

# A novel thermo- ethanol tolerant *Acetobacter okinawensis* KBMNS-IAUF-1 isolated from Iranian nectarine as a potential for nectarine vinegar production in food biotechnology

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## Research article

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# Abstract

**Background** Recently production of several types of vinegar using new strains of acetic acid bacteria is challenging. **Results** In this research we isolated a new strain of *A. okinawensis* from Iranian nectarine we obtained from nectarine garden at Isfahan, Iran. According to 16s-rDNA molecular analysis we named that *Acetobacter okinawensis* strain KBMNS-IAUF-1 and its partial 16s-rDNA sequence was deposited in GenBank, NCBI under the accession number of MG544095.1. The tolerance of *A. okinawensis* KBMNS-IAUF-1 against ethanol concentrations of 2%-10% and high temperatures of 34-38°C was investigated. This strain had good growth and acid production in 2%-5% ethanol at high temperature of 38°C and as a thermo-tolerant AAB had good growth in 5% ethanol at 38°C. Also produced 6% acetic acid in an economical industrial culture medium at high temperature of 38°C using a fermentor apparatus we designed for vinegar production. **Conclusions** This is the first report of isolation and identification of *Acetobacter okinawensis* KBMNS-IAUF-1 from Iranian nectarine as a high thermo- ethanol- tolerant AAB capable of high acetic acid production in a short period of time and is a very good candidate for production of a new type of vinegar, nectarine vinegar, in high temperatures and ethanol concentrations. The thermo- ethanol-tolerant AAB as potential for production of new types of vinegar with suitable flavor could be an asset in food microbiology as well as industrial biotechnology.

# Background

The US Food and Drug Administration (FDA) has defined the sour wine or vinegar as a 4% liquid of acetic acid (4 g of acetic acid in each cm<sup>3</sup>) that has been generated through alcoholic fermentation of sugary and sweet precursors [1, 2]. Natural vinegar as a popular food flavoring and supplement has several essential amino acids according to its fruit source and could be applied for relieving the pains especially those occurred in the human gastrointestinal tract [3, 4]. The acetic acid bacteria (AAB) are Gram negative aerobic rods that are responsible for vinegar production through biological oxidation [5, 6]. While more than 30 years ago the AAB were divided to two genera of *Acetobacter* and *Gluconobacter* but recently this classification has been considerably changed. According to modern molecular classification of 16s ribosomal DNA analysis, the AAB are related to family of *Acetobacteriaceae* and classified in 19 genera of *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Bombella*, *Commensalibacter*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia* and *Tantichar-oenia* [3, 7, 8, 9]. The use of identified pure AAB could increase the production of vinegar and the industrial vinegar producers are looking for new AAB capable for manufacturing attractive types of natural vinegars socially [2, 5, 10]. Various primary substrates have been used for vinegar production such as bee honey [11], rice [12], sugarcane [4] and balsam [13-15]. Among AAB, *Acetobacter* spp. As the main responsible microorganism for vinegar production have been isolated from several natural resources such as grape, date and coconut [16-18], Iranian white-red cherry [19, 20], Iranian peach [1, 21], Iranian apricot [22], Jamaican cherry, pineapple, rambutan, mango and longan [23, 24], palm wine and palm tree [25-27] and Iranian date palm, Rotab [5]. The aims of this research were isolation and identification of AAB from

Iranian nectarine as well as investigation of their tolerance against high temperatures and ethanol concentrations. Also the production of acetic acid by newly isolated AAB in a miniature fermentor was discussed.

## Results

**Primary isolation of AAB from Iranian nectarine extract.** The culture of Iranian nectarine extract to Frateur medium after 48 hours' incubation at 30°C resulted in the appearance of colonies that could make acid and transparency around individual colonies. The growth of isolate in this medium and acid production after incubation time confirmed that the isolated bacterium was related to AAB (Figure 1A).

**Screening of *Acetobacter* spp. from Iranian nectarine extract.** The passage of AAB individual colonies from Frateur medium to Carr medium and incubation at 30°C for 24 hours showed the colonies that could convert the blue color of bromocresol green of Carr medium to yellow with acetic acid fermentation (Figure 1B). The reemerging of blue color in Carr medium after 96 hours' incubation (Figure 1C) confirmed the over-oxidation ability of isolate and proposed that the isolated sp. from Iranian nectarine was related to the genus *Acetobacter* and the family of *Acetobacteriaceae*.

