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Isolation and Cultural Properties of Acetic Acid Bacteria for Production of Onion Vinegar

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Abstract In order to produce vinegar using onions, 12 acetic acid bacteria were screened from the juice of fallen peaches, and a strain showing the highest acetic acid productivity among them was selected and identified as *Acetobacter tropicalis* No. 22. The culture broth containing 2.5%(w/v) of initial sugar concentration showed maximum acetic production after 10 days of cultivation, and the acetic acid was produced at the highest rate and reached the maximum acidity after 2 to 6 days of cultivation when the residual sugar and the ethanol concentration were in the range of 1.6 to 2%(w/v) and 0.6 to 1.8%(v/v), respectively. Also optimum conditions for acetic acid production by response surface method using the fractional factorial design with 3 variables and 5 levels were involved with initial ethanol content of 4.67%(v/v), initial acidity of 0.03%, and initial glucose concentration of 2.35%(w/v) and predicted level of acetic acid production at these conditions was 3.77%.

Keywords: onion juice, acetic acid, vinegar, response surface method, *Acetobacter tropicalis* No. 22

Introduction

Onions (*Allium cepa* L.) are the most widely eaten plants among the organosulfur compound-containing vegetables belonging to the family Alliaceae. Main ingredients of onions are sugars such as glucose, fructose, and sucrose, which give onions a unique sweet taste. Onions are known to develop one's stamina by promoting the absorption of vitamin B_1, when taken together with other food, thereby speeding up metabolism and helping to overcome fatigue (1).

Due to the physiological or therapeutic activities of onions, there have been reported the prevention of cardiovascular diseases (2), lowering of blood glucose level (3), anti-oxidative effect (4), neutralization of the toxicity of lead (5), lowering of serum lipid level and allaying of CCl_4 toxicity (6), repression of liver lipid peroxidation (7), and inhibition of DNA damage (8). In recent studies, various physiological activities of onions such as an anti-bacterial activity (9), anti-cancer activity (10-12), anti-oxidative activity (11,13), and removal of heavy metals (14) have been described, which confirms the efficacy of folk remedies using onions. These physiological activities of onions are mainly attributed to sulfur-containing compounds such as *S*-alkyl or *S*-alkenyl thiosulfinates and sulfides (15,16) contained therein. In addition, one of the flavonoids, quercetin, has been reported to have inhibitory activities on breast cancer, colon cancer, ovarian cancer, stomach cancer, lung cancer, and bladder cancer as well as cardiovascular diseases.

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However, because of their short shelf lives, 10-20% of the total production of onions loses their values as commodities due to the rot and softening of tissues, and are used as compost or dumped. Accordingly, in order to guarantee the stable income of onion-cultivating farmers, it is urgently needed to prepare a measure for promoting the consumption of onions and to develop onion processing and storage technologies.

Meanwhile, vinegars are traditional foods used in both the East and the West from ancient times (17). They are representative aerobic fermented foods having unique aroma and sour taste, and contain a small amount of volatile and non-volatile organic acids, sugars, amino acids, esters, and the like components. The sour taste of vinegars stimulates the digestive organs to increase the secretion of digestive juices, thereby promoting the digestive process. Furthermore, it gives a feeling of refreshment, thereby stimulating appetite. Moreover, vinegars have been known to make blood weak alkaline state, be effective in diet, prevent atherosclerosis by decomposing lipid peroxide, promote the secretion of corticosteroid hormones, and have various physiological activities such as lowering of blood pressure, recovering from fatigue, and promotion of calcium absorption.

It is known that brewing vinegars show differences in their chemical components depending on whether the fermentation of alcohols, which are the substrates for acetic acid bacteria, is carried out or not. It is also known that sprit vinegars and fruit or cereal vinegars have different kinds and contents of components according to the raw materials, bacterial strains, preparation methods, and conditions of fermentation and aging used for the preparation thereof. Especially, researches for the preparation of vinegar by employing onions require a wide range of

studies considering preparation method, physicochemical analyses and sensory properties for the harmonization of the strong taste of onions with vinegar. However, only a limited range of studies have been reported (18,19) and, accordingly, there is a great need of such wide range of studies.

In this study, as a part of basic research for enhancing the availability of onions having short shelf-life and for the development of a commercial onion vinegar having functionalities of both of onions and vinegar, we isolated acetic acid bacteria having excellent acetic acid productivity from fallen peaches, and have found the optimum culture condition by using the properties of the bacteria and response surface method (RSM).

Materials and Methods

Screening of bacterial strains having high acid productivity In order to screen bacterial strains having high acid productivity, sourly smelling juices extracted from fallen peaches grown in Janghowon area were incubated for 4 days at 30°C for the enrichment of bacteria. One mL of peach juice was serially diluted by 10 fold using saline solution and 100 μL of diluted peach juice was spread on a modified YCE plate, of which composition was shown in Table 1, for the isolation of acetic acid bacteria. The isolation processes were repeated to obtain purely isolated strains. Ten strains which forming large clear halo were selected as acetic acid-producing bacteria.

Monitoring the growth of isolated strains Growth was determined by checking the optical density of culture broth at 660 nm using spectrophotometer (Du 530; Beckman Coulter, Inc., Fullerton, CA, USA).

