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Isolation and identification of a novel strain of *Acetobacter ghanensis* KBMNS-IAUF-6 from banana fruit, resistant to high temperature and ethanol concentration

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Abstract

Background and Aims: The use of ethanol- temperature-resistant *Acetobacter* strains to produce vinegar on an industrial scale is important due to their sensitivity to high ethanol concentration as substrate and high energy consumption for acetator cooling. The aims of this study were to isolate and identify high temperature and ethanol resistant *Acetobacter* strains as starter for production of vinegar.

Materials and Methods: The banana alcoholic extract was transferred to the fermenter medium and after 24 h incubation at 30°C, colonies with transparent zone were purified as acetic acid bacteria and examined macroscopically and microscopically. The resistance to high temperature in constant ethanol and time as well as resistance to high ethanol in constant temperature and time were investigated.

Results: The studies of AAB isolate grown in the Carr medium showed that it was a *Acetobacter* strain. According to the single-factor optimization, this species was able to grow in a Carr medium containing 9% ethanol at 40°C after 72 h.

Conclusion: This is the first report of an AAB isolation from banana in Iran. This bacterium, as a new resistant strain to high levels of ethanol and temperature, was identified as *Acetobacter ghanensis* KBMNS-IAUF-6 and its 16S-rDNA sequence was deposited in GenBank, NCBI, under the accession number of MK968570. This new strain can be suggested as a high temperature and ethanol resistant strain for producing banana vinegar on a semi-industrial and industrial scale.

Keywords: Acetic acid bacteria, *Acetobacter ghanensis*, Industrial and food biotechnology, Temperature and ethanol resistant strain

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Introduction

According to the US Food and Drug Administration, vinegar is a sour-tasting solution containing at least 4% acetic acid. Acetic acid in vinegar is produced by acetic acid bacteria. The quality of vinegar production depends on the type of acetic acid, the substrate and the method used in its production (1-3). Recently vinegar factories are searching for new varieties of vinegar using different types of acetic acid bacteria as starter. Because each microbial starter, in addition to acetic acid, produces 50 other types of aromatic substances. So, the vinegar produced by these strains will have a new flavor. Acetic acid bacteria are isolated from a variety of natural substrates such as fruits, potatoes and rice. Various kinds of vinegar known so far include wine vinegar, white vinegar, sherry vinegar, balsamic vinegar, beer vinegar, malt vinegar, barley vinegar, rice vinegar, onion vinegar and potato vinegar (4, 5). Acetic acid bacteria are obligatory Gram-negative and aerobic rods. There are currently 12 genera in this family, among which three genera are more important industrially including *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (6). Among the acetic acid bacteria, *Acetobacter* species are more suitable for the production of vinegar on an industrial scale because they directly use ethanol as a cheap substrate and also produce no other product than acetic acid. *Acetobacter* species are chemolithotrophic, motile or non-motile, catalase positive, oxidase negative, mesophilic and capable of super-oxidation. In *Acetobacter* species, ethanol is oxidized to acetic acid and then acetic acid to carbon dioxide in the process of oxidation and super-oxidation. The redox process occurs when the oxygen content is high but ethanol is not present in the medium. Therefore, the concentration of ethanol as a substrate is very important in the vinegar production process (7). The traditional method, as the first industrial process of vinegar production, was carried out in open barrels. Despite the high quality of the product, this method was slow. In the nineteenth century, surface fermentations emerged as faster methods. One of these methods was the drop generator, which is still used today. At the beginning of 1949, submerged fermentation methods were developed. Therefore, the goal of new technologies is to produce high quality, inexpensive vinegar in a short period of time. Thus, recent methods of vinegar production include the use of acetator submerged methods (4, 8, 9). Because of the high energy consumption for cooling the acetator, isolation of thermo-tolerant *Acetobacter* strains is necessary. These isolated strains must be highly resistant to ethanol because when the ethanol concentration is low the Acetobacter strains have to oxidize the acetate for survival, but super-oxidation does not occur in the presence of high ethanol levels. Due to the mesophilic nature and susceptibility of the bacteria to high ethanol concentrations, it is needed to optimize different varieties of *Acetobacter* spp. in vinegar production in order to increase their resistance to high temperature and ethanol (10). The purposes of this study were to isolate and identify new strains of high ethanol and temperature resistant *Acetobacter* strains from banana fruit to produce a new flavored vinegar.

