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Isolation and Identification of an Acetobacter Strain from Iranian White-Red Cherry with High Acetic Acid Productivity as a Potential Strain for Cherry Vinegar Production in Food and Agriculture Biotechnology

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***Abstract*—**According to FDA (Food and Drug Administration of the United States), vinegar is definedas a sour liquid containing at least 4 grams acetic acid in 100 cubic centimeter (4% solution of acetic acid) of solution that is produced from sugary materials by alcoholic fermentation. In the base of microbial starters, vinegars could be contained of more than 50 types of volatile and aromatic substances that responsible for their sweet taste and smelling. Recently the vinegar industry has a great proportion in agriculture, food and microbial biotechnology. The acetic acid bacteria are from the family *Acetobacteraceae*. Regarding to the latest version of Bergy's Mannual of Systematic Bacteriology that has categorized bacteria in the base of their 16s RNA differences, the most important acetic acid genera are included *Acetobacter* (genus I), *Gluconacetobacter* (genus VIII) and *Gluconobacter* (genus IX). The genus *Acetobacter* that is primarily used in vinegar manufacturing plants is a gram negative, obligate aerobe coccus or rod shaped bacterium with the size 0.6 - 0.8 X 1.0 - 4.0 µm, nonmotile or motile with peritrichous flagella and catalase positive – oxidase negative biochemically.

Some strains are overoxidizer that could convert acetic acid to carbon dioxide and water.In this research one *Acetobacter* native strain with high acetic acid productivity was isolated from Iranian white – red cherry. We used two specific culture media include Carr medium [yeast extract, 3%; ethanol, 2% (v/v); bromocresol green, 0.002%; agar, 2% and distilled water, 1000 ml], Frateur medium [yeast extract, 10 g/l; CaCO3, 20 g/l; ethanol, 20 g/l; agar, 20 g/l and distilled water, 1000 ml] and an industrial culture medium. In addition to high acetic acid production and high growth rate, this strain had a good tolerance against ethanol concentration that was examined using modified Carr media with 5%, 7% and 9% ethanol concentrations. While the industrial strains of acetic acid bacteria grow in the thermal range of 28 – 30 oC, this strain was adapted for growth in 34 – 36 oC after 96 hours incubation period. These dramatic characteristics suggest a potential biotechnological strain in production of cherry vinegar with a sweet smell and different nutritional properties in comparison to recent vinegar types. The lack of growth after 24, 48 and 72 hours incubation at 34 – 36 oC and the growth after 96 hours indicates a good and fast thermal flexibility of this strain as a significant characteristic of biotechnological and industrial strains.

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I. Introduction

VINEGAR is defined as a 4% acetic acid solution that is originated from an alcoholic fermentation processing using sugary substances [1] – [4]. Recently the vinegar industry has been developed to produce several vinegar types using various qualified native or engineered acetic acid bacteria [2]- [3]. As originally defined, the acid acetic bacteria comprised a group of gram-negative, aerobic, motile rods that carried out incomplete oxidation of alcohol and sugars, leading to the accumulation of organic acids as end products. The acetic acid bacteria (AAB) are heterogenous assemblage organisms [5] - [6]. There are several genus in AAB group but *Gluconobacter sp.* and *Acetobacter sp.* are more discussed as bacteria that can produce acetic acid industrially.

Acetic acid bacteria are commonly associated with some fruits such as grape and are normally present in must deteriorated fruits. These have more acetic acid bacteria population whereas unspoiled fruits have less [7].

There are several factors that affect the growth and survival of AAB that amongst, ethanol concentration, acetic acid concentration, oxygen, temperature and nutrient availability are the most important factors that can affect the survival of AAB.

Acetic acid concentration below 10 g/l is resulted in significantly increased growth rate (particularly at low ethanol concentration) above 20 g/l acetic acid, however growth is severely restricted and virtually inhibited at an acetic acid concentration in the region of 50 g/l, whatever the amount of ethanol present [8]. There is no doubt that the presence or absence of oxygen greatly impacts the growth of acetic acid bacteria and in industrial vinegar fermentation (wine and rohm).

Most of actic acid bacteria are mesophilic but recently a novel strain has been isolated which can tolerate up to 40ºC, this strain can be used for industrial production of vinegar in regions with warm climates.

Among the most important acetic acid bacteria, the strains of genus *Acetobacter* are mainly involved in vinegar production [5], [9]. *Acetobacter* is a gram negative, obligate aerobe coccus or rod shaped bacterium with the size of 0.6 - 0.8 X 1.0 - 4.0 µm, nonmotile or motile with peritrichous flagella, catalase positive and oxidase negative biochemically. Some strains are overoxidizer that could convert acetic acid to carbon dioxide and water. *Acetobacter* use ethanol as carbon source preferably and is increased during the wine fermentation processing [1] - [2], [9] - [10]. *Acetobacter* strains have been isolated from several natural origins such as grape, date and palm resources and coconut [9] and have been applied for production of several vinegar types from various substrates e.g. sugarcane [3], rice [8] and balsam [11] - [12].

The aims of this study were characterization of the isolated strain from novel food and agricultural resources that could grow at high temperatures and tolerate against high concentrations of ethanol and produce high levels of acetic acid.