Identification of isolated strains Colonies on the modified YCE plate were streaked again on the same agar plate for pure isolation, and then tested for their biochemical traits according to the Bergey's manual of systematic bacteriology (20). For confirmation of the biochemical identification data, 16S rRNA gene was cloned by polymerase chain reaction (PCR) and its nucleotide sequence was determined. PCR experiment was carried out using AccuPower® PreMix (Bioneer, Daejeon, Korea), genomic DNA as a template and 1 set of primers, 8F (5'-AGAGTTTGATCATGGCTCAG-3'), 15R (5'-AAGGAGG TGATCCAACCGCA-3'). Thermal cycler (Hybaid Ltd., London, UK) was programmed at 94°C/2 min for 1 cycle and 94°C/30 sec, 60°C/1 min, 72°C/1 min for 35 cycles and finally 72°C/7 min. After agarose gel electrophoresis of PCR product, the amplified 16S rRNA gene fragment was eluted from the gel using AccuPrep® Gel Purification kit (Bioneer).

Table 1. Medium composition for isolation of acetic acid bacteria

Modified YCE medium		GYP medium	
Glucose	3.0 g	Glucose	1.5 g
Peptone	1.0 g	Peptone	0.3 g
Beef extract	1.0 g	Yeast extract	0.2 g
Ethanol	4.0 mL	Ethanol	3.0 mL
$CaCO_3$	1.0 g	Acetic acid	1.0 g
Agar	1.5 g	Distilled water	94.0 mL
Distilled water	88.3 mL		

The nucleotide sequence of 16S rDNA was determined using PTC-225 Peltier Thermal Cycler and ABI PRISM 3730 XL Analyzer (MJ Reserch, Reno, NV, USA), and analyzed with Vector NTI Suite 7.1 program (InfoMax Inc., Bethesda, MD, USA). Nucleotide sequence homology search was done with BLASTN online program (http://www.ncbi.nlm.nih.gov/blast/) and phylogenetic tree was plotted using MEGA4 program (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA).

Measurement of pH and titratable acidity pH of 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.

Effect of initial acidity, initial ethanol concentration, and initial glucose concentration In order to find out the optimum condition of initial acidity (0, 1, 2, 3, and 4%), initial ethanol concentration (3, 4, 5, 6, and 7%, v/v), and initial glucose concentration (0, 0.5, 1, 1.5, 2, 2.5, 3, 6, and 8%, w/v) on the production of acetic acid, *A. tropicalis* No. 22 strain was cultured at 30°C for 10 and 18 days in glucose/yeast extract/peptone (GYP) broth.

Measurement of reducing sugar The reducing sugar of culture broth was measured by Somogyi-Nelson method (21). One mL of centrifuged culture broth, mixed with 1 mL of copper reagent, was heated for 10 min in the boiling water. After cooling with tap water, 1 mL of arsenomolybdate reagent and 3 mL of distilled water were added and then optical density at 520 nm was measured with a spectrophotometer (Du 530; Beckman Coulter, Inc.). The reducing sugar content was determined using glucose standard curve, and the measured values were triplicated.

Analysis of organic acids Organic acid composition of the centrifuged culture broth supernatant was analyzed by high performance liquid chromatography (HPLC, 1200 Series; Agilent Technologies, Inc., Santa Clara, CA, USA) after removing pigments and proteins with Sep-pak C18 (Waters Co., Milford, MA, USA) and filtering through the 0.25-μm membrane filter. The analysis condition of HPLC is as follows: The column was μ Bondapak C18 column (Waters Co.), and the mobile phase (1 mL/min of flow rate), 1%(v/v) acetonitrile solution (20 mM of $NaHPO_4$), was adjusted to pH 2.0 with phosphoric acid. The injection volume was 20 μL and the UV detector was used at 210 nm.

Ethanol gas chromatography (GC) analysis by solid phase microextraction (SPME) A half mL of culture broth supernatant and 9.4 mL of distilled water were put into a 20-mL screw cap vial, into which 0.1 mL of butanol was added as internal standard. After 10 min absorption of

ethanol into the SPME fiber in the 40°C water bath by the immersion method, ethanol and butanol were detected from the SPME fiber containing alcohol with gas chromatography (6890 Series; Agilent Technologies, Inc.).

The analysis condition of GC is as follows: The column was Innowax HP and the detector was flame-ionization detector (FID). The temperatures of the injector and the detector were 200 and 250°C, respectively. The temperature of the column was kept at 45°C for 3 min, thereafter the temperature was increased up to 180°C with the rate of 20°C/min, of which state was kept for 3 min. The carrier gas was helium (50 mL/min of flow rate), and the gases for detection were air (250 mL/min of flow rate) and hydrogen (30 mL/min of flow rate). As standard materials, ethanol (99.5%) and butanol (99.5%) purchased from Sigma-Aldrich (St. Louis, MO, USA) were used. The amount of ethanol was measured by the response factor of ethanol peak area/butanol peak area identified by each retention time.

Efficiency of acetic acid production The ratio of the amount of the produced acetic acid to the theoretical amount of the acetic acid was represented as a percentage.

Experimental design In order to optimize acetic acid production from onion, experiments were planned according to the central composite design using SAS program.

