermentation, the acidity showed a steady increase without any increase of dry cell weight of *A. tropicalis* 11476P, and finally, the acidity reached 5.72% after 78 h of fermentation.

Onion vinegar production in the pilot scale Onion vinegar was fermented in the 300 L of the pilot scale as an essential step prior to the mass production of onion vinegar. The initial ethanol concentration was 6% when the working volume of onion extract was 120 L out of the 300 L working volume of the pilot scale fermenter, which was set to 300 rpm and 1.5 vvm (Fig. 5). While an initial ethanol concentration over 4% inhibited the acetic acid production when fermentation was carried out in a flask due to the restricted aeration, there is no inhibitory effect of 6% ethanol on the fermentation when the fermenter was applied. Therefore, 6% of ethanol concentration was used for the higher acetic acid production.

In Fig. 5, acidity did not increase in the beginning of fermentation despite significant decreases in ethanol concentration. In this experiment, the growth of acetic acid bacteria showed 20 h of lag phase and during that time it seems that acetic acid bacteria assimilates ethanol rather than ferments acetic acid. Ethanol was depleted after 48 h of fermentation when the acidity was 4.6%, which was similar to the result of the 2 L fermenter. Considering the fact that the fermentation period needed for the production of commercial vinegar of which acidity ranged 4–29% is generally considered to be about 10 days, the result of this study is economically superior in that the fermentation period of this study is five times shorter than the general standard (24).

Vinegar and onions have their own health beneficial effects such as anti-oxidative activity and when vinegar is produced using onions, it is expected that the fermentation product, onion vinegar, would have synergistic effects on the health beneficial activities. Furthermore, a fed-batch fermentation of onion vinegar optimized in this study shortens the fermentation time and this will enable the production of onion vinegar more economical.

Disclosure The authors declare no conflict of interest.

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