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Characterization of an *Acetobacter* Strain Isolated from Iranian Peach that Tolerates High Temperatures and Ethanol Concentrations

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***Abstract*—**Vinegar is a precious food additive and complement as well as effective preservative against food spoilage. Recently traditional vinegar production has been improved using various natural substrates and fruits such as grape, palm, cherry, coconut, date, sugarcane, rice and balsam. These neoclassical fermentations resulted in several vinegar types with different tastes, fragrances and nutritional values because of applying various acetic acid bacteria as starters. Acetic acid bacteria include genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* according to latest edition of Bergy's Manual of Systematic Bacteriology that classifies genera on the basis of their 16s RNA differences. *Acetobacter* spp as the main vinegar starters belong to family *Acetobacteraceae* that are gram negative obligate aerobes, chemoorganotrophic bacilli that are oxidase negative and oxidize ethanol to acetic acid. In this research we isolated and identified a native *Acetobacter* strain with high acetic acid productivity and tolerance against high ethanol concentrations from Iranian peach as a summer delicious fruit that is very susceptible to food spoilage and decay. We used selective and specific laboratorial culture media such as Standard GYC, Frateur and Carr medium. Also we used a new industrial culture medium and a miniature fermentor with a new aeration system innovated by Pars Yeema Biotechnologists Co., Isfahan Science and Technology Town (ISTT), Isfahan, Iran. The isolated strain was successfully cultivated in modified Carr media with 2.5% and 5% ethanol simultaneously in high temperatures, 34 - 40º C after 96 hours of incubation period. We showed that the increase of ethanol concentration resulted in rising of strain sensitivity to high temperature. In conclusion we isolated and characterized a new *Acetobacter* strain from Iranian peach that could be considered as a potential strain for production of a new vinegar type, peach vinegar, with a delicious taste and advantageous nutritional value in food biotechnology and industrial microbiology.

Keywords*—Acetobacter*, Acetic Acid Bacteria, Vinegar, Peach, Food Biotechnology, Industrial Microbiology, Fermentation

I. INTRODUCTION

VINEGAR is a delicious food additive that is produced by Acetic Acid Bacteria (AAB) and contains essential nutrients such as amino acids regarding its fruit source [1]. FDA (Food and Drug Administration, USA) has explained the vinegar as a 4% acetic acid solution that is synthesized from sweet or sugary substances through alcoholic fermentation. Currently the vinegar manufacturers are seeking for new types of vinegar using different AAB as their starter [2]. AAB are classified into genera *Acetobacter, Gluconobacter, Fratueria, Acidomonas* and *Gluconacetobacter* [2]-[6]. Among AAB, *Acetobacter* strains are the major bacteria that are dealing with vinegar production industrially [2]-[3], [7]. *Acetobacteria* that are living on certain fruits and flowers are resulted in beer and wine spoilage as well as fruit decay. These motile / nonmotile gram negative rods are catalase positive, oxidase negative with optimum growth temperature of 25 – 30° C and typically oxidize ethanol to acetic acid and sequentially to carbon dioxide and water (over oxidation). *Acetobacter* strains have been isolated from several resources such as Iranian white-red cherry [2], palm tree and palm wine [8]-[10], apple, Jamaican cherry, longan, mango, pineapple and rambutan [11]-[12], grape, date and coconut [3], [13]-[14]. Several vinegar types have been synthesized using AAB and various precursors such as balsam [15]-[17], sugarcane [1] and rice [18]. AAB strains specially those of *Acetobacter* and *Gluconobacter* spp could produce various polysaccharides e.g. cellulose, dextran and levan that affect the quality of vinegar produced. Some mutated *Acetobacter* strains could release heterogeneous exopolysaccharides combined from glucose, mannose, rhamnose and glucuronic acid [3]. The goals of this research were investigating natural origins for producing new vinegar types as well as isolating and identifying new AAB strains that could be used in large scale industrial procedures and those could resist formal stresses such as alcohol and acetic acid concentrations, temperature fluctuations and other conditional alterations.