Materials and Methods

The **Chemicals and culture media**

The materials used in this study included Gram staining kit (Taligene Pars Co., ISTT, Iran), hydrogen peroxide and oxidase disk (Merck, Germany). Also the culture media used in this study included Carr culture medium [yeast extract, 3%; agar, 2%; bromocresol green, 0.002% (Merck, Germany); sterile distilled water, 100 ml; ethanol, 2% (Merck, Germany)] and Frateur medium [yeast extract, 2%; calcium carbonate, 2%; agar, 2%; sterile distilled water, 100 ml; ethanol, 2% (v/v)].

Initial isolation of different strains of acetic acid bacteria

The ripe and sweet bananas were kept in a clean cabinet at room temperature until the fruit flies were seen and the smell of the scent arose. After peeling and squeezing the banana structure by sterile mortar, the extract obtained from the banana was transferred to a 2-liter sterile bottle. Also, a few holes were created to prevent the bottle from exploding due to the yeast' production of carbon dioxide. The bottle was kept in a clean cabinet at room temperature for 7 to 10 days (7). Then 50 µl of 10^{-1} to 10^{-5} dilutions of this extract were cultured in Frateur culture medium. After incubation at 30°C for 48 to 72 hours, the colonies that were surrounded by clear halo zone were purified in the same medium. Then, the macroscopic and microscopic characteristics of the isolates were studied after 24-hour culture in Frateurr and Carr medium (11).

Screening of different *Acetobacter* spp.

For the screening of *Acetobacter* species from other acetic acid bacteria, in Carr and Frateur media, the yellowish colonies with oxidase-negative and catalase positive reactions were selected (11).

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Molecular identification of *Acetobacter* isolated strains

Pure colonies were transferred to 50 ml sterile distilled water from the culture medium for 24 h. 10 ml of this suspension was transferred to a sterile 15 ml Falcon and centrifuged at 5000 g for 15 minutes. The supernatant was discarded and the pellet was transferred to a sterile tube. DNA extraction kit (Bioneer, South Korea) was used for DNA extraction from 1 mg of bacterial mass. In this study, general primers designed and constructed by Taligene Pars Company, Isfahan, Iran. The OF BUI and OR BUI, were as forward and reverse primers respectively. Their sequences were 5´-AACTGGAGGAAGGTGGGGAT-3´ as forward primer and 5'- AGGAGGTGATCCAACCGCA -3´ as reverse primer.

The PCR program consisted of initial temperature of 96°C for 4 minutes followed by 30 cycles with temperatures of 94°C for 2 minutes, 55°C for 1 minute and 72°C for 1 minute, respectively. The final stages consisted of 72°C for 4 minutes and 4°C for 10 minutes, respectively. The expected molecular weight of the PCR product was 370 bp (12). The PCR product and primers were sent to Taligene Pars for sequencing. These sequences were analyzed using Finch TV V.1.4.0 and Mega 6 software and compared with the genomic sequences available in GenBank, NCBI using blast software (http://blast.ncbi.nlm.nih.gov). Finally, 16S-rDNA sequence of the isolate was deposited in the GenBank, NCBI.

Single-factor optimization of high ethanol resistance and temperature *Acetobacter* isolate

The experiment was performed to select high-alcohol and temperature-resistant *Acetobacter* strains. In this study, acid production and growth rate of the isolate in terms of colony forming unit (CFU) in Carr medium with different amounts of ethanol (2 to 10%) at constant temperatures of 34, 36, 38 and 40°C after 24, 48, 72 and 96 hours were performed. Also, the growth and acid production of this isolate at different temperatures of 34, 36, 38 and 40°C in the culture medium with constant ethanol content (5, 7 and 9%) after 24, 48, 72 and 96 hours were compared (6).