In the 250-mL Erlnmeyer flasks, each of which contains 100 mL of GYP medium, 2%(v/v) of *Acetobactor tropicalis* No. 22 was inoculated and cultured. Independent variables of initial ethanol concentration, initial acetic acid concentration, and initial glucose concentration of variables of 5 levels (–2, –1, 0, 1, 2) were set according to the central composite design, and the experiments were conducted by 16 conditions. The dependent variable (Y1) related to culture properties was acidity.

Onion vinegar The supernatant (8-10°Bx) obtained from the onion, which was ground and centrifuged at 15,000×g, was sterilized. As the onion juice turned brown after 15 min sterilization at 121°C, the sterilization condition was set for 10 min at 110°C. When the temperature of the sterilized onion juice reached below 50°C, 4%(v/v) of ethanol was added and then acetic acid bacteria was inoculated at the concentration of 2%(v/v). Onion juice was fermented for 18 days in the shaking incubator at 150 rpm at 30°C, and the acidity, remaining ethanol, and reducing sugar were measured periodically.

Results and Discussion

Screening of acetic acid-producing bacteria and strain identification For the screening of acetic acid-producing bacteria, fallen peaches grown in Janghowon area were collected and juices extracted from them were incubated at 30°C for 4 days. When peace juices were spread onto modified YCE agar plate, a selective medium for acetic acid bacteria, several hundred colonies were grown on a plate. Among them, 10 strains which showed large clear halo were selected as acetic acid-producing bacteria.

In order to identify the selected 10 strains according to their molecular genetic properties, nucleotide sequences of their 16S rRNA gene were determined and analyzed. As a result, nucleotide sequences (1,450 base pairs each) of 16S rRNA genes of strain No. 1, 3, 6, 7, 10, and 16 showed 99% identity with those of *A. tropicalis* LMG 1663 and *A. tropicalis* NRIC 0312 isolate No. 39, while that of strain No. 22 exhibited 98% identity with those of *A. tropicalis* LMG 1663 and *A. tropicalis* NRIC 0312 isolate No. 39. In addition, nucleotide sequence of 16S rRNA gene of strain No. 2 showed 99% identity with those of *Acetobactor orientalis* 4B-S16D and *A. orientalis* B-7SC, respectively, while that of strain No. 27 had 99% identity with that of *Gluconobacter cerinus* NBRC 3269. A phylogenetic tree was prepared by employing the nucleotide sequence information of 16S rRNA genes of the selected 10 strains (Fig. 1), which exhibited that strain No. 20 and 22 have very close genetic relation with each other. Further, it was also confirmed that strain No. 3 and 7 were very similar strains, while strain No. 27 was genetically different from other strains.

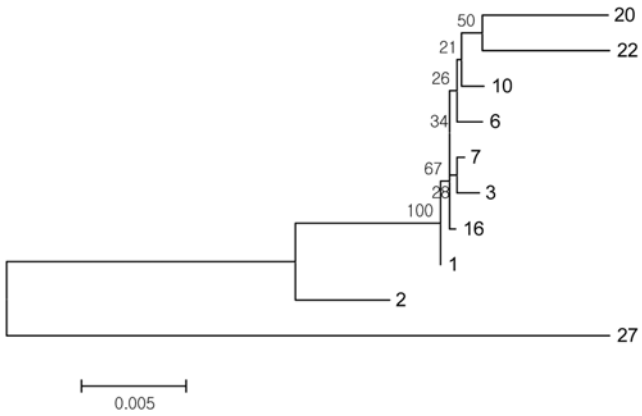

Fig. 1. Phylogenetic tree of 10 isolates based on nucleotide sequence of 16S rRNA gene.

Based on the results of the molecular genetic analyses as discussed above, strain No. 1, 3, 6, 7, 10, 16, 20, and 22 were identified as *A. tropicalis*; strain No. 2 as *A. orientalis*; and strain No. 27 as *G. cerinus*.

Selection of vinegar-producing strain The selected 10 strains were cultured on GYP medium at 30°C for 8 days with shaking at 150 rpm, and acetic acid productivity and growth of the strains were measured (Table 2). Among the tested strains, *A. tropicalis* No. 1 grew best and No. 22 was next. *A. tropicalis* No. 22 showed highest acid production, 3.88%, and *G. cerunus* No. 27 showed acidity of 3.83%. Based on the results shown in Table 2, *A. tropicalis* No. 22 was finally selected as an acetic acid-producing starter strain in this study due to its high acidity and excellent bacterial growth.

Morphological and physiological characteristics of the selected strain To confirm the identification of the selected strain, the morphological and physiological characteristics of *A. tropicalis* No. 22 were examined. Strain No. 22 was observed as a rod-type under an optical microscope (data not shown), confirmed as Gram-negative bacteria in the Gram staining, and exhibited no motility. The strain was identified as non-spore forming bacteria due to its negative

Table 2. Growth, acidity, and pH of acetic acid bacteria isolates

Acetic acid bacteria strain No.	$OD_{660\,nm}$	Acidity (%)	pH
Acetobacter tropicalis No. 1	0.334	3.73	1.8
Acetobacter orientalis No. 2	0.000	1.07	2.4
Acetobacter tropicalis No. 3	0.257	3.80	1.9
Acetobacter tropicalis No. 6	0.274	3.54	2.1
Acetobacter tropicalis No. 7	0.280	3.76	1.9
Acetobacter tropicalis No. 10	0.301	3.26	1.9
Acetobacter tropicalis No. 16	0.194	3.38	1.8
Acetobacter tropicalis No. 20	ND[1)]	1.15	2.4
Acetobacter tropicalis No. 22	0.303	3.88	1.9
Gluconobacter cerinus No. 27	0.264	3.83	1.9

[1)]Not detectable.