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II. MATERIAL AND METHODS

Iranian peach extract preparation: The heterogeneous samples of both intact and spoilaged summer fruit, Iranian peach, were gathered from various fruit stores in Isfahan, Iran using sterile containers and incubated in a good ventilated cabinet at room temperature, 25-30° C, for 2 weeks. After rising sour smelling from incubated container that suggested the activity of AAB, the Iranian peach spoilages were pressed,

squeezed, homogenized and passed through a sterile basket with recognized pore size. The cores of peaches were isolated and extracts were transferred to sterile 2 liter bottles, capped and incubated at 30° C for 7 day anaerobically. For inhibiting bottle's explosion due to alcoholic fermentation, a few tiny punctures were made on top of the bottles for extra gas drainage.

Microbial culture media and instruments: The laboratorial culture media that we used were included GYC standard medium [yeast extract, 10 g/l; D-glucose, 50 g/l; $CaCO_3$, 30 g/l; agar, 25 g/l; distilled water, 1000 ml], Frateur medium [yeast extract, 10 g/l; $CaCO_3$, 20 g/l; ethanol, 20 g/l; agar, 20 g/l; distilled water, 1000 ml], Carr medium [yeast extract, 3%; agar, 2%; bromocresol green, 0.002%; ethanol, 2% (v/v); distilled water, 1000 ml] and modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol concentrations. The industrial culture medium [ethanol, 2%; acetic acid, 2%; yeast extract, 1%] was applied for initial enrichment and isolation of AAB from Iranian peach. All the chemicals were from the Merck. The main instruments that we used were incubator (Heraus, Germany), shaker incubator (Jahl, JSH 20L,Iran), autoclave (Iran Tolid, Iran), miniature glass fermentor (Pars Yeema Biotechnologists Co., ISTT, Isfahan, Iran), Spectrophotometer (Milton Roy, USA), high speed refrigerated centrifuge (Hitachi, 20PR, Japan), microscope (Nikon, Japan) and stereomicroscope (Wild, Germany).

AAB enrichment and isolation from Iranian peach: Fifty milliliters of homogenized Iranian peach extract after 7 days incubation at 30° C were collected and centrifuged at 10000 rpm for 20 minutes. The supernatants were discarded and the pellets were added to miniature glass fermentors containing of 400 ml industrial broth medium. The fermentors then were placed in a 30° C incubator while aeration was carrying out through an appropriate sparging system for 7 days. Each 24 hours the acetic acid elevations were examined through titration assay. The containing of fermentors with acetic acid percentage more than 4% were cultured on AAB selective culture media using streak plate method and incubated at 30° C for 4 days.

Macroscopic, microscopic and biochemical properties of isolated strains: The macroscopic traits of isolated colonies from Iranian peach, morphological characteristics of colonies were investigated using stereomicroscopy. Microscopic and biochemical examinations of pure individual colonies were carried out using gram staining, catalase and oxidase reactions, ability of overoxidizing in Carr medium and ability of consuming calcium carbonate (transparenting around of colonies) in Frateur medium.

Investigation of strain's tolerance to various ethanol concentrations: The isolated strains of AAB from Iranian peach were cultured on modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol concentrations using streak plate method and incubated at 30° C for 96 hours.

Investigation of strain's resistance to high temperatures: The isolated strains of AAB from Iranian peach were cultivated on Carr medium and modified Carr media with 2.5% and 5% ethanol concentrations and incubated at high temperatures of 34° C, 36° C, 38° C and 40° C for 96 hours.

Acetic acid titration assay: In all industrial broth media, both before and after inoculation with isolated strains from Iranian peach, the titration assay of acetic acid percentage were took placed as follow: five milliliters of the broth medium were added to 20 ml of distilled water in a 250 ml flask and mixed thoroughly with 5 drops of phenol phetalein [phenol phetalein, 0.1 g; ethanol, 60 g; distilled water, 40 g] and then 0.5 normal sodium hydroxide [NaOH, 20 g/l; distilled water, 1000 ml] were added using 200 ml burette to mentioned mixture 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 computed.

