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## A novel high ethanol-thermo-tolerant *Acetobacter pasteurianus* KBMNS-IAUF-2 strain and the optimization of acetic acid production using the Taguchi statistical method

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### Abstract

Because of the high energy consumption for fermentor cooling, the isolation of thermo-tolerant *Acetobacter* strains for vinegar production has a high priority. The aims of this study were the isolation and identification of a high ethanol-thermo-tolerant *Acetobacter* spp. from grapes as well as the optimization of conditions for increasing the acetic acid production. The grape extract was cultured on Frateur and Carr media. The isolates were characterized against macroscopic, microscopic, and molecular traits. The resistance of the isolates against different concentrations of ethanol as well as high temperatures were measured. The best ethanol-thermo-tolerant isolate using ribotyping was identified as *Acetobacter pasteurianus* KBMNS-IAUF-2 and its 16s-rDNA sequence was deposited in GenBank, NCBI under the accession number MG547344. This strain was able to grow in Carr medium with 9% (v/v) ethanol after 48h incubation at 40°C. A Taguchi design with L9 analysis revealed that the highest yield of acetic acid production occurred at 34°C in the medium containing of 2% (v/v) ethanol and 2% (w/v) yeast extract. The temperature and yeast extract concentration with 76.70% and 6.29% were the most and least effective factors in acetic acid production, respectively. The acetic acid production yield after 24h under the optimized condition was measured as 97%. This thermo-ethanol-tolerant strain could be applied as an industrial starter for producing vinegar with grapevine flavor.

### 1. Introduction

According to the US Food and Drug Administration, vinegar, a seasoned and sour-tasting additive, contains at least 4% acetic acid at a temperature of 20°C (Diba et al. 2015). The varieties of vinegar are including the white

vinegar, Sherri vinegar (Spanish), Balsamic vinegar (Italian), beer vinegar, Malta vinegar, rice vinegar, and potato vinegar (Mas and Torija 2014). Wine vinegar is produced in most Mediterranean countries and is widely used as a spice and food preservative (JuanYe et al. 2004). White vinegar is also the most common type of vinegar around the world due to the direct conversion of ethanol to

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acetic acid (Horiuchi et al. 2000). The quality of vinegar production depends on the type of substrate, the Acetic Acid Bacteria (AAB), the method used in vinegar production, and the aging process (Gullo and Giudici 2008). The AAB are Gram negative and aerobic rods among which, the *Acetobacter* species are more suitable for vinegar production because they can directly use ethanol as a carbon source and don't need other substrates such as glucose. These spp. also produce less side effects than other AAB used in industrial processes (Beheshti Maal and Shafiee 2011). *Acetobacter* species are catalase positive and oxidase negative with a super-oxidation ability. During the process of oxidation, acetate is transformed into carbon dioxide and water by the tri-carboxylic cycle (Diba et al. 2015). Traditional vinegar production requires many years to get high steaks and, consequently, is converted to an expensive product. Therefore, the goal of the vinegar industries is to produce high quality vinegars in a short period of time. These methods include the use of continuous and semi-continuous immersion culture methods (Tsfaye et al. 2002).

Today, the use of cell stabilization methods for vinegar production is important. Since the bacteriophages of AAB in the industrial acetator are considered problematic, the production of acetic acid can be increased by the immobilizing of AAB. Fixation methods include the absorption of cells by physical and chemical bonds as well as stacking intra-carrier cells (Ori et al. 2004). The resistance of an AAB against high ethanol concentrations and high temperatures during acetic acid fermentation are accounted as two significant rate limiting factors. These two factors affect both the AAB growth and acetic acid production negatively (Vashisht et al. 2019; Chen et al. 2016). The variation of temperature during acetic acid fermentation can result in the failure of vinegar production because formal AAB grow in mesophilic temperatures. Also, due to the costly cooling of the acetators, the use of the thermo-tolerant strains of *Acetobacter* is considered as an asset in the vinegar industry (Shafiei and Delvigne

2018). Introducing various strains of *Acetobacter* as microbial starters in the vinegar industry is challenging because each starter, in addition to acetic acid, produces 50 different volatile and aromatic substances that confer different flavors as well as nutritional values to vinegar. Therefore, optimization procedures in acetic acid production using *Acetobacter* strains are necessary in order to obtain high acetic acid production in the presence of high ethanol concentrations and temperatures in a short time (Beheshti Maal and Shafiee 2011). The purposes of this study were to isolate and identify the thermo-ethanol-tolerant species of AAB from Iranian grapevine as well as to optimize their culture conditions using the Taguchi statistical method to increase the acetic acid production.