**Macroscopic, microscopic and biochemical characterization of *Acetobacter* isolate.** The macroscopic characteristics of isolated AAB from Iranian nectarine in Frateur and Carr media including the colony morphology, color and smell were evaluated. The Gram staining of the AAB isolate showed the Gram negative bacilli and coccobacilli (Figure 2). The primary results of oxidase and catalase tests indicated that the isolated AAB from Iranian nectarine was related to *Acetobacter* spp. The results of biochemical examinations confirmed that the isolated *Acetobacter* sp. was *Acetobacter okinawensis*. The macroscopic, microscopic and biochemical examinations of the isolated AAB were indicated in Table 1.

**Molecular identification of *Acetobacter* strain isolated from Iranian nectarine.** The amplification of 16s-rDNA gene from extracted Iranian nectarine *Acetobacter* DNA using universal primers of OF BUI and OR BUI showed a 370 bp product after electrophoresis and transillumination. The amplification of 16s-rDNA genes of extracted DNA from two standard strains of *Escherichia coli* ATCC25922 and *Acetobacter aceti* ATCC23746 as positive controls showed the same band of 370 bp (Figure 3). The BLASTN analysis of 16s-rDNA sequence from *Acetobacter* strain showed 92% similarity and 96% query coverage with *Acetobacter okinawensis* strain SZCPY2 (GenBank accession number: KU555380.1). These molecular analyses indicated that the isolated AAB strain from Iranian nectarine sample was related to the species of *Acetobacter okinawensis*. This strain was named *Acetobacter okinawensis* strain KBMNS-IAUF-1 and its 16s-rDNA gene partial sequence was deposited in GenBank, NCBI under the accession number of MG544095.1. Figure 4 shows the phylogenetic tree of different spp. of *Acetobacter* and the position of currently identified *Acetobacter okinawensis* KBMNS-IAUF-1.

**The tolerance of *Acetobacter okinawensis* KBMNS-IAUF-1 against ethanol concentrations and high temperatures.** The results of growth rates and acid production of *Acetobacter okinawensis* KBMNS-IAUF-1 in modified Carr media with different ethanol concentrations of 2%-10% after 24-96 hours' incubation at

34°C has been indicated in Table 2. The effects of ethanol concentrations of 2%-10% and the high temperature of 36°C against the growth rates and acid production by *Acetobacter okinawensis* KBMNS-IAUF-1 was shown in Table 3. The results of growth rates and acid production of *Acetobacter okinawensis* KBMNS-IAUF-1 in modified Carr media with different ethanol concentrations of 2%-10% after 24-96 hours' incubation at 38°C has been indicated in Table 4.

**Acetic acid production using isolated *Acetobacter okinawensis* KBMNS-IAUF-1 from Iranian nectarine in industrial culture medium.** The cultivation of *Acetobacter okinawensis* KBMNS-IAUF-1 in miniature glass fermentor containing 500 ml industrial medium was led to production of a new type vinegar with a good smell, flavor and appearance after 24 hours of fermentor operation. The acetic acid titration assay of fermented industrial culture medium containing this newly discovered AAB strain after 24, 48, 72, 96, 120, 144 and 168 hours of incubation at 38°C and 16 LPM of aeration speed indicated the elevation of acetic acid percentage of 3.6%, 3.72%, 4.77%, 5.36%, 5.58%, 6% and 6% respectively. The results confirmed that *Acetobacter okinawensis* KBMNS-IAUF-1 isolated from Iranian nectarine could produce a considerable amount of acetic acid in a short period of time. Figure 5 shows the production of acetic acid by *Acetobacter okinawensis* KBMNS-IAUF-1 in industrial culture medium using a miniature glass fermentation vessel.