III. RESULTS

Incubation of industrial culture media inoculated with Iranian peach extracts at 30° C and for 4 days resulted in turbidity and acetic acid enhancement (data not shown). Streak plate method cultivation of turbid broth on Carr medium and Frateur medium resulted in emerging of pure, individual colonies that were similar to *Acetobacter* genus. The differential macroscopic, microscopic and biochemical examinations revealed that the isolated strain was related AAB. The capability of strains in converting the blue color of Carr medium to yellow color and growth in 2% ethanol after 48 hours suggested that they were related to *Gluconobacter* or *Acetobacter* spp but color returning of Carr medium from yellow to blue after 96 hour incubation at 30° C showed that the isolated bacterium was an overoxidizer strain and confirmed that related to *Acetobacter* spp. Growth in Frateur culture medium and transparenting the zones around the individual colonies after 96 hour incubation at 30° C showed that the same strain has utilized calcium carbonate in Frateur medium, the property that is exclusively related to genus *Acetobacter*, so reconfirmed that the isolated strain was *Acetobacter*. A summary of main experiments and their results that led to identification of *Acetobacter* strain isolated from Iranian peach were shown in table 1.

TABLE I
MACROSCOPIC, MICROSCOPIC AND BIOCHEMICAL TESTS OF ISOLATED ACETOBACTER STRAIN FROM IRANIAN PEACH AFTER 24-96 HOUR INCUBATION AT 30° C

Culture Media Examinations		Carr Medium Agar	Frateur Medium Agar
Macroscopic Tests	Colony Characterizations	Blue – yellowish, needle-tip to small with < 3 mm diameter, circular, convex and regular shape with brownish center, vinegar smelling	Pale gray – greenish, small colonies with ≤ 3 mm diameter, circular, convex and regular shape, clearance zone around the individual colonies

Microscopic Tests	Gram Reaction	Gram negative	Gram negative
	Morphology	Short rods/coccobacilli	Short rods/coccobacilli
	Arrangement	Mono, diplo and a few streptobacilli	Mono and diplobacilli
Biochemical Tests	Aerobic Growth	Positive	Positive
	Anaerobic Growth	Negative	Negative
	Catalase Reaction	Positive	Positive
	Oxidase Reaction	Negative	Negative
	Overoxidation Capability	Positive	Negative
	CaCO3 Utilization	Negative	Positive

The cultivation of *Acetobacter* strain isolated from Iranian peach on modified Carr media with 3-10% ethanol concentrations at 30° C for 4 days showed that it could tolerate against high ethanol concentrations. The tables 2 – 5 summarize the results of experiments accessed the strain tolerance in various ethanol concentrations on modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol after 24 – 96 hour incubation at 30° C. The growth rates of isolated *Acetobacter* strain in modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol after 24 hours incubation at 30° C were 4+, 2+, 1+, -, -, - and - respectively (Table.2).

TABLE II
THE TOLERANCE OF ISOLATED ACETOBACTER STRAIN FROM IRANIAN PEACH AGAINST VARIOUS ETHANOL CONCENTRATIONS ON MODIFIED CARR MEDIA AT 30° C AFTER 24 HOURS

Ethanol Concentrations	3%	4%	5%	6%	7%	8%	9%	10%
Growth Rates	4+	2+	1+	Neg	Neg	Neg	Neg	Neg
Acetic Acid Production	4+	2+	1+	Neg	Neg	Neg	Neg	Neg

The isolated *Acetobacter* strain after 48 hour incubation at 30° C in modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol showed the growth rates of 4+, 3+, 2+, 1+, -, -, - and – respectively as were shown in Table 3.

TABLE III
THE TOLERANCE OF ISOLATED ACETOBACTER STRAIN FROM IRANIAN PEACH AGAINST VARIOUS ETHANOL CONCENTRATIONS ON MODIFIED CARR MEDIA AT 30° C AFTER 48 HOURS

Ethanol Concentrations	3%	4%	5%	6%	7%	8%	9%	10%
Growth Rates	4+	3+	2+	1+	Neg	Neg	Neg	Neg
Acetic Acid Production	4+	3+	2+	1+	Neg	Neg	Neg	Neg

The same strain on modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol at 30° C after 72 hours indicated the growth rates of 4+, 3+, 3+, 2+, 1+, 1+, - and - respectively (Table 4).