Table 3. Morphological and physiological characteristics of isolate *A. tropicalis* No. 22

Characteristics	No. 22
Morphology	Rod-shaped
Gram stain	-
Catalase test	+
Decarboxylase test	-
MR test	-
VP test	-
Motility	-
Spore test	-
Oxidase test	-
Indole test	-
Gelatin test	-
H_2S formation	-

response in the spore staining test. Further, it showed positive response in the catalase test and negative responses in the decarboxylase test, MR test, VP test, oxidase test, indole test, and gelatin test. As confirmed by these test results, the strain has the typical characteristics of *Acetobacter* spp. (Table 3).

Effect of initial acid concentration on the growth and acetic acid production of the selected strain In order to find out optimum conditions for the highest acetic acid productivity with *A. tropicalis* No. 22 strain, the bacterial cells were cultured in GYP broth containing 3%(v/v) ethanol at 30°C for 10 days, while the initial acidity of the broth being adjusted to 0, 1, 2, 3, and 4%, respectively. Then, the growth of bacteria and the acid production were measured and the results are shown in Fig. 2. Generally, in conventional acetic acid fermentations, the medium has initial acidity for the prevention of contamination by unwanted microorganisms and studies on the acetic acid fermentation include such initial acidity to total acetic acid production. In our study, however, the amount of acetic acid added for the adjustment of initial acidity was excluded from the total acetic acid production, considering that it is reasonable to compare the net amounts of acetic acid produced by acetic acid bacteria.

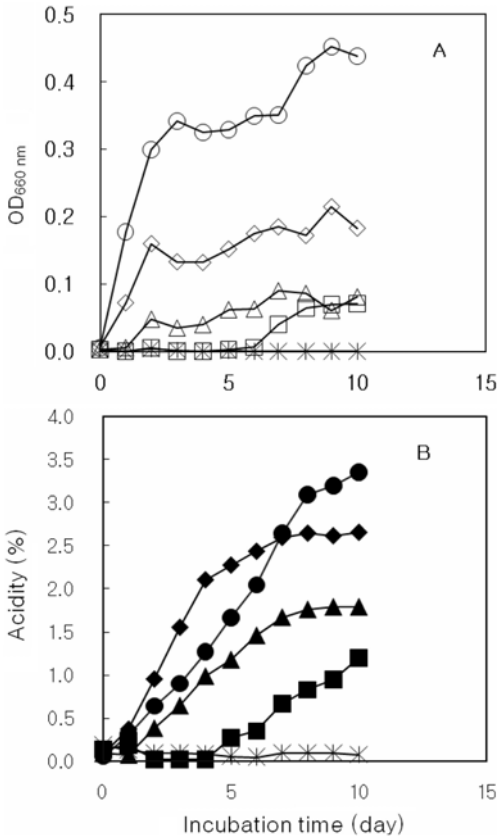

Fig. 2. Growth (A) and acid production (B) of *A. tropicalis* No. 22 by initial acetic acid concentration at 30°C. Acetic acid concentration: ● and ○, 0%; ◆ and ◇, 1%; ▲ and △, 2%; ■ and □, 3%; ✳, 4%.

Upon comparing the net acetic acid production, the acidity of the fermentation broth was highest until day 7 when the initial acidity was 1%, while the fermentation broth using no initial acidity showed the highest acidity from day 8 (Fig. 2B). At day 8, the acidity of the fermentation broth using no initial acidity was 3.36%, which is much higher than 2.66% of the fermentation broth using 1% initial acidity. The total acetic acid production sensitively reduced as the initial acidity increased. Upon calculating the fermentation efficiencies at day 10, that of the fermentation broth with no initial acidity was 86%, while that of fermentation broth with 1% initial acidity was 42%, and the fermentation broth with 3 or 4% initial acidity showed negative value. Kang *et al.* (22) and Oh (23) reported that acetic acid fermentation poorly progressed due to the contamination of film forming bacteria at initial acidity of 0.5 and 1%, and, accordingly, optimum initial acidity was 2%. These results were obtained by including the amount of acetic acid added for the adjustment of initial acidity in total acetic acid production, and, accordingly, it does not correspond to the results comparing only the amount of acetic acid produced by acetic acid bacteria. Further, as can be seen from the growth curve of acetic acid bacteria (Fig. 2A), the growth of acetic acid bacteria was inhibited depending on the initial acidity. Accordingly, it is considered that the condition of no initial acidity is preferable for the growth of bacteria, because the mass