## Discussion

Manufacturing high acetic acid concentration in acetators is challenging and finding new ethanol-tolerant AAB strains could be an asset in vinegar industry as well as food biotechnology [3, 28]. The most *Acetobacter* spp. that have been reported for vinegar production are *A. aceti*, *A. cerevisiae*, *A. malorum*, *A. oeni*, *A. pasteurianus* and *A. pomorum* [7, 29]. At the first time *Acetobacter okinawensis* was isolated from sugarcane stem in 2004 at Okinawa, Japan. Then isolated from grape, Japanese plum and oriental melon in 2007 at Okayama, Japan [30]. Chen et al. (2016) have reported the isolation of a AAB as *A. okinawensis* from Tibetan kefir [31]. In another study was reported that *A. okinawensis* has been frequently isolated in samples related to apple resources. Also suggested that the specific gene-based sequence analysis is effective for AAB discrimination [29]. In this research we isolated a new strain of *A. okinawensis* from Iranian nectarine we obtained from nectarine garden at Isfahan, Iran. According to 16s-rDNA molecular analysis we named that *Acetobacter okinawensis* strain KBMNS-IAUF-1 and its partial 16s-rDNA sequence was deposited in GenBank, NCBI under the accession number of MG544095.1. Sharafi et al. (2010) used GYC culture medium for isolation and screening of AAB from several fruits samples [21]. Klawpiyapamornkun et al. (2015) used a synthetic medium of normal saline and ethanol for enrichment and isolation of AAB from fruits and fermented fruit juices [9]. In this research we used Frateur culture medium for screening AAB and then Carr medium for AAB overoxidation activity and isolating the *Acetobacter* spp. as the most privileged AAB spp. for industrial production of vinegar. Both mentioned media were economic and easy to handle biotechnologically. In a research study, AAB growth in the presence of 4%-10% ethanol concentrations were examined. There were indicated that all of AAB isolates were able to grow optimally in the concentrations of 4%-6% ethanol [9]. In a research an *Acetobacter* sp. that was isolated from Iranian peach could successfully tolerate against 2.5%-5% ethanol

in high temperatures of 34-40°C after 96 hours' incubation [1]. In another study was indicated that the elevation of ethanol concentration from 2% to 9% in culture medium is led to the high sensitivity of an *Acetobacter* sp. isolated from Rotab to high temperatures of 34-38°C [5]. So far there is no report of optimization of *A. okinawensis* acid production and growth rates using cultivation in high ethanol concentration and temperatures simultaneously. In the present study, the tolerance of *A. okinawensis* KBMNS-IAUF-1 against ethanol concentrations of 2%-10% and high temperatures of 34-38°C was investigated. The results suggested that at 34°C, the 2%-5% ethanol had no effects on the growth of *A. okinawensis* KBMNS-IAUF-1. The 6% ethanol between 24-72 hours' incubation decreased the growth rate and acetic acid production a little but after 96 hours we had the maximum growth. The ethanol concentrations of 7%-8% reduced the growth and acid production considerably. There was no growth at ethanol concentrations of 9%-10% at 34°C. At higher temperatures of 36°C the sensitivity of isolated AAB strain to higher ethanol percentages was more obvious so that the isolated strain had good growth and acetic acid production in 2%-5% ethanol but in 6% ethanol the growth rate was declined and had no growth in 7%-10% ethanol at 36°C. The *A. okinawensis* KBMNS-IAUF-1 had good growth and acid production in 2%-5% ethanol at high temperature of 38°C but in 6% ethanol lost the growth obviously. There was no growth and acetic acid production in  $\geq 6\%$  ethanol. These results suggested that the extreme conditions of high ethanol concentrations and temperatures could prevent the growth rate and subsequently the acetic acid production by *A. okinawensis* KBMNS-IAUF-1 isolated from Iranian nectarine. However, this strain as a thermo-tolerant AAB had good acetic acid production in 5% ethanol at 38°C. Klawpiyapamornkun et al. (2015) showed that using rotary shaker incubator and a medium containing 2% ethanol and 2% yeast extract, their AAB isolates of P1, P4, P6, P12 and *Acetobacter aceti* produced acetic acids percentage of 1.78, 1.80, 1.80, 1.81 and 1.81 respectively [9]. Diba et al. (2015) reported that their isolated AAB from decomposed fruits in YGEA medium containing yeast extract, glucose, ethanol and acetic acid at 37°C after 72 hours' incubation made 3-6% acetic acid. While the glucose has induced the acetic fermentation in a considerable manner in their research but is not economic in industrial purposes [32]. While in previous studies we reported the optimal growth of AAB in 2-5% ethanol, the AAB were identified as *Acetobacter* spp. and they were not identified at the levels of species and strain using biochemical test and then molecular identifications [1, 5, 19, 20,22] so the identification of a novel AAB at the molecular level is the first important aspect of this study in comparison to previous researches. The second importance of this work is the isolation of *Acetobacter okinawensis* from nectarine fruit that is reported for the first time in the world. The third importance of this study was finding a AAB could grow in 6-8% ethanol concentrations and simultaneously in high temperatures of 36-38°C. As it was indicated in Table 2, the *A. okinawensis* KBMNS-IAUF-1 had very good growth in 6-8% of ethanol concentrations at 34°C in modified Carr media. Also Table 2 and 3 indicated that the isolated strain had good growth in 6-7% of ethanol concentrations at 36°C and 38°C respectively. The *Acetobacter* spp. usually grow in mesophilic temperatures of 28-30°C while the keeping of industrial acetators in these temperatures is costly so finding the thermo-tolerant *Acetobacter* spp. is considered as an asset biotechnologically. The growth of *A. okinawensis* KBMNS-IAUF-1 in high temperatures if could be translated to industrial concepts and in a 100,000 liter acetator means a very high amount of energy saving during a vinegar production batch that takes long 1-2 weeks in an advanced high-tech facility. The