TABLE IV
THE TOLERANCE OF ISOLATED ACETOBACTER STRAIN FROM IRANIAN PEACH AGAINST VARIOUS ETHANOL CONCENTRATIONS ON MODIFIED CARR MEDIA AT 30° C AFTER 72 HOURS

Ethanol Concentrations	3%	4%	5%	6%	7%	8%	9%	10%
Growth Rates	4+	3+	3+	2+	1+	1+	Neg	Neg
Acetic Acid Production	4+	3+	3+	2+	1+	1+	Neg	Neg

The *Acetobacter* strain isolated from Iranian peach after incubation at 30° C for 96 hours and on different modified Carr media with 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol showed the growth rates of 4+, 4+, 4+, 4+, 4+, 3+, 2+ and 2+ respectively as have been indicated in Table 5.

TABLE V
THE TOLERANCE OF ISOLATED ACETOBACTER STRAIN FROM IRANIAN PEACH AGAINST VARIOUS ETHANOL CONCENTRATIONS ON MODIFIED CARR MEDIA AT 30° C AFTER 96 HOURS

Ethanol Concentrations	3%	4%	5%	6%	7%	8%	9%	10%
Growth Rates	4+	4+	4+	4+	4+	3+	2+	2+
Acetic Acid Production	4+	4+	4+	4+	4+	3+	2+	2+

The comparison of growth rates of isolated *Acetobacter* strain from Iranian peach in increasingly ethanol concentrations from 3% to 10% at 30° C and in different incubation times, 24-96 hours, were shown in Figure 1.

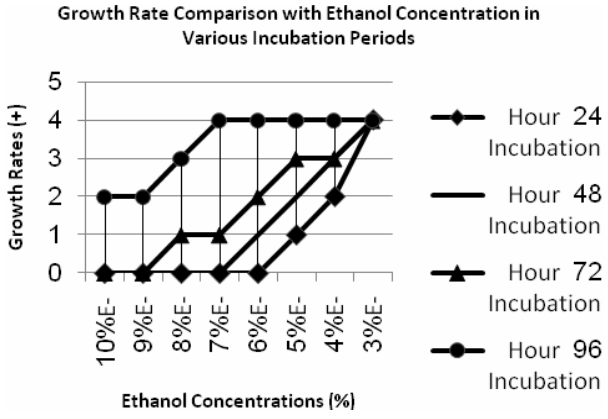

Fig. 1 The comparison of growth rates of *Acetobacter* strain isolated from Iranian peach and it's tolerance against various ethanol concentrations in different incubation periods, 24 -96 hours, at 30° C on modified Carr media with 3-10% ethanol

The tolerance examinations of *Acetobacter* strain isolated from Iranian peach against high temperatures such as 34, 36, 38 and 40° C indicated that this strain could grow on 2.5% and 5% ethanol concentrations in these high temperatures. Figure 2 shows the growth of isolated *Acetobacter* strain on modified Carr mediums with 2.5% and 5% ethanol concentrations at 34° C after 96 hour incubation period.

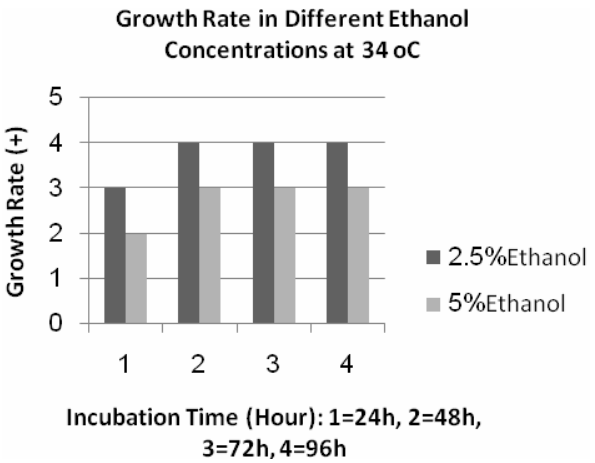

Fig. 2 The growth rate of isolated *Acetobacter* strain from Iranian peach after 24-96 hour incubation in 2.5% and 5% ethanol concentrations at 34° C