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## 2. Materials and methods

### 2.1. Chemicals and culture media

The materials used in this research were as follows. The Gram staining kit was purchased from Taligene Pars Co., ISTT, Iran. The other chemicals, including hydrogen peroxide 30% (w/w), oxidase disk, phenol-phthalein, and NaOH, were provided by Merck, Germany. Also, the culture media used included the Carr culture medium (yeast extract, 3%; agar, 2%; bromocresol green, 0.002% (w/v); ethanol, 2% (v/v); sterile distilled water, 100 (ml); Frateur Culture medium (yeast extract, 2% (w/v); calcium carbonate, 2% (w/v); ethanol, 2% (v/v); agar, 2% (w/v); sterile distilled water, 100 (ml); industrial culture medium (yeast extract, 2% (w/v); ethanol, 2% (v/v); acetic acid, 2% (v/v); distilled water, 100 (ml) (Sharafi et al. 2010), and Brain Heart Infusion (BHI) broth provided by Taligene Pars Co., Iran.

### 2.2. Preparation of extracts and isolation of AAB

The grapes were kept in a cabinet for 2 weeks, and after a rustiness smell emerged and their structure were loosened they were compressed and

transferred to a sterilized bottle. The bottle was then placed at a temperature of 30°C for at least a week (Beheshti Maal 2014). Then, 50 µl of different dilutions of grapevine extract were transferred to a Frateur culture medium using the streak plate method. The plates were incubated at 30°C for 48 hours. Lastly, the colonies that had a clear zone were purified in the same culture medium (Sharafi et al. 2010).

### 2.3. Screening of *Acetobacter* Species

Colonies containing a clear and transparent area were transferred from the Frateur culture medium to the Carr medium. After 24 hours incubation at 30°C, the yellow colonies that had ability to change their color to bluish after 72 hours incubation at the same conditions were isolated and purified. Also in this medium, macroscopic, microscopic and biochemical characteristics of the isolates were examined as previously described in Beheshti Maal (2014).

### 2.4. Molecular identification of *Acetobacter* strain isolated from Iranian grapes using 16s-rDNA analysis

An individual colony of the *Acetobacter* isolate in the 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 a DNA extraction kit (Bioneer, South Korea). The universal primers were OF BUI as forward primer with the sequence of 5'AACTGGAGGAAGGTGGGGAT3' and OR BUI as reverse primer with the sequence of 5'AGGAGGTGATCCAACCGCA3' provided by the Taligene Pars Co., ISTT, Isfahan, Iran.

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. 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. 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 phylogenetic tree of 16s-rRNA gene of isolated AAB from grapevine was drawn using the NCBI software of "BLAST pairwise alignments" (Pedramfar et al. 2017; Beheshti-Maal et al. 2014).

### 2.5. One-factor optimization

This test was conducted to select ethanol resistant AAB strains that could grow in high temperatures. The growth rate and production of acetic acid were compared by isolates under different concentrations of ethanol at constant temperature and time, as well as different temperature conditions in constant ethanol concentration and time. This experiment was designated as a primary step before Taguchi examination. In this experiment, 3 variables of ethanol concentrations of 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v), temperatures of 34, 36, 38 and 40°C and incubation times of 24 and 48 hours were applied for evaluating the AAB performance as well as selecting the suitable range (amount) of variable in the Taguchi test. At a constant temperature of 34°C, the growth rate of isolated AAB in different concentrations of ethanol (2-10%) (v/v) at 24 and 48 hours were evaluated. This experiment was repeated at 36, 38 and 40°C. After finding the best temperatures and ethanol concentrations, these variables and their levels in the Taguchi test were applied (Klawpiyapamornkun et al. 2015).

## 2.6. Taguchi statistical design

In this experiment, the levels with the highest efficiency in one-factor optimization were used in the design of the Taguchi statistical tests. According to the measurements of acetic acid production by grapevine AAB isolates using titration after 24 hours, three factors and three levels in the industrial culture medium were considered (Table 1). The variables and levels were entered into Qualiteck 4 software and then the production of acetic acid was investigated in nine designated experiments by Taguchi test's L9 design (Table 2). The experiments were designed to be randomized to each of the levels determined in the production of acetic acid in each experiment. The data were analyzed using an ANOVA variance analysis and the effects of these factors and their interactions were measured. For validation of the Taguchi design results, the best condition proposed by the Qualiteck 4 software as the optimum condition for acetic acid production was reexamined five times and their results were compared with the Taguchi output.

**Table 1.** The factors and levels affecting the acetic acid production of *Acetobacter* sp. isolated from grapevines by Taguchi's statistical design.

Factors No.	Levels		
	1	2	3
1 Temperature (°C)	34	36	38
2 Ethanol % (v/v)	2	3	4
3 Yeast Extract % (w/v)	1	1.5	2

**Table 2.** The L9 experiments applied for acetic acid production by *Acetobacter* sp. isolated from grapevines designated by Qualiteck 4 software.