Results

Macroscopic, microscopic and biochemical properties of *Acetobacter* isolates

In this experiment, an acetic acid bacterium was isolated by creating a transparent area around colonies in the Frateur medium after 72 h (Fig. A1). Investigation of macroscopic characteristics of 24-hour culture of this bacterium in Carr and Frateur media showed that the colonies grown were round, fine, colorless, translucent, soft and had an odor of vinegar. Also microscopic characteristics of the isolates in these two media showed that these bacteria were rod-shaped and Gram-negative. According to the results of the screening, this catalase-positive and oxidase-negative isolate had oxidation (Figure B1) and super-oxidation (Figure C1) properties. Therefore, this isolate was identified as an *Acetobacter* species.

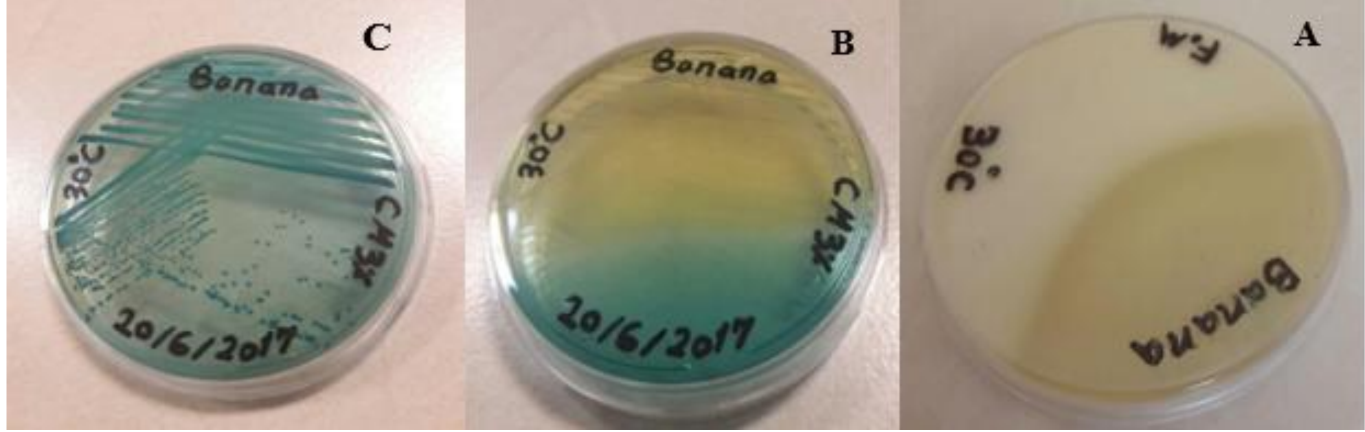

Figure 1. Calcium carbonate consumption in Frateur medium by strain isolated from bananas after 72 h (A), oxidation process by isolate after 24 h in Carr medium (B) and super-oxidation process by isolate after 72 h in Carr medium (C).