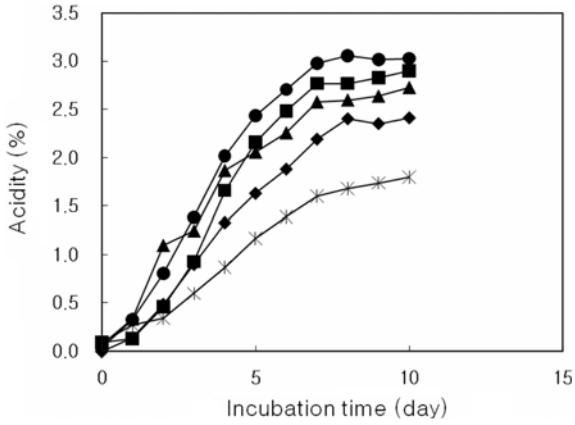

Fig. 3. Acid production profile of *A. tropicalis* No. 22 by initial ethanol concentration during the culture period of 10 days at 30°C. Initial ethanol concentration: ✳, 3%; ●, 4%; ▲, 5%; ◆, 6%; ■, 7%.

production of acetic acid requires cultivating bacterial cells at a high concentration. The bacterial growth curve exhibited that the growth of bacterial cells reduced due to acetic acid produced by themselves, while it increased again after the period of adaptation to acid (3 to 7 days).

Acidity and growth according to the initial ethanol concentration of the selected strain Acetic acid bacteria are microorganisms producing acetic acid by oxidizing ethanol and, accordingly, initial ethanol concentration in the medium is very important in the acetic acid production. In order to examine the effect of initial ethanol concentration on the acetic acid production by acetic acid bacteria, *A. tropicalis* No. 22 was cultured on GYP medium with initial acetic acid concentration of 1% at 30°C for 10 days, while varying the initial ethanol concentration to 3, 4, 5, 6, and 7%, respectively. Then, the acetic acid production by the bacteria was measured and the results are shown in Fig. 3.

A. tropicalis No. 22 showed the highest acidity at day 8, wherein the case of initial ethanol concentration of 4% exhibited the highest acidity of 3.06%. This result corresponds to the results of Hong *et al.* (24), Son *et al.* (25), and Yang and Choi (26). Hong *et al.* (24) reported that the fermentation broth with initial ethanol concentration of 4%(v/v) showed an excellent acid productivity and luscious flavor, while the fermentation broth with initial ethanol concentration of 6%(v/v) had a strong stimulating odor and insufficient luscious flavor due to the inhibition of acid production. Yang and Choi (26) reported that, when the ethanol concentration reached high, the growth of cells became retarded due to the extension of the lag phase and the acid productivity decreased, and that optimum concentration of ethanol for the acetic acid fermentation is about 4%(v/v). In the present study, it was also observed that the lag phase of acetic acid production was extended above 4%(v/v) of initial ethanol concentration. Meanwhile, Lee *et al.* (27), Park *et al.* (28) and Sim *et al.* (29) reported that the highest acid productivity was observed at ethanol concentration of 6%(v/v) in the production of aloe and persimmon vinegar, and Kang *et al.* (22) reported that

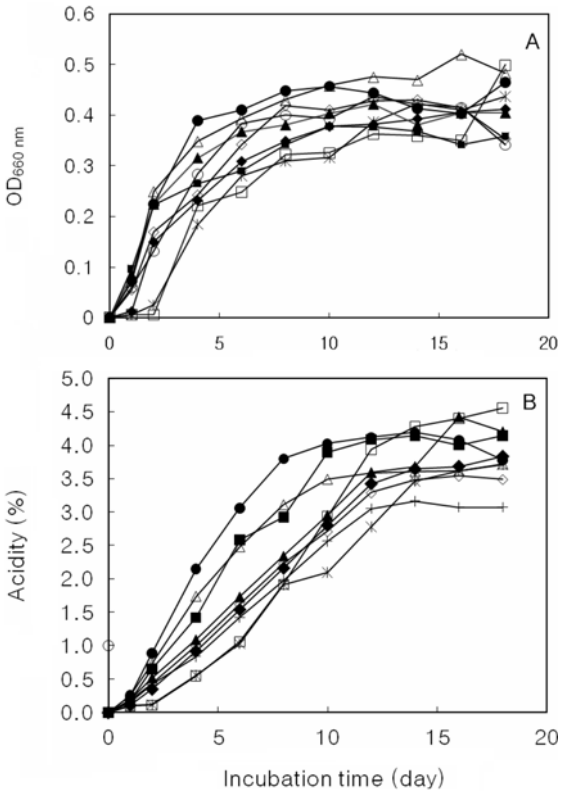

Fig. 4. Growth (A) and acid production (B) of *A. tropicalis* No. 22 by initial glucose concentration during the incubation period at 30°C. Initial glucose concentration: □, 0%; ✳, 0.5%; ◆, 1.0%; ■, 1.5%; ▲, 2.0%; ●, 2.5%; △, 30.%; ○, 6.0%; ◇, 8.0%.

initial ethanol concentration of 5%(v/v) was most efficient in the production of citron vinegar. Jeong *et al.* (30) reported that the lag phase became longer at the initial ethanol concentration of 8%(v/v), while the fermentation progressed well at a later stage.