tables 2 to 4 have shown the growth of *A. okinawensis* KBMNS-IAUF-1 in solid culture media but the most important aspect of this study was the thermal and acetic acid tolerance of this new strain in a simulated industrial broth medium. As it was inferred from Figure 5, this strain was able to grow at high temperature of 38°C in 6% acetic acid concentration. The acetic acid was obtained from ethanol during the acetic fermentation of AAB, so the ability of this strain to grow in broth industrial medium in the extreme condition of high temperature of 38°C in the presence of 2% ethanol and 6% acetic acid in 7 days' incubation is similar to grow in a broth medium containing 8% ethanol and is considered as a very significant trait of *A. okinawensis* KBMNS-IAUF-1 that is reported for the first time in the world.

## Conclusions

This is the first report of isolation and identification of *Acetobacter okinawensis* KBMNS-IAUF-1 from Iranian nectarine. Also the optimization of growth rate as well as acetic acid production in the extreme conditions of high temperatures and ethanol concentrations have been investigated for the first time. The *A. okinawensis* KBMNS-IAUF-1 had maximum growth rate and acid production in 6% ethanol at 34°C after 96 hours' incubation. The maximum acid production and growth of this newly discovered AAB strain at 36°C and 38°C were obtained in ethanol concentrations of 4% and 3% respectively after 96 hours' incubation. The results of this research confirmed that *A. okinawensis* KBMNS-IAUF-1 isolated from Iranian nectarine not only could tolerate against high ethanol concentrations and high temperatures but also resist in high concentrations of acetic acid and could be considered as a potential AAB strain for production of vinegar with high rancidity of 6% in a very short period of time, 5 days, industrially. So the *A. okinawensis* KBMNS-IAUF-1 as a thermo- ethanol-tolerant AAB strain is a very good candidate for production of a new type of vinegar, nectarine vinegar, in high temperatures and ethanol concentrations. The thermo- ethanol-tolerant AAB could be considered as potential for vinegar production in food microbiology as well as industrial biotechnology.

## Methods

**Chemicals and microbiological culture media.** The main chemicals we used were ethanol 96% (Razi, Iran), yeast extract (Taligene, Iran), acetic acid, agar agar, D-glucose, CaCO<sub>3</sub>, bromocresol green, NaOH and phenolphthalein (all from Merck, Germany). The microbiological culture media were Brain Heart Infusion Broth (BHI) from Himedia, India, Frateur medium (CaCO<sub>3</sub>, 20 g/l; yeast extract, 10 g/l; ethanol, 20 g/l; agar agar, 20 g/l; distilled water, 1000 ml), Carr medium (yeast extract, 3%; bromocresol green, 0.002%, ethanol 2%, agar agar, 2%; DW, 1000 ml) and modified Carr media with different ethanol concentrations of 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%. Also an industrial culture medium (yeast extract, 1%; acetic acid, 2%; ethanol, 2% and DW, 1000 ml) was used.