Experiment	Temperature (°C)	Ethanol % (v/v)	Yeast Ex % (w/v)
1	34	2	1
2	34	3	1.5
3	34	4	2
4	36	2	1.5
5	36	3	2
6	36	4	1
7	38	2	2
8	38	3	1
9	38	4	1.5

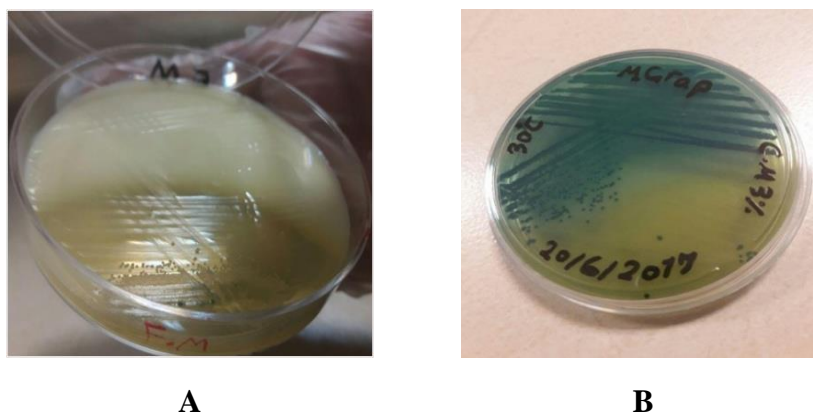
## 2.7. The preparatory steps for acetic acid production and assay

The purified colonies from the Carr medium were transferred to the BHI medium until the OD<sub>600</sub> of suspension reached 0.5. Then, 4 ml of suspension was transferred to 96 ml of industrial culture medium with a specified ingredient (Table 2) in a 250 ml miniature acetator equipped with a sparging aeration system. Next, the system was heated to the desired temperature. Every 24 hours, 2% ethanol (v/v) was gradually added to the miniature acetator for 120 hours. After 24, 48, 72, 96 and 120 hours, the acetic acid titration was measured as previously described by Beheshti-Maal et al. (2010).

## 3. Results and Discussion

### 3.1. Screening and identification of AAB

Three different individual colonies were isolated in the Frateur culture medium after 24 hours' incubation at 30°C, two of these colonies did not make the transparent zone around their colonies. The first isolate had convex, intermediate, white and rough colonies. The second isolate had irregular, small, gray and smooth colonies. These two isolates were removed from further examinations in the Carr medium because they did not produce acetic acid in the Frateur medium and so were not AAB. As shown in Figure 1A, only one AAB strain was capable of dissolving the calcium carbonate and acetic acid production from ethanol in the Frateur culture medium isolated from Iranian grapevines. After 24 hours' incubation at 30°C on the Carr medium, the AAB isolated from grapevines during the oxidation process of ethanol, changed the medium color from blue to yellow. Also after 72 hours, the over-oxidation phenomenon occurred and the color of the Carr medium was converted from yellow to blue again (Figure 1B).



**Fig.1.** (A): The clearance of the Frateur culture medium after passage of Iranian grapevines extract and 24 hours' incubation at 30°C. (B) The isolated AAB after the same culture condition on the Carr medium with over-oxidation ability. The yellow colonies were converted to blue ones after 48 hours' incubation.

The macroscopic examinations in the Frateur and Carr media indicated very tiny convex, smooth and shiny yellow to blue colonies. Biochemical examinations confirmed that the isolated AAB was oxidase negative and catalase positive, capable of acid production in the Frateur medium and over-oxidation in the Carr medium. Thus, the isolated AAB from Iranian grapevines was identified as *Acetobacter* sp. Isolation and identification of different *Acetobacter* spp. from various resources, such as peach (Beheshti-Maal and Shafiee 2010b; Beheshti-Maal and Shafiee 2011), white-red cherry (Beheshti-Maal and Shafiee 2010a), apricot (Beheshti-Maal et al. 2010) and Rotab (Beheshti-Maal 2014), have been previously reported. Sharafi et al. (2010) isolated colonies that contained a clear zone of acetic acid bacteria around them, separated by GYC medium containing glucose, yeast extract and calcium carbonate. They also used the Carr medium to check the oxidation and redox properties. The bacteria grown in the GYC medium were capable of oxidizing the glucose to acid; therefore, they proposed a method for separating different types of AAB. Klapayamvrkan et al. (2015) isolated various species of AAB after enrichment in a medium containing normal salts and ethanol and then cultured them in a medium containing glucose, yeast extract, peptone, glycerol, potato extract, ethanol, agar, and bromocresole purple. Cyclohexamide was added to this culture medium