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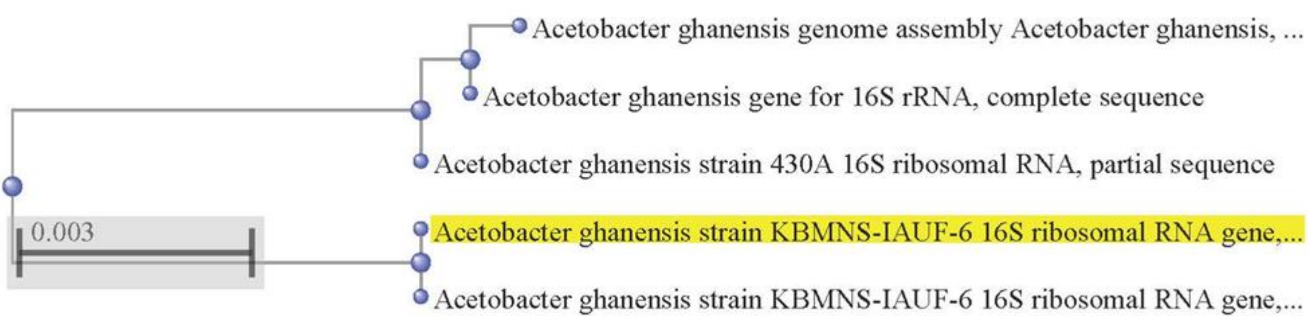

Figure 2. The phylogenetic tree of *Acetobacter ghanensis* KBMNS-IAUF-6 16S-rDNA sequence deposited in GenBank, NCBI under the accession number of MK968570.

Table 1. Growth rate of Acetobacter ghanensis KBMNS-IAUF-6 in different ethanol concentration after 24 h incubation at constant temperatures of 34, 36, 38 and 40°C.

No	Ethanol (%)	2	3	4	5	6	7	8	9	10
1	34°C	4+	4+	3+	2+	2+	2+	1+	-	-*
2	36°C	4+	4+	3+	2+	2+	1+	1+	-	-
3	38°C	4+	3+	3+	2+	2+	1+	1+	-	-
4	40°C	3+	3+	3+	2+	1+	1+	1+	-	-

*: -: non-growth, 1+: 103>CFU/ml>101, 2+: 105>CFU/ml>103, 3+: 107>CFU/ml>105, 4+: 109>CFU/ml>107

Table 2. Growth rate of Acetobacter ghanensis KBMNS-IAUF-6 in different ethanol concentration after 48 h incubation at constant temperatures of 34, 36, 38 and 40°C.

No	Ethanol (%)	2	3	4	5	6	7	8	9	10
1	34°C	4+	4+	4+	3+	3+	2+	2+	-	-*
2	36°C	4+	4+	4+	3+	2+	2+	2+	-	-
3	38°C	4+	4+	4+	3+	2+	2+	2+	-	-
4	40°C	4+	4+	4+	3+	2+	2+	1+	-	-

*: -: non-growth, 1+: 10^3>CFU/ml>10^1, 2+: 10^5>CFU/ml>10^3, 3+: 10^7>CFU/ml>10^5, 4+: 10^9>CFU/ml>10^7

Table 3. Growth rate of Acetobacter ghanensis KBMNS-IAUF-6 in different ethanol concentration after 72 h incubation at constant temperatures of 34, 36, 38 and 40°C.

No	Ethanol (%)	2	3	4	5	6	7	8	9	10
1	34°C	4+	4+	4+	4+	3+	3+	2+	1+	-*
2	36°C	4+	4+	4+	4+	3+	3+	2+	1+	-
3	38°C	4+	4+	4+	4+	3+	3+	2+	1+	-
4	40°C	4+	4+	4+	4+	3+	3+	2+	1+	-

*: -: non-growth, 1+: 10^3>CFU/ml>10^1, 2+: 10^5>CFU/ml>10^3, 3+: 10^7>CFU/ml>10^5, 4+: 10^9>CFU/ml>10^7

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Table 4. Growth rate of Acetobacter ghanensis KBMNS-IAUF-6 in different ethanol concentration after 96 h incubation at constant temperatures of 34, 36, 38 and 40°C.