**Iranian nectarine sampling and preparation.** The both intact and spoiled Iranian nectarine samples were provided from nectarine garden, Isfahan, Iran. Using sterile container, the fruits were transferred at 4°C to the laboratory of microbiology, R&D laboratories complex, Falavarjan Branch, Islamic Azad University, Isfahan, Iran. The fruits were placed in sterile plastic bag with openings at room temperature and

appropriate ventilating condition for 7-10 days. After emerging fruit flies and vinegar smelling from container, the fruits were pressed, scrutinized and homogenized and then transferred to a sterile plastic bottle and its cap was closed. For preventing the bottle burst during alcoholic fermentation and CO<sub>2</sub> accumulation, some tiny openings were made with needle on the top of bottle. The bottle was incubated at 30°C for 7 days [5].

**Primary isolation of AAB from Iranian nectarine extract.** Ten milliliters of nectarine extract were added to 90 ml sterile distilled water to make 10<sup>-1</sup> dilution. One standard loop of mentioned dilution was cultured on Frateur medium using streak plate method. The medium was incubated at 30°C for 24-48 hours. The individual colonies with transparent surrounding were collected and purified in the same condition [1, 5].

**Screening of *Acetobacter* spp. from Iranian nectarine extract.** The purified AAB from previous stage were cultured on Carr media using streak plate method and incubated at 30°C for 24-48 hours. Those yellow colonies after 24 hours' incubation that were converted to blue ones after 48 hours' incubation were selected and purified using the same condition and preserved at -70°C in Carr medium included 50% glycerol for next experiments [21].

**Macroscopic, microscopic and biochemical characterization of *Acetobacter* isolate.** The colony characterization of isolated *Acetobacter* strain from Iranian nectarine was performed using stereomicroscopy. The microscopic traits were detected after Gram staining of purified colonies in Frateur and Carr media. The individual purified colonies were examined against catalase and oxidase reaction, acid production and making transparent in Frateur medium and overoxidation ability in Carr medium [22]. For identification of the genus and species of the isolated AAB, the complement biochemical examinations were fulfilled. These tests were production of ketogluconic acids, production acid from D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose and growth on 30% D-glucose. All tests were done in triplicate [33]. For confirmation of biochemical examinations all the aforementioned tests were simultaneously performed using a standard strain of *Acetobacter okinawensis* NRIC 0659 as positive control, that was provided from Taligene Pars Co., ISTT, Isfahan, Iran.

**Molecular identification of *Acetobacter* strain isolated from Iranian nectarine using 16s-rDNA analysis.** An individual colony of the *Acetobacter* isolate in Carr medium was transferred to 50 ml of sterile distilled water. Ten milliliters of suspension were transferred to 15 ml sterile falcon and centrifuged at 3000g for 15 minutes. The supernatant was discarded and 1 mg of bacterial biomass was used for DNA extraction by DNA extraction kit (Bioneer, South Korea). The universal primers used, were OF BUI as the forward primer with the sequence of 5'AACTGGAGGAAGGTGGGGAT3' and OR BUI as the reverse primer with the sequence of 5'AGGAGGTGATCCAACCGCA3'. PCR was performed in an Eppendorf Thermal Cycler. The PCR program encompassed initial denaturation at 96°C for 4 minutes, followed by 30 cycles of 94°C for 2 minutes, 55°C for 1 minute and 72°C for 1 minute respectively. The final steps were 72°C for 4 minutes and incubation at 4°C for 10 minutes. The expected molecular weight of PCR product was 370 bp [21, 27]. The PCR product and primers were sent to Taligene Pars Co., Isfahan Science and Technology Town (ISTT), Isfahan, Iran for DNA sequencing. The DNA sequence was reviewed using Finch TV V.1.4.0 and

Mega 6 software and its similarity to GenBank genomic sequences was investigated using BLASTN software (<http://blast.ncbi.nlm.nih.gov>). The isolated strain was identified after bioinformatics analysis and its 16s-rDNA sequence was deposited in GenBank, NCBI.