to prevent the growth of yeasts and molds (Klawpiyapamornkun et al. 2015). This method is more cost effective than the method used in the present experiment due to the high diversity of the contents of the selected culture media. In a previous study, different strains of AAB were isolated from different fruits, enriched in a culture medium containing distilled water and 4% (v/v) ethanol for 3-5 days at 37°C and then in potatoes agar containing 0.03% bromocresole purple (Moryadee & Pathom Aree 2008). Given the mesophilic nature of acetic acid bacteria, the use of an enrichment medium at 37°C may eliminate acetic acid bacteria that has high potential for growth in mesophilic conditions. In the present study, the enrichment of various species of AAB was carried out at the temperature of 30°C. The fruit extract was also considered as a natural enrichment medium without the addition of any other material because the ethanol and acetic acid in this environment were produced by yeast and natural AAB, respectively. Diba et al. (2015) enriched different isolates of AAB from different fruits from Bangladesh in a culture medium containing 3% (v/v) acetic acid and 4% (v/v) ethanol and then in a medium of YPG agar (yeast extract, poly-peptone, glycerol, agar) containing 100 milligrams of bromocresole green, 4% (v/v) acetic acid and 5% (v/v) ethanol (Diba et al. 2015).

### **3.2. Molecular identification of *Acetobacter* sp. isolated from Iranian grapevines**

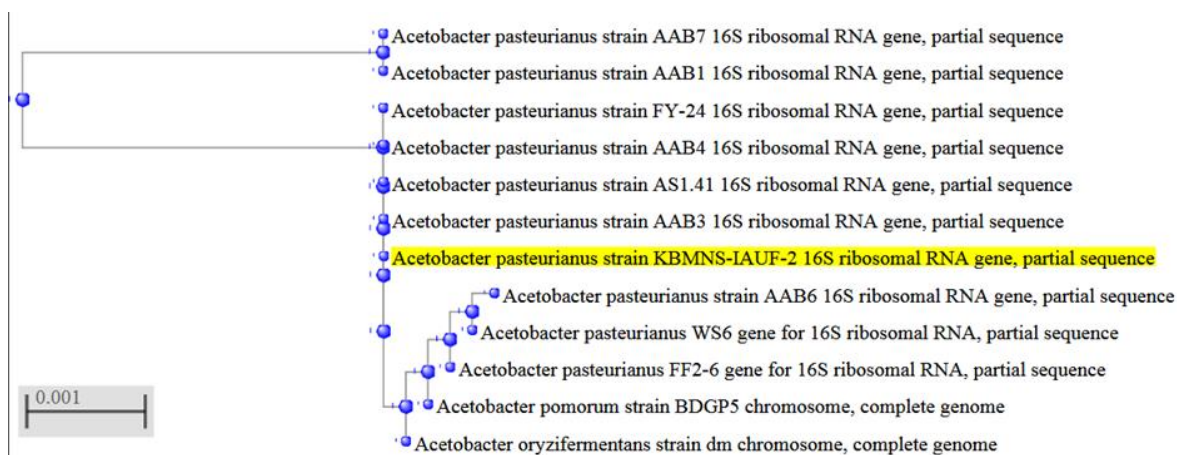
The electrophoresis of PCR products from *Acetobacter* sp. isolated from Iranian grapevine showed that the 16s-rDNA amplified product MW was measured as 370 bp. Following the matching of its 16S-rDNA sequence to all sequences found in GenBank genomic databases using BLASTN, it was similar to the *Acetobacter pasteurianus* strain FY-24 (GenBank accession number KT283054) with 97% and 100% of query coverage and similarity, respectively. So this strain was named *Acetobacter pasteurianus* strain KBMNS-IAUF-2 and its 16s-rDNA partial sequence was deposited in GenBank, NCBI under the accession number MG547344. Figure 2 shows the phylogenetic tree of 16s ribosomal RNA gene partial sequence of identified *Acetobacter pasteurianus* strain KBMNS-IAUF-2.

### 3.3. One factor optimization

#### 3.3.1. Strain resistance to different ethanol concentrations at constant temperature after 24 hours' incubation

While the strain had an acceptable growth in 10% (v/v) ethanol concentration after 24 hours' incubation at 34°C, the maximum growth rate was achieved in 6% (v/v) ethanol concentration. The growth rate of *Acetobacter pasteurianus* KBMNS-