The examinations showed that the *Acetobacter* strain isolated from Iranian peach grew in modified Carr media with 2.5% and 5% ethanol concentrations after 24-96 hours at 36° C as shown in figure 3.

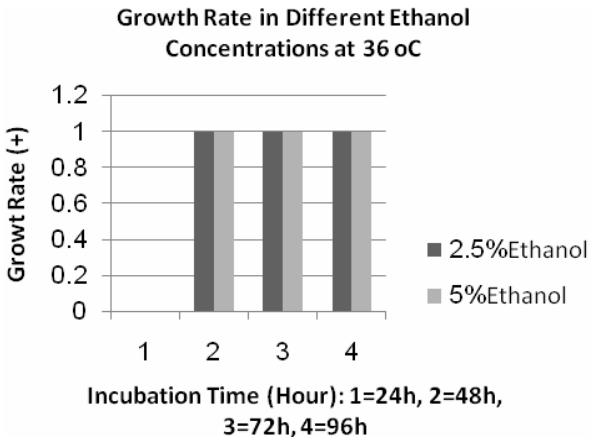

Fig. 3 The growth rate of isolated *Acetobacter* strain from Iranian peach after 24-96 hour incubation in 2.5% and 5% ethanol concentrations at 36° C

Figure 4 shows the growth rate of isolated *Acetobacter* strain from Iranian peach on modified Carr mediums with 2.5% and 5% ethanol concentrations at 38° C after 24-96 hour incubation period.

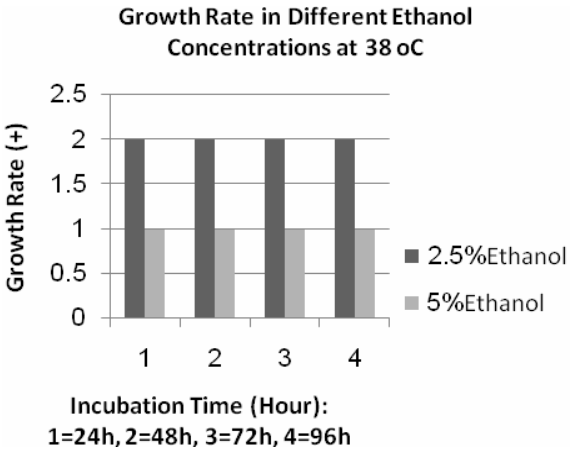

Fig. 4 The growth rate of isolated *Acetobacter* strain from Iranian peach after 24-96 hour incubation in 2.5% and 5% ethanol concentrations at 38° C

The results of examinations showed that the same strain could grow at 40° C simultaneously on modified Carr media with 2.5% and 5% ethanol concentrations after 24-96 hour incubation period (Figure 5).

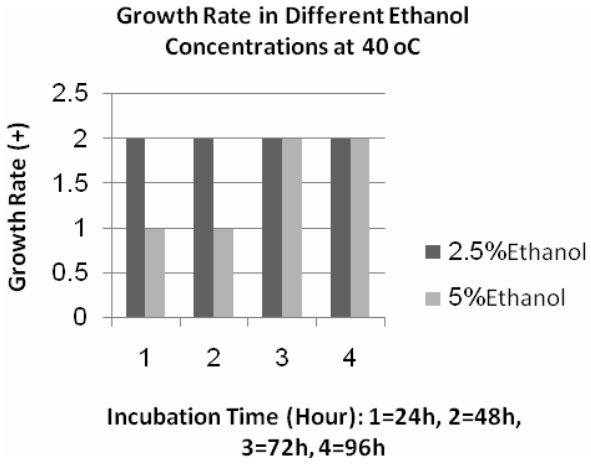

Fig. 5 The growth rate of isolated *Acetobacter* strain from Iranian peach after 24-96 hour incubation in 2.5% and 5% ethanol concentrations at 40° C