**The tolerance of Iranian nectarine *Acetobacter* strain against ethanol concentrations and high temperatures.** The isolated *Acetobacter* strain from Iranian nectarine was cultured on modified Carr media with 3, 4, 5, 6, 7, 8, 9 and 10% ethanol concentrations using streak plate method and incubated at high temperatures of 34, 36 and 38°C for 24-96 hours. Every 24 h, the growth rate and acid production of isolated *Acetobacter* strain on culture media was measured. All experiments were fulfilled in triplicate and the mean of growth rate in each test was considered as the growth rate. The growth of bacterium in Carr medium with 2% ethanol was considered as control [1, 5].

**Acetic acid titration assay.** The titration assay of the produced acetic acid by isolated strain from Iranian nectarine was done as follow. Five milliliters of the broth medium were added to 20 ml of distilled water in an Erlenmeyer flask and then a couple of phenolphthalein drops [phenolphthalein, 0.1 g; ethanol, 60 g; distilled water, 40 g] were added. The 0.5 normal sodium hydroxide [NaOH, 20 g/l; distilled water, 1000 ml] were added using 50 ml burette to acetic acid solution until appearance of pale pink color in the flask. The volume of consumed NaOH was measured and the acetic acid percentage in each medium was calculated [21, 22].

**Acetic acid production using isolated *Acetobacter* strain from Iranian nectarine in industrial culture medium.** For assessment of isolated AAB from Iranian nectarine as a potential agent for production of nectarine vinegar, the strain was cultured in 50 ml of industrial broth medium for vinegar production [ethanol, 2%; acetic acid, 2%, yeast extract, 1%, DW, 1000 ml] and incubated at 38°C and 120 rpm for 24 hours. After emerging the vinegar smell from the medium, the whole 50 ml transferred to 1000 ml miniature glass fermentor (Taligene Pars Co., ISTT, Iran) containing 500 ml of industrial culture medium. The sterile air sparging pump speed was set on 16 LPM and fermentor was incubated at 38°C for 168 hours. Every 24 hours the acetic acid elevation was measured using previously described acetic acid titration assay. After titration completed, 2% extra-ethanol were added to fermentor using aseptic method and the fermentor set up to the same condition i.e was let to continue to ferment ethanol to acetic acid until the maximum acetic acid titer was reached [19, 20].

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and material



The datasets about the bacterial strain characterization generated and analyzed during the current study are available in the GenBank, NCBI at <https://www.ncbi.nlm.nih.gov/nuccore/MG544095.1>

### Competing interests

The authors declare that they have no competing interests

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### Authors' contributions

KBM reviewed literatures and chose the subject. KBM and NSH conducted experiments. KBM wrote the paper and proofed data and text. All authors read and approved the final manuscript.