IAUF-2 at 34°C in ethanol concentrations of 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v) were measured as  $10^8$ ,  $10^8$ ,  $10^8$ ,  $10^8$ ,  $10^6$ ,  $10^6$ ,  $10^4$  and  $10^4$  CFU, respectively (Figure 3-A). The *Acetobacter pasteurianus* KBMNS-IAUF-2 at 36°C had a maximum growth rate in ethanol concentrations of 2%-5%, and its growth decreased a little in 6%-8% (v/v) ethanol. The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 at 36°C in ethanol concentrations of 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v) were measured as  $10^8$ ,  $10^8$ ,  $10^8$ ,  $10^8$ ,  $10^6$ ,  $10^6$ ,  $10^6$ ,  $10^0$  and  $10^0$  CFU, respectively (Figure 3-B). *Acetobacter pasteurianus* KBMNS-IAUF-2 at 38°C had a very good growth in ethanol concentrations of 2%-5% (v/v). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 at 38°C in ethanol concentrations of 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v) were measured as  $10^8$ ,  $10^6$ ,  $10^6$ ,  $10^6$ ,  $10^4$ ,  $10^4$ ,  $10^2$ ,  $10^0$  and  $10^0$  CFU, respectively (Figure 3-C). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 at 40°C in ethanol concentrations of 2, 3, 4, 5, 6, 7, 8, 9 and 10% (v/v) were measured as  $10^6$ ,  $10^6$ ,  $10^6$ ,  $10^4$ ,  $10^2$ ,  $10^2$ ,  $10^2$ ,  $10^0$  and  $10^0$  CFU, respectively (Figure 3-D). This strain had a very good growth rate at 40°C in 2%-4% (v/v) ethanol and tolerated the ethanol concentrations of 5%-8% (v/v) at 40°C so, it was considered as a thermo- ethanol- tolerant AAB

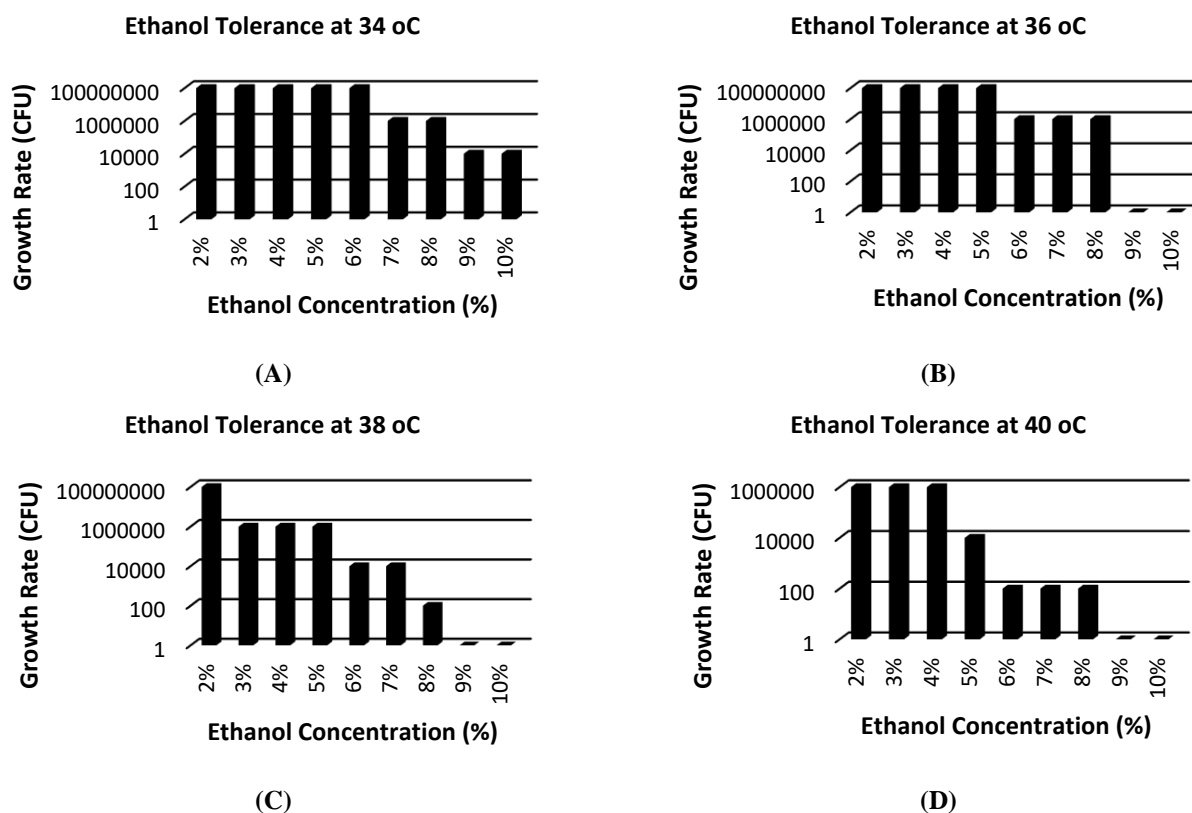


**Fig.2.** The phylogenetic tree of *Acetobacter pasteurianus* KBMNS-IAUF-2 16s-rDNA partial sequence. The 16s-rRNA gene of isolated AAB was similar to the *Acetobacter pasteurianus* strain FY-24 (GenBank accession number KT283054) with 97% and 100% of query coverage and similarity, respectively. The distance tree was drawn using NCBI software of "BLAST pairwise alignments".



strain. As shown in Figures 3, the growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 in the Carr medium was decreased by increasing the

ethanol concentration in constant temperatures of 34, 36, 38 and 40°C.