IV. DISCUSSION

Sossou et al. (2009) have isolated *Acetobacter* sp. (ASVO3) from pineapple juice and produced vinegar after 23-25 days at 30° C. They studied their strain tolerance against glucose but reported that the concentration of ethanol had no effect on growth of AAB [12]. Beheshti Maal et al. (2010) have reported the isolation and characterization of an *Acetobacter* strain from Iranian white red cherry. They have specified that the mentioned isolated strain had growth at 34-36° C in high ethanol, 5-9%, concentrations and after 4 day incubation duration [2]. Moryadee et al. (2008) have isolated

thermotolerant *Acetobacter* spp. from apple, Jamaican cherry, longan, mango, pineapple and rambutan using a culture medium containing of 4% ethanol. They reported an *Acetobacter* strain isolated from rambutan that produced the highest acetic acid amount at 37° C after 7 day incubation period [11]. IIha et al. (2000) have utilized honey for vinegar production using a mixed AAB and in room temperature but didn't report any suggestion for tolerance of their responsible strains against high temperature or ethanol concentrations [19]. Kocher et al. (2006) have studied the production of vinegar by sugarcane using corn cobs, bagasse and wood shavings as immobilizers and *Acetobacter aceti* NRRL746. They showed that the fermentation time was reduced to 13 days by proposed recycling method [1]. According to Kadere et al. (2008) the coconut toddy (mnazi) could be applied for production of vinegar using an *Acetobacter* strain that they isolated from coconut. They reported that the isolated strain had grew at 25, 30 and 40° C [3]. Ndoye et al. (2007) have presented a new species of *Acetobater*, *A. senegalensis*, a thermotolerant *Acetobacter* that they isolated from mango (Mangifera indica L.). They have reported that their strain had exhibited growth at 35° C as an optimum temperature [20]. We isolated a thermotolerant *Acetobacter* strain from Iranian peach that we obtained from Isfahan, Iran. We showed that this strain could grow at high ethanol concentrations, 7-10%, at 30° C after 96 hours on modified Carr media. We indicated that the isolated *Acetobacter* has grew on 2.5% and 5% ethanol concentrations at 34° C after 24 hours but had no growth on the same ethanol concentrations at 36° C after 24 hours, instead had growth after 48 hours at 36° C. Also this strain had an acceptable growth on 2.5% and 5% ethanol concentrations at 38° C and 40° C, unusual temperatures for AAB industrially, after 24 hours. We suggested that the elevation of ethanol concentration resulted in extending log phase of growth curve in isolated *Acetobacter* strain from Iranian peach but after 96 hours in Carr medium with more than 6% ethanol concentration, the problem was overcome. These observations showed that the isolated *Acetobacter* stain from Iranian peach could rapidly adapt itself to extreme conditions such as high temperatures of 38-40° C as well as high concentrations of 5% ethanol simultaneously. We suggested this strain could be used in pilot-plant and large-scale fermentation of vinegar specially a new type of vinegar, peach vinegar. While the peach is a very delicious summer fruit with nutritional advantages, but it is a very susceptible fruit against microbial spoilage. We showed that this strain could use mentioned spoilages as precursor for producing of peach vinegar as a superior food additive. These properties, parallel and rapid adaptations to high temperatures and ethanol concentrations, could be considered as very dramatic characteristics for industrial strains that are qualified for large-scale commercial fermentations in vinegar industry and industrial microbiology. In conclusion, this is the first report of isolation and identification of an *Acetobacter* strain from the Iranian peach. The isolated thermotolerant *Acetobacter* strain could grow at 5% ethanol concentrations and 38-40° C that suggests a very suitable strain for large-scale vinegar fermentation in microbial biotechnology as well as a new amenable strain for bioremediation uses in environmental microbiology.

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