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# Figures

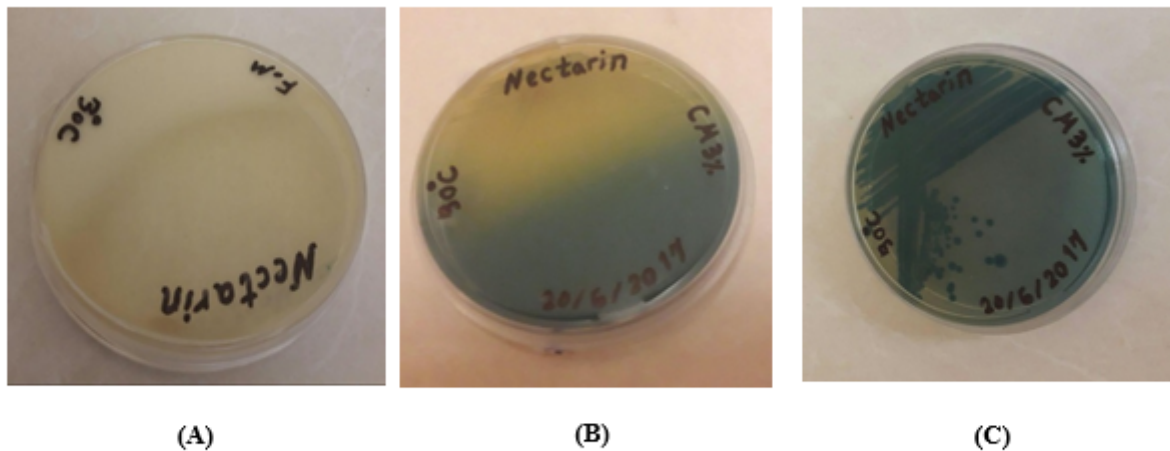


Figure 1

## Figure 1

The isolation of AAB from Iranian nectarine extract on selective culture media after 48 hour's incubation at 30°C on (A) Frateur culture medium and (B) Carr medium. (C) Overoxidation of *Acetobacter* sp. After 96 hours' incubation.

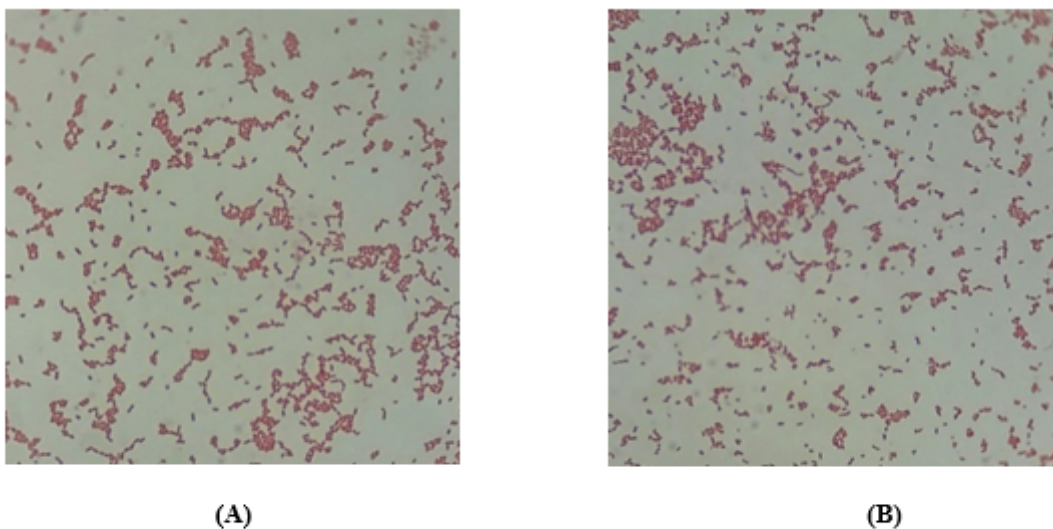


Figure 2

## Figure 2

The Gram staining of Isolated *Acetobacter* from Iranian nectarine after 24 hours incubation at 30°C on (A) Carr medium and (B) Frateur medium.

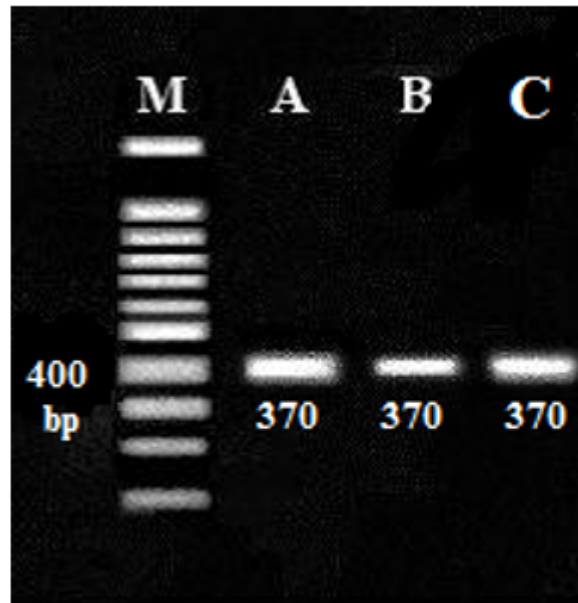


Figure 3

### Figure 3

The PCR amplification of *Acetobacter* DNA isolated from Iranian nectarine using universal primers of OF BUI and OR BUI. M: 1500 bp marker, A: *Acetobacter* isolated from Iranian nectarine, B: positive control 1, *Escherichia coli* ATCC25922, C: positive control 2, *Acetobacter aceti* ATCC23746.