**Fig.3.** The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 24 hours' incubation against different ethanol concentrations of 2%-10% (v/v) in the modified Carr media at 34°C (A), 36°C (B), 38°C (C) and 40°C (D).

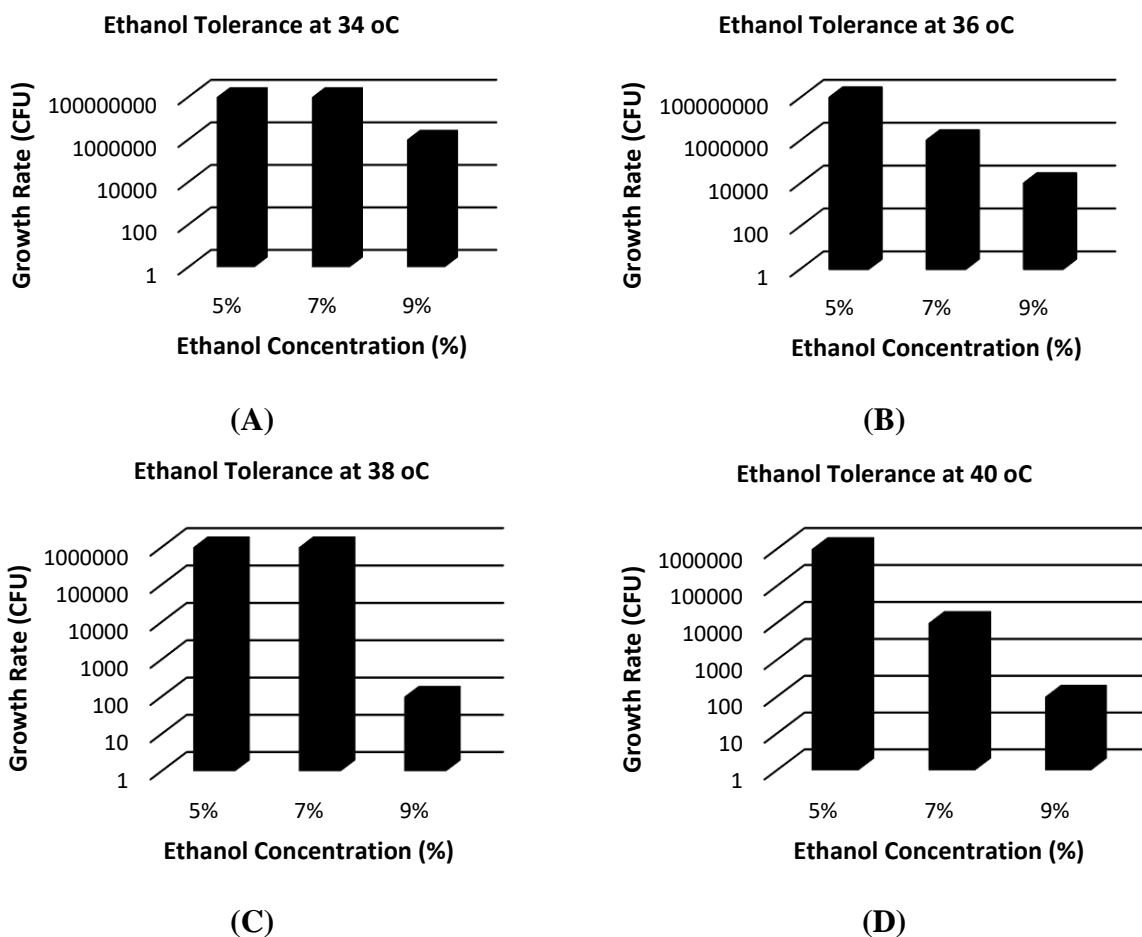
### 3.3.2. Strain resistance to different ethanol concentrations at constant temperature after 48 hours' incubation

The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation at 34°C in ethanol concentrations of 5, 7 and 9% (v/v) were measured as  $10^8$ ,  $10^8$  and  $10^6$  CFU, respectively (Figure 4-A). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation at 36°C in ethanol concentrations of 5, 7 and 9% (v/v) were measured as  $10^8$ ,  $10^6$  and  $10^4$  CFU, respectively (Figure 4-B). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation at 38°C in ethanol concentrations of 5, 7 and 9% (v/v) were measured as  $10^6$ ,  $10^6$  and  $10^2$  CFU,