Acidity according to the initial glucose concentration of the selected strain In order to examine the optimum conditions of the acetic acid production using *A. tropicalis* No. 22 according to initial glucose concentration, the bacteria were cultured on GYP medium with 4%(v/v) ethanol concentration, which had induced the highest acidity, and initial acetic acid concentration of 0% at 30°C for 18 days, while the concentration of glucose, which can enhance the growth of bacteria, was adjusted to 0, 0.5, 1, 1.5, 2, 2.5, 3, 6, and 8%(w/v), respectively. The growth of bacteria and total acid production by the bacteria were determined and the results are shown in Fig. 4. The acidity rapidly increased until day 8, and reached the highest one at day 10 (Fig. 4A). With regard to the effect of glucose on acidity, Kim *et al.* (31) reported that, in case of the Japanese apricot juice medium for the acetic acid production for preparing Japanese apricot vinegar, addition of 0.2%(w/v) glucose induced the highest acidity of 6.5%, while the addition of 0.3%(w/v) or less of glucose reduced the acidity; namely, the acetic acid production was inhibited by high initial glucose concentration. Hong *et al.* (24) reported that the addition of glucose to the medium in the preparation

of persimmon vinegar is not appropriate for the reason that, when glucose, a substrate of alcohol fermentation, is added to the medium, only a part of added glucose was converted into alcohol and the reminder was participated in the proliferation of harmful bacteria, generation of abnormal odor and coloring, thereby deteriorating the quality of vinegar. Son *et al.* (25) reported that the optimum glucose concentration was 8.42%(w/v) when determined by the response surface methodology using central composite design.

According to the results of our study, until day 8 of the fermentation, the fermentation broth with initial glucose concentration of 2.5%(w/v) showed higher bacterial growth and acidity than the other fermentation broth with initial glucose concentration of more than or less than 2.5%(w/v) (Fig. 4B). Accordingly, it is considered that initial glucose concentration of 2.5%(w/v) is the optimum condition for the growth of acetic acid bacteria and acetic acid production. In addition, acetic acid bacteria grown in a medium with the addition of ethanol without addition of glucose showed low growth and acidity until day 6, while the acidity increased consistently thereafter.

Composition of organic acids in the culture broth of acetic acid bacteria *A. tropicalis* No. 22 was cultured for 18 days in GYP medium and onion juice, each containing 4%(v/v) ethanol, respectively, and the composition of organic acids in the culture broth showing the maximum acidity was determined and shown in Table 4. Among the standard organic acids used in the analysis, oxalic acid, citric acid, tartaric acid, malic acid, succinic acid, lactic acid, and acetic acid were identified, wherein the content of acetic acid was the highest and, then, in the order of citric acid, malic acid, and succinic acid. Further, when the same acetic acid bacteria was employed in the experiment, the compositions of organic acids obtained in the GYP medium and onion juice culture broth were similar to each other, but the contents of organic acids from 2 culture broth were different from each other.

Lee and Yuk (32) reported that, based on the result of analysis of the organic acids in a persimmon vinegar, acetic acid, lactic acid, and citric acid were detected in the order of their contents, but malic acid and oxalic acid were not detected. Moon *et al.* (33) reported that, based on the result of organic acid analysis, oxalic, citric, tartaric, malic, succinic, lactic, and acetic acids were detected in the commercialized vinegars. And the kinds of generated organic acids varied slightly according to the kind of vinegars tested, wherein oxalic acid was detected in a trace amount in all of the vinegars, and malic acid was present below 0.08% in a brewing vinegar, unpolished rice vinegar, and persimmon vinegar, contrary to its significantly high content of 0.08 to 0.29% in an apple vinegar. Shin *et al.* (18) reported that acetic, oxalic, citric, tartaric, malic, and succinic acids were detected as organic acids of onion vinegar obtained by 2-stage fermentation, wherein the contents of acetic, succinic, and malic acids were high, such result being similar to that of our study. Moreover, Moon *et al.* (33) reported that, based on the results of sensory tests on commercialized vinegars, no correlation was found between the contents of saccharides and amino acids, and the organoleptic properties, because the contents of saccharides and amino acids were too low. In case of organic acids, lactic acid showed a negative correlation with overall taste preference at 1% significance level, while acetic acid showed a positive correlation. It was stated that as the ratio of acetic acid to total organic acids (A/T) increased, the taste preference was evaluated better. In this point of view, because the acetic acid bacteria of the present study produced lactic acid in a trace amount and because A/T ratio of the culture broth obtained by culturing the bacteria in an onion juice for 18 days was only 0.65, in contrast to 0.89 of the culture broth obtained by culturing the bacteria in GYP medium for 10 days, it was noticed that acetic acid production in an onion juice should be enhanced for a higher preference.

Effect of initial and residual glucose and ethanol concentrations on acetic acid production *A. tropicalis* No. 22 was inoculated at 5%(v/v) in 2 kinds of GYP media, which commonly had initial acidity of 0% and initial ethanol concentration of 4%(v/v) with different initial glucose concentration of 0 or 2.5%(w/v), and in an onion juice containing 5.1%(w/v) of reduced sugars in itself, respectively, and cultured for 18 days. Then, the changes of glucose and ethanol concentrations, and the acidity were determined and the results are shown in Fig. 5.