No	Ethanol (%)	2	3	4	5	6	7	8	9	10
1	34°C	4+	4+	4+	4+	4+	3+	3+	2+	-*
2	36°C	4+	4+	4+	4+	4+	3+	3+	1+	-
3	38°C	4+	4+	4+	4+	4+	3+	3+	1+	-
4	40°C	4+	4+	4+	4+	4+	3+	3+	1+	-

*: -: non-growth, 1+: 10^3>CFU/ml>10^1, 2+: 10^5>CFU/ml>10^3, 3+: 10^7>CFU/ml>10^5, 4+: 10^9>CFU/ml>10^7

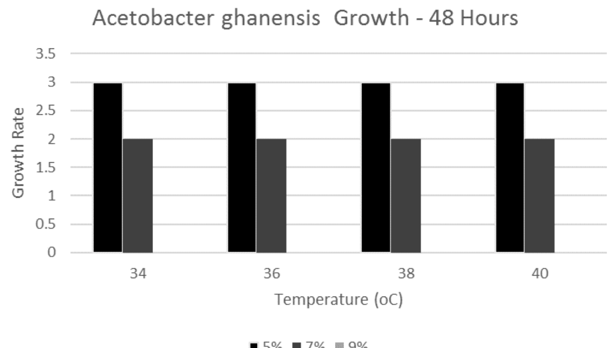

Figure. 4. *A. ghanensis* KBMNS-IAUF-6 growth rate in different ethanol concentrations and temperatures after 48 hours

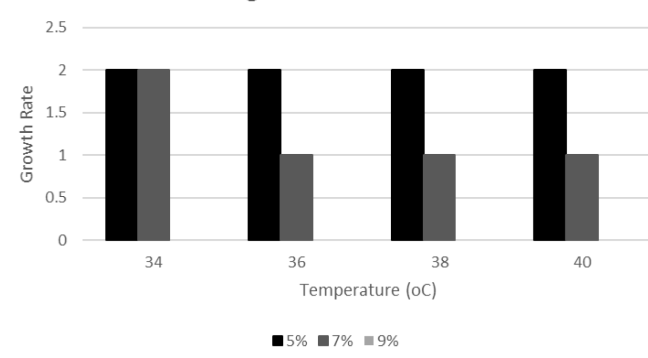

Figure. 3. *A. ghanensis* KBMNS-IAUF-6 growth rate in different ethanol concentrations and temperatures after 24 hours

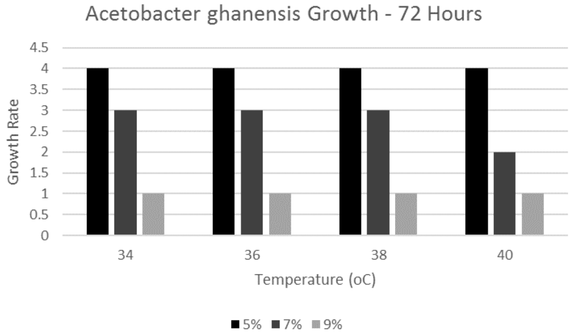

Figure. 5. *A. ghanensis* KBMNS-IAUF-6 growth rate in different ethanol concentrations and temperatures after 72 hours

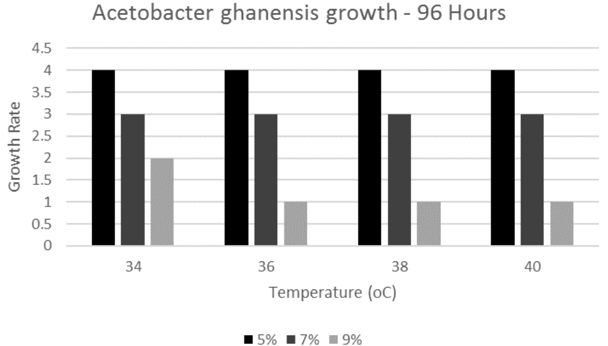

Figure. 6. *A. ghanensis* KBMNS-IAUF-6 growth rate in different ethanol concentrations and temperatures after 96 hours