respectively (Figure 4-C). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation at 40°C in ethanol concentrations of 5, 7 and 9% (v/v) were measured as  $10^6$ ,  $10^4$  and  $10^2$  CFU, respectively (Figure 4-D). The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation at different temperatures of 34, 36, 38 and 40°C in different ethanol concentrations of 5%, 7% and 9% (v/v) confirmed that this strain was a thermo-ethanol-tolerant AAB. This strain had a maximum growth rate in 5% (v/v) ethanol at 34 and 36°C and a very good growth at 38 and 40°C at the same ethanol concentration. Also, it had a maximum growth rate in 7% (v/v) ethanol at 34°C and very good growth at 36-40°C after 48 hours' incubation. *Acetobacter pasteurianus* KBMNS-IAUF-2 as an

ethanol-resistant AAB had an acceptable growth rate in 9% (v/v) ethanol at 34, 36, 38 and 40°C. As shown in Figure 4, by increasing temperature, the growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 in the Carr medium from 5% to 9% (v/v) ethanol decreased after 48 hours. In a study, the ability of AAB to grow in yeast extract agar (0.5% yeast extract and 2% agar) containing different ethanol concentrations of 4-10% (v/v) were measured. They concluded that these isolates were able to grow in an environment containing 4-6% (v/v) ethanol (Klawpiyamapurm et al. 2015). In the present research, the growth rate and acid production of *Acetobacter pasteurianus* KBMNS-IAUF-2 decreased as the ethanol concentration increased at constant temperature. This strain was

able to grow in a culture medium containing 10% (v/v) ethanol at 34°C after 24 hours. Also, it was able to grow at 40°C in a medium containing 9% (v/v) ethanol after 48 hours. It has been shown that increasing the ethanol concentrations resulted in temperature sensitivity of an *Acetobacter* sp. isolated from Date palm. The effect of increasing the temperature on the growth of that AAB isolate in a constant concentration of ethanol has also been investigated (Beheshti Maal 2014). In the present experiment, increasing the ethanol percentage increased the sensitivity of the bacterial cell to temperature, i.e., the sensitivity of bacterial cells to 9% (v/v) ethanol was higher than that of 7% (v/v) ethanol at 40°C.



**Fig.4.** The growth rate of *Acetobacter pasteurianus* KBMNS-IAUF-2 after 48 hours' incubation against different ethanol concentrations of 5%, 7% and 9% (v/v) in modified Carr media at 34°C (A), 36°C (B), 38°C (C), and 40°C (D).



### 3.4. Taguchi statistical optimization

The Taguchi statistical optimization confirmed that the amount of acetic acid produced by *Acetobacter pasteurianus* KBMNS-IAUF-2 in experiment 1 was higher than the other tests. According to the results of the L9 analysis, *Acetobacter pasteurianus* KBMNS-IAUF-2 produced the highest yield in the production of acetic acid, 1.60%, under conditions of 34°C, 2% (v/v) ethanol, and 2% (w/v) yeast extract concentrations. Also, the ANOVA analysis showed that the temperature factor (with 76.7% of influence) and the yeast extract concentration factor (with 6.2% of influence) were the most and the least effective factors, respectively, in acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2 (Table 3). The results of the Taguchi validation test showed the mean of 1.59%

of acetic acid production after 24 hours' incubation in the optimum condition proposed by the Taguchi design, conditions of 34°C, 2% (v/v) ethanol and 2% (w/v) yeast extract concentrations. The mean of the validation test, 1.59% of acetic acid production, was near to the result proposed by Taguchi, 1.60%. So, the validation test confirmed the optimum results suggested by Qualiteck 4 software (Table 4). Optimization by the Taguchi method was performed for the first time on acetic acid producing bacteria. Temperature and ethanol percentages had the greatest effect on acetic acid production and the least interaction with other factors, respectively. Also, the yeast extract concentration factor had the least effect on acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2 and had the highest interaction with other factors.

**Table 3.** ANOVA statistical analysis related to the influence of different factors in acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2.

Col # / Factor	DOF (f)	Sum of Sqrs. (S)	Variance (V)	F – Ratio (F)	Pure Sum (S')	Percent P (%)
Temperature	2	0.332	0.166	348.021	0.331	76.705
Ethanol %	2	0.07	0.035	73.895	0.069	16.112
Yeast Extract %	2	0.028	0.014	29.489	0.027	6.297
Other Error	2	-0.001	-0.001			0.886
<b>Total</b>	<b>8</b>	<b>0.431</b>				<b>100.00%</b>

**Table 4.** The results of Taguchi validation test

Validation Test Repeat	AA* Production (%)	AA Production Mean (%)	AA OPT Production Proposed by Taguchi Design (%)	Validation of Taguchi Design
1	1.58			
2	1.57			
3	1.63	1.59	1.60	OK
4	1.58			
5	1.59			