II. Materials and Methods

Preparation of Iranian white – red cherry extract: The spoilages of Iranian white – red cherry were collected from several areas in Esfahan, Iran. The samples were placed in a safe cabinet with good ventilation and at room temperature for 10 days. After appearance of fruit flies around the samples, the fruits were pressed and scrutinized with a sterile metal plate and were poured into a sterile 2 liters plastic bottle so that 2/3 of bottle was filled. The bottle was closed and for preventing bottle explosion, due to alcoholic fermentation and gas production, some tiny openings were made in the top of bottle through a sterile needle. The bottle then was incubated at 30º C for 4 days. The bottle was being examined every 24 hours according to alcoholic fermentation and sour smelling appearance.

Isolation of bacterial strain: For isolating bacterial strains that were responsible for vinegar smelling, 20 ml of Iranian white-red cherry extract were transferred to sterile tubes aseptically and centrifuged at 3000 rpm for 5 minutes. The pellets were injected into industrial culture mediums and incubated at 30º C with aeration speed of 120 rpm for 7 days. After enrichment of acetic acid bacteria in mentioned medium, the isolation process was followed using specific culture media.

Culture media and ingredients: The industrial culture medium that was used at the first isolation and enrichment phase includes yeast extract, 2%; ethanol, 2%; acetic acid, 1% and distilled water, 1000 ml. At the second isolation phase, the Carr medium [yeast extract, 3%; agar, 2%; bromocresol green, 0.002%; ethanol, 2% and distilled water, 1000 ml] and Frateur medium [yeast extract, 10 g/l; CaCO3, 20 g/l; ethanol, 20 g/l; agar, 20 g/l and distilled water, 1000 ml] and modified Carr media with 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol were used [4].

Characterization and biochemical tests: After isolation of acetic acid bacteria from Iranian white-red cherry, macroscopic examinations, microscopic and morphological properties, gram reaction, oxidase and catalase tests, over oxidation and lactate utilization capability on Carr medium were investigated.

Titration assay and measurement of acetic acid percentage: The phenol phetalein reagent [phenol phetalein, 0.1 g; ethanol, 60 g and distilled water, 40 g] and 0.5 normal NaOH [NaOH, 20 g and distilled water 1000 ml] were made. For titration assay, 5 ml of culture solution were being added to a flask and after addition of 20 ml distilled water and 5 drops phenol phetalein, the amount of acetic acid in solution was being titrated.

Bacterial growth: Bacterial growth was quantified by measuring the absorbance of industrial culture media at 540 nm.

Measurement of the tolerance of strains against different ethanol concentrations: The tolerance of isolated strain to ethanol concentration stress was investigated using modified Carr media with 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol at 30º C and after 24 and 48 hours incubation periods.

Assessment of stains in growth at extreme like conditions: The isolated strain was cultured in modified Carr media using 5%, 7% and 9% ethanol concentrations and at high unusual temperatures, 34 ºC and 36 ºC, simultaneously.

III. Results

The industrial culture media after 24, 48, 72, 96, 120, 144 and 168 hours incubation at 30º C and with 120 rpm aeration speed were examined according to their acetic acid production by titration assay and their turbidity due to bacterial growth. The results showed their acetic acid percentage after mentioned incubation periods were 2%, 2.3%, 3.6%, 5.4%, 6.5%, 7.3%, 9.5% respectively as are shown in Fig. 1.

The bacteria were cultured from industrial culture media to Carr medium and after 24 hours incubation at 30º C showed tiny blue conducted smooth colonies with shiny reflex and after 48 hours were initiating to convert the color of the Carr medium to yellowish indicating that isolated strain was an acid producing bacterium. Microscopic examinations confirmed that this strain was a gram negative coccobacillus to rod with mono, diplo and streptobacillus arrangements. Biochemical tests showed that the catalase was positive and the oxidase was negative. The next examinations in measuring the ethanol tolerance of isolated strain showed that it could be cultured in different ethanol concentrations, 4%-10%, in modified Carr media after 24 and 48 hours incubation periods and was capable to produce acetic acid increasingly (Table I and Table II).

TABLE I
GROWTH AND ACETIC ACID PRODUCTION IN DIFFERENT ETHANOL CONCENTRATION AT 30° AFTER 24 HOURS

Time	24 Hours						
Ethanol %	4	5	6	7	8	9	10
Growth	+4	+4	+3	+2	-	-	-
Acid Production	+4	+4	+3	+2	-	-	-

TABLE II
GROWTH AND ACETIC ACID PRODUCTION IN DIFFERENT ETHANOL CONCENTRATION AT 30° AFTER 48 HOURS

Time	48 Hours						
Ethanol %	4	5	6	7	8	9	10
Growth	+4	+4	+4	+4	+3	+2	+1
Acid Production	+4	+4	+4	+4	+3	+2	+1

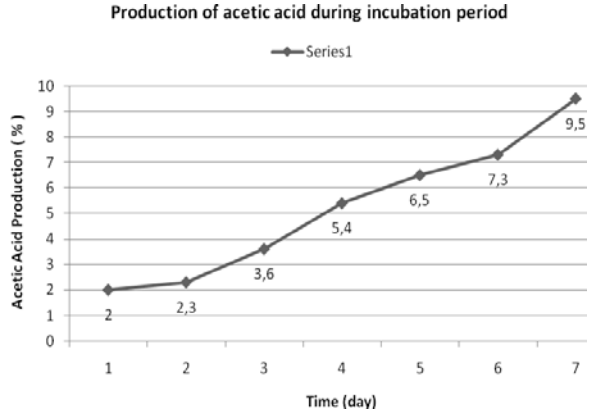

Fig. 1 The high acetic acid productivity of Acetobacter strain isolated from Iranian white red cherry after 24, 48, 72, 96, 120, 144 1nd 168 hours at 30 oC and 120 RPM aeration speed