Molecular characterization of *Acetobacter* isolate. Molecular identification was used to identify this bacterium at species level. The PCR product in this isolate was electrophoresed with the primers of OF BUI and OR BUI. The marker used was 100 bp and the product ranged from 370 to 380 bp. After matching the 16S-rDNA sequence to all sequences in the GenBank genomic database, BLAST software determined that the isolate was identical as *Acetobacter ghanensis* genomic sequence LN309609.1, 98.77% similarity and 94% coverage. The phylogenetic tree of the sequenced fragment showed that this bacterium was genetically most closely related to Acetobacter ghanensis (Fig. 2). Due

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to the genomic similarities found, the 16S-rDNA sequence of the isolate obtained at the GenBank, Acetobacter ghanensis KBMNS-IAUF-6, was deposited under the accession number of MK968570.

Evaluation of *Acetobacter ghanensis* KBMNS-IAUF-6 resistance to different ethanol concentrations at constant temperatures

The growth intensity and acetic acid production of *Acetobacter ghanensis* KBMNS-IAUF-6 by increasing ethanol content at constant temperatures of 34, 36, 38 and 40°C and constant times of 24, 48, 72 and 96 h decreased in the culture medium (Tables 1 to 4). These results were repeated three times. In Tables 1 to 4, 4+ indicates very high growth rate of AAB equal to 10^9>CFU/ml>10^7, 3+ indicates high growth rate equivalent to 10^7>CFU/ml>10^5, 2+ indicates moderate growth rate equal to 10^5>CFU/ml>10^3, 1+ indicates low growth rate, equivalent to 10^3>CFU/ml>10^1 and - (negative) indicates no growth of AAB in the medium.

Comparison of acetic acid production at different temperatures under constant ethanol content and time

With increasing temperature, growth and acid production in *Acetobacter ghanensis* KBMNS-IAUF-6 decreased in the culture medium (with constant ethanol levels of 5, 7 and 9%) and at constant times (24, 48, 72 and 96 h) (Figures 3 to 6). These results were repeated three times. In Figures 3 to 6, 4+ indicates very high growth rate of AAB equal to 10^9>CFU/ml>10^7, 3+ indicates high growth rate equal to 10^7>CFU/ml>10^5, 2+ indicates moderate growth rate equivalent to 10^5>CFU/ml>10^3, 1+ indicates low growth and equivalent to 10^3>CFU/ml>10^1 and - (negative) indicates no growth of AAB in the medium.

Discussion and Conclusion

So far, high temperature and ethanol resistant strains of *Acetobacter* have been isolated and identified from fruits such as peaches **(7, 13)**, cherry **(14, 15)**, apricot **(16)** and Rotab-Date palm (10). In the present experiment, banana fruit was used as a source of isolation. In a 2015 study, Klawpiyapamornkun et al., Enriched acetic acid species in medium containing sodium chloride and ethanol and then in medium containing glucose, yeast extract, peptone, glycerol, potato extract, ethanol, agar and bromocruzol. They selected the colonies that turned yellow on their culture medium as acetic acid bacteria. Cyclohexamide was also added to this medium to prevent fungal growth **(6)**. This method was costlier than the method used in this study because of the high diversity of media contents and the potential for acetic acid bacteria to be lost due to the use of ethanol-enriched medium. Sharafi et al. isolated AAB from different fruits after fruit extract by GYC medium (10% glucose, 1% yeast extract, 1.5% agar and 2% calcium carbonate) and also screened by Frateur culture medium. In this way, bacteria were grown that were able to produce acetic acid and dissolve calcium carbonate by oxidizing the glucose in the culture medium **(11)**. This method was suitable for the isolation of different types of AAB, but it is not suitable for isolation of *Acetobacter* species alone because *Acetobacter* spp. are only able to use alcohol and the use of glucose-containing medium makes them difficult to be isolated. In 2008, Moryadee and Pathom-Aree enriched the AAB spp. from various fruits in media containing 4% ethanol for 3 to 5 days at 37°C and then agar medium with purple bromocresol. They selected the colonies with yellow surrounded area as AAB. Due to the mesophilic nature AAB, the use of 37°C in the enrichment medium may kill bacteria that have a high potential for thermo-tolerance **(17)**. In a research, Diba et al. (2015) strains of AAB after enrichment in medium containing 3% acetic acid and 4% ethanol, were cultured in a medium containing yeast extract, polypeptone, glycerol, agar, purple bromocruzol and ethanol. They isolated the colonies containing yellow halo around them as AAB. This method was more expensive than the one used in the present experiment because of the presence of peptone as a nitrogen source **(1)**. The extract obtained in the present experiment was considered a natural enrichment medium for AAB because yeast and acetic acid bacteria in fruit inhibited the growth of other secondary microorganisms, respectively, by producing alcohol and acid. *Acetobacter* species were able to produce acetic acid and dissolve calcium carbonate by oxidizing ethanol in the Frateur medium. These bacteria also changed the color of the bromocresol green medium in the Carr medium, during the oxidation and production of acetic acid, from green to yellow, as well as due to the redox and acetic acid use, from yellow to blue again. In one study, the ability of AAB to grow in yeast extract agar medium containing 4 to 10% ethanol was determined and most of these bacteria were able to grow in medium containing 4 to 6% ethanol but only a few of them were able to grow in medium containing 10% ethanol **(6)**. Beheshti-Maal et al. Reported in 2010 the production of apricot vinegar by an *Acetobacter* strain