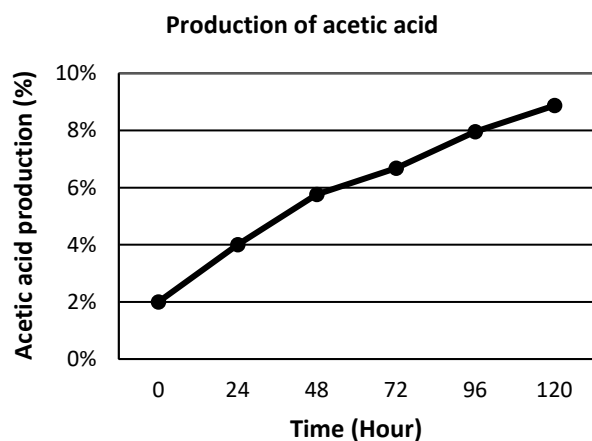
\* Acetic Acid

### 3.5. Production of acetic acid by *Acetobacter pasteurianus* KBMNS-IAUF-2

The amount of acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2 under optimal conditions in an industrial culture medium containing 2% (v/v) ethanol, 2% (w/v) yeast extract at 34°C after 24, 48, 72, 96 and 120 hours'

incubations were 4%, 5.76%, 6.68%, 7.96% and 8.87%, respectively. The production of acetic acid by the isolated AAB from grapevine during 120 hours' incubation at 34°C is indicated in Figure 5. In a report, the amount of acetic acid production by AAB isolates of P1, P4, P6, P12 in yeast-ethanol extract (2% (v/v) ethanol and 2% (w/v)

yeast extracts) using rotary shaker aeration was calculated to be 1.78, 1.80, 1.80, 1.81, and 1.81% after 24 hours (Klawpiyapamornkun et al. 2015). Diba et al. (2015) reported the amount of acetic acid produced by their isolates in the YGEA medium (yeast extract, glucose, ethanol and acetic acid) at 37°C after 72 hours as 3-9.6%. In the present study, 2% (v/v) ethanol was added to the acetator after 24 hours of incubation and then again after 48 hours, and this approach was continued for 72 hours. The amount of acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2 in an industrial culture medium (2% (v/v) ethanol, 2% (v/v) acetic acid, and 2% (w/v) yeast extract) (Sharafi et al., 2010) at 34°C after 48 hours was measured as 4% and after 72 hours as 6.68%. These measurements indicated that the yield of acetic acid production by this newly identified strain was 97%. Therefore, the isolated AAB strain from Iranian grapevine produced a high acetic acid at relatively high temperatures.



**Fig.5.** The production of acetic acid by *Acetobacter pasteurianus* KBMNS-IAUF-2 isolated from grapevine during 120 hours' incubation at 34°C in industrial culture medium.

#### 4. Conclusion

This is the first report of the identification of *Acetobacter pasteurianus* KBMNS-IAUF-2 isolated from Iranian grapevine. Figure 3 shows the growth of *Acetobacter pasteurianus* KBMNS-IAUF-2 in a modified Carr medium containing

10% ethanol. Figure 4 also show the growth of this strain on a modified Carr medium with 9% ethanol. These results of one factor optimizations confirmed that the *Acetobacter pasteurianus* KBMNS-IAUF-2 is an ethanol tolerant AAB strain. After detection and isolation of a thermo-ethanol tolerant AAB from the first set of experiments, the Taguchi method was designed to optimize the acetic acid production using the isolated AAB. During acetic acid production ethanol is gradually added to the broth culture medium, usually up to 2% each time. The ethanol is oxidized to 2% acetic acid or less, and the ethanol is added to the medium again to increase the percentage of acetic acid and it usually takes up to a week. Since a constant 24-hour incubation time was considered in all the Taguchi experiments, fermentation time was not considered as a variable. So, it is reasonable that in the Taguchi test the best production of acetic acid was achieved at 2% ethanol concentration because the addition of more than 2% ethanol (5-10%) to the broth medium in the same time will resulted in the AAB death. Therefore, the 2% ethanol concentration proposed by Taguchi was related to the acetic acid production and had no relationship to the strain tolerance against ethanol. The L9 analysis in the Taguchi optimization, which was first reported in Iran to increase the yield of acetic acid production by an AAB, confirmed that the temperature and ethanol percentage were the significant factors in the acetic acid production by *Acetobacter pasteurianus* KBMNS-IAUF-2. Thus, small changes in temperature and the amount of ethanol significantly affected the percentage of acetic acid production. The analysis of Qualiteck 4 software confirmed that the best condition for producing the acetic acid by *Acetobacter pasteurianus* KBMNS-IAUF-2 after 24 hours were incubation at 34°C using an industrial culture medium containing 2% (v/v) ethanol and 2% (w/v) yeast extract. Also, the yield of acetic acid production by this strain was measured as 97% after 24 hours. Therefore, this thermo-ethanol-tolerant strain could be applied as

an industrial starter for producing vinegar with grapevine flavor.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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### Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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