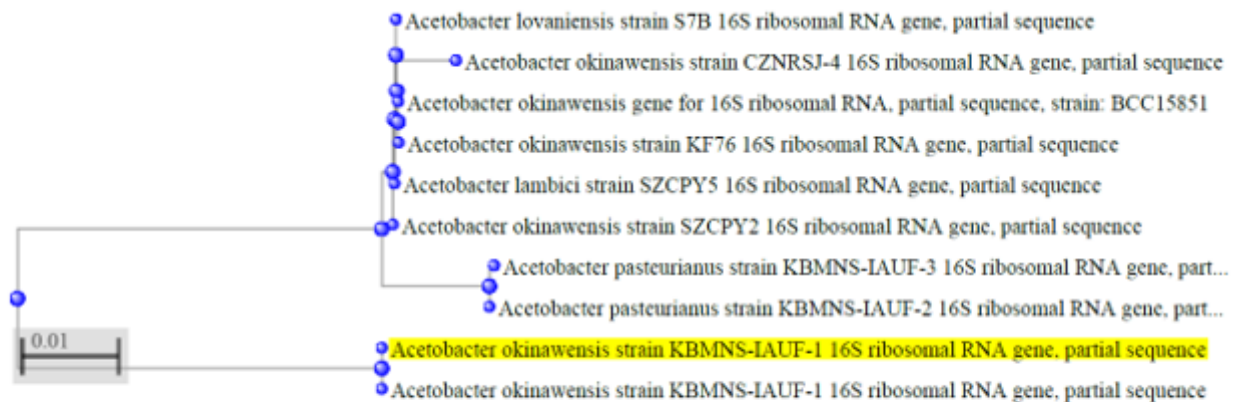
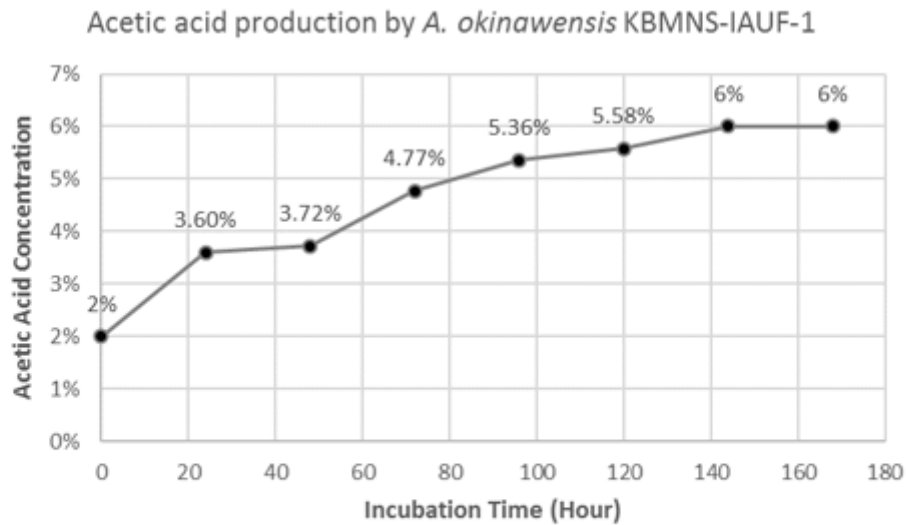


Figure 4

### Figure 4

The phylogenetic tree of different spp. of *Acetobacter* and the position of currently identified *Acetobacter okinawensis* KBMNS-IAUF-1.



**Figure 5**

## Figure 5

Acetic acid production by *Acetobacter okinawensis* KBMNS-IAUF-1 isolated from Iranian nectarine after 168 hours' incubation at 38°C and 16 LPM aeration speed in industrial culture medium using miniature glass fermentor.

## Supplementary Files

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