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isolated from Iranian apricot. The target *Acetobacter* was able to grow in concentrations of 5% -9% ethanol at 30°C **(16)**. In 2009 and 2010, Beheshti-Maal and Shafiei isolated and identified an *Acetobacter* species from Iranian white-red cherry fruit. The species was able to produce acetic acid at concentrations of 5%-9% ethanol at 34-36°C after 72 hours. As the concentration of ethanol increased, the growth rate and consequently the production of acetic acid decreased by the mentioned isolate **(14, 15)**. In 2010, Beheshti-Maal and Shafiei isolated and identified an *Acetobacter* species from Iranian peach fruit. The isolated AAB was able to grow in the presence of 2.5% -5.5% ethanol at 34-40°C after 96 h of incubation. In this study, increasing the percentage of ethanol increased bacterial susceptibility to high temperatures **(7, 13)**. In all of these studies, acetic acid producing isolates were identified at the level of genus and were identified as *Acetobacter.* With the exception of *Acetobacter* isolated from cherry, other isolates were not able to withstand concentrations of more than 5% ethanol at temperatures above 34°C **(7, 13-16)**. In the present experiment, the growth rate of *Acetobacter ghanensis* KBMNS-IAUF-6 decreased with increasing ethanol at constant temperature due to shock and increased bacterial cell sensitivity. Also, *Acetobacter ghanensis* KBMNS-IAUF-6 was able to grow in culture medium containing 9% ethanol at 40°C after 72 h. In 2014, it was shown that increasing the amount of ethanol increased the temperature sensitivity in a species of *Acetobacter* isolated from Rotab. Also, with increasing the temperature, the growth of this strain decreased in a constant amount of ethanol **(10)**. In the present experiment, with increasing ethanol content, the susceptibility of *Acetobacter ghanensis* KBMNS-IAUF-6 to higher temperature and its growth was decreased. For example, the cells' sensitivity to 9% ethanol was greater than that of 7% ethanol at 40°C. Also, with increasing temperature the growth of this strain decreased in constant amounts of ethanol. This is the first report of the isolation of an acetic acid producing bacterium from banana fruit in Iran. According to the results of single-factor optimization, *Acetobacter ghanensis* KBMNS-IAUF-6, which was able to grow in a medium containing 9% ethanol at 40°C, was identified as a high temperature and ethanol resistant strain. Therefore, this new strain could be suggested as a viable option for producing banana vinegar on a semi-industrial and industrial scale.

Acknowledgments

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Conflict of Interest

There is no conflict of interest reported between authors.

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