Upon comparing the results of 10 day-cultures conducted in 2 GYP media, the acidity obtained in GYP medium with initial glucose concentration of 2.5%(w/v) at day 8 was 3.88% approaching the highest acidity, while the acidity obtained in GYP medium without glucose was 2.98%. These results demonstrate that initial glucose concentration, as well as initial ethanol concentration, is required for the rapid production of acetic acid. The acidity of culture broth containing initial glucose concentration of 2.5%(w/v) did not increase after day 8, probably due to the exhaustion of ethanol. The residual ethanol concentration at a period of day 2 to day 8, when the increase rate of acidity was high, was in a range of 0.6 to 1.8%(v/v), and the residual glucose concentration at this period was in a range of 1.6 to 2.0%(w/v). In case of the culture broth using the onion juice containing 5.1%(w/v) of reduced sugars in itself, it

Table 4. Contents of organic acids in culture broth of *A. tropicalis* No. 22

Broth	Organic acids (%)									
	Oxalic acid	Citric acid	Tartaric acid	Malic acid	Succinic acid	Lactic acid	Acetic acid	fumaric acid	Total	A/T[1)]
GYP medium broth	0.01	0.05	0.08	0.10	0.10	0.21	4.26	Trace	4.81	0.89
Onion juice	0.01	0.7	0.09	0.69	0.62	Trace	2.67	Trace	4.08	0.65

[1)]Ratio of acetic acid compared to total organic acids.

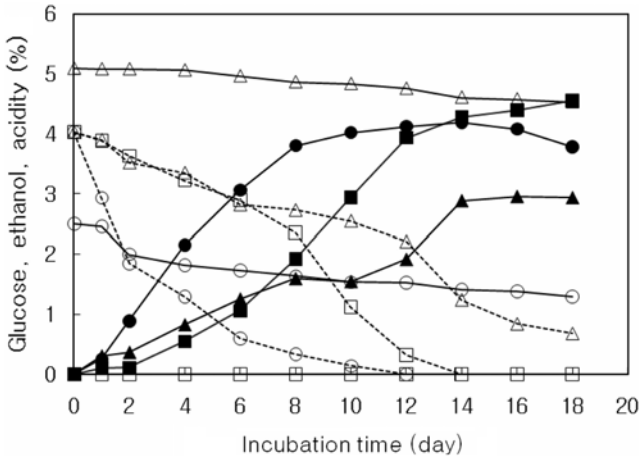

Fig. 5. Effect of residual glucose, ethanol concentration for growth, and acid production of *A. tropicalis* No. 22 by initial acetic acid concentration during the incubation period at initial ethanol concentration of 4%. Initial glucose concentration 0%: -■, acidity; -□, residual glucose; ···□, residual ethanol concentration. Initial glucose concentration 2.5%: -●, acidity; -○, residual glucose; ···○, residual ethanol concentration. Onion juice: -▲, acidity; -△, residual glucose; ···△, residual ethanol concentration.

showed an acidity as low as 1.6% at day 8, and 0.68%(v/v) of ethanol was still remained in the broth at day 18, not being completely consumed (acidity 2.94%). These results may be attributed to the residual sugar concentration as high as 4.53%(w/v), which inhibited efficient ethanol consumption. Such phenomenon was also observed in case of the GYP medium with initial glucose concentration of 6%(w/v) and being cultured for 18 days (Fig. 4), wherein the residual glucose concentration was 5.2% and the residual ethanol concentration was 0.76%(v/v). Lee *et al.* (34) reported that a vinegar having an acidity of 16% was produced in a fed-batch culture, wherein ethanol was added in a fed-batch type under the condition of 0.5%(v/v) residual ethanol concentration and the temperature was lowered gradually by 1°C during the fermentation period of 40 hr. Based on this result, we concluded that it is possible to produce onion vinegar showing high acidity by carrying out the fermentation under the conditions that residual glucose concentration is within the range of 1.6-2.0%(w/v), and ethanol was added continuously to maintain the residual ethanol concentration of 0.6-1.8%(v/v). The overriding prerequisite for the production of onion vinegar showing high acidity is to reduce the sugar content of the onion juice. From these results, it may be concluded that a high-acidity onion vinegar can be produced by a fed-batch culture, wherein ethanol is not added to the initial medium but added after the residual glucose is consumed of by acetic acid bacteria for some extent, or the sugar content of

Table 5. Experimental data for acidity under different conditions of initial ethanol concentration initial glucose concentration, initial acetic acid concentration of *A. tropicalis* No. 22

Experimental No.	Culture condition			Physicochemical property
	Initial ethanol concen. (%)	Initial acetic acid concen. (%)	Initial glucose concen. (%)	Acidity (%)
1	4(-1)	1(-1)	1.5(-1)	3.19
2	4(-1)	1(-1)	3.5(1)	2.72
3	4(-1)	3(1)	1.5(-1)	1.37
4	4(-1)	3(1)	3.5(1)	1.33
5	6(1)	1(-1)	1.5(-1)	2.41
6	6(1)	1(-1)	3.5(1)	2.35
7	6(1)	3(1)	1.5(-1)	1.08
8	6(1)	3(1)	3.5(1)	1.04
9	5(0)	2(0)	2.5(0)	1.27
10	5(0)	2(0)	2.5(0)	1.27
11	7(2)	2(0)	2.5(0)	1.12
12	3(-2)	2(0)	2.5(0)	1.1
13	5(0)	4(2)	2.5(0)	0
14	5(0)	0(-2)	2.5(0)	3.56
15	5(0)	2(0)	4.5(2)	1.23
16	5(0)	2(0)	0.5(-2)	0.86

medium is reduced to a range of 1.6 to 2.0%(w/v) by culturing yeasts, followed by inoculating acetic acid bacteria when the concentration of ethanol reached to about 4%.

Optimization of acetic acid production by response surface methodology In order to optimize acetic acid production, independent variables of initial ethanol concentration, initial acetic acid concentration and initial glucose concentration and variables of 5 levels (-2, -1, 0, 1, 2) were set according to a central composite design and supplementary experiments were carried out. Acetic acid production by *A. tropicalis* No. 22 was measured and the results are shown in Table 5 and 6.

As can be seen in Table 6 showing the effects of independent variables on the response variable, i.e., acetic acid production, the initial acetic acid concentration out of the independent variables has the highest effect on the acid production, and the initial acetic acid concentration was acknowledged as being significant at the level of 5% ($p<0.05$).

The result of analysis of variance (ANOVA) conducted on the established regression is shown in Table 7. In the response surface formed by quadratic regression, R^2 was 0.8482. When initial ethanol concentration, initial acetic

Table 6. Analysis of variance showing significant effects of culture variables on acetic acid production of *A. tropicalis* No. 22

Culture variables	Degree of freedom	Sum of squares	F value	Probability>F
Initial ethanol concentration (%)	4	0.27	0.19	0.9323
Initial acetic acid concentration (%)	4	10.84	7.95	0.0141
Initial glucose concentration (%)	4	0.09	0.07	0.9882

Table 7. Analysis of variance showing effects of treatment variables as linear or quadratic terms and interaction (cross product) effect on response variables

Source	Degree of freedom	Sum of squares	R^2	Probability >F
Linear	3	10.69	0.7927	0.0085
Quadratic	3	0.66	0.0493	0.6111
Cross product	3	0.09	0.0064	0.9657
Total model	9	11.45	0.8484	0.0615

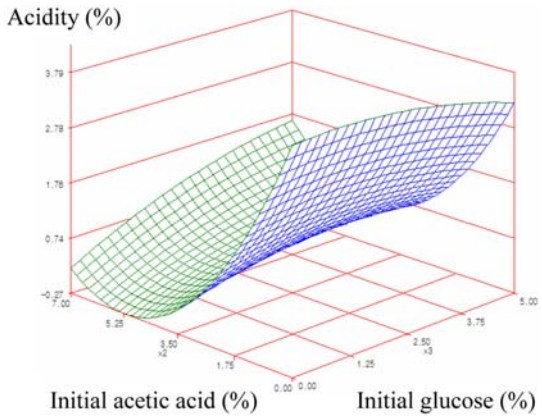

Fig. 6. Response surface for acetic acid production of *A. tropicalis* No. 22.

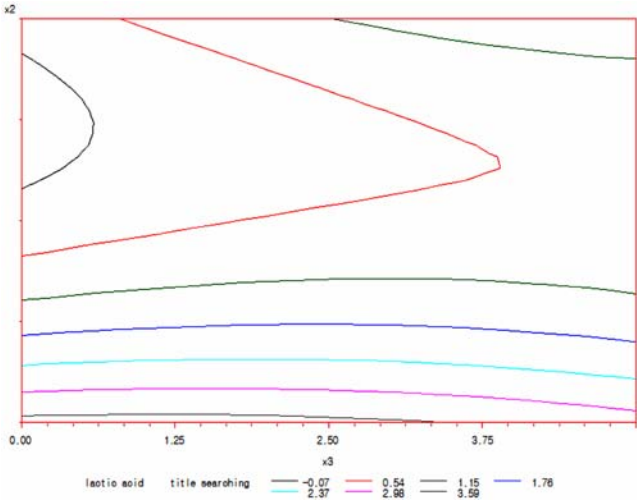

Fig. 7. Contour map for acetic acid production of *A. tropicalis* No. 22.

acid concentration, and initial glucose concentration were selected as independent variables for the acetic acid production, their degrees of change were determined with response surface and contour map. When contour lines were drawn using each of initial ethanol concentration, initial acetic acid concentration and initial glucose concentration as a function, conditions according to the response variables did not coincide. Accordingly, we fixed initial ethanol concentration, which was disclosed as having smallest effect regardless of concentrations, among the 3 experimental variables, and tried to find out an optimum condition between initial acetic acid concentration and initial glucose concentration. An analysis was carried out by fixing initial ethanol concentration to 5% and employing only initial acetic acid concentration and initial glucose concentration as variables, and response surface and contour map prepared for analyzing the degree of variance are shown in Fig. 6 and 7, respectively. Because the stationary points of each response variables were exhibited as stable points, a ridge analysis was conducted to find out conditions for the highest acetic acid production. As can be seen from the results shown in Table 8, initial ethanol concentration of 4.67%(v/v), initial acetic acid concentration of 0.03%, and initial glucose concentration of 2.35%(w/v) were predicted as culture conditions for optimum responses of acetic acid production, and the acidity under these conditions was predicted as 3.77%, which approximates that of the experimental result.

Table 8. Predicted levels of culture conditions for optimum responses of acetic acid production of isolated *A. tropicalis* No. 22

Culture variables	Levels for optimum responses of acetic acid production
Initial ethanol concentration (%)	4.67
Initial acetic acid concentration (%)	0.03
Initial glucose concentration (%)	2.34
Morphology	saddle point
Predicted value at stationary point (%)	3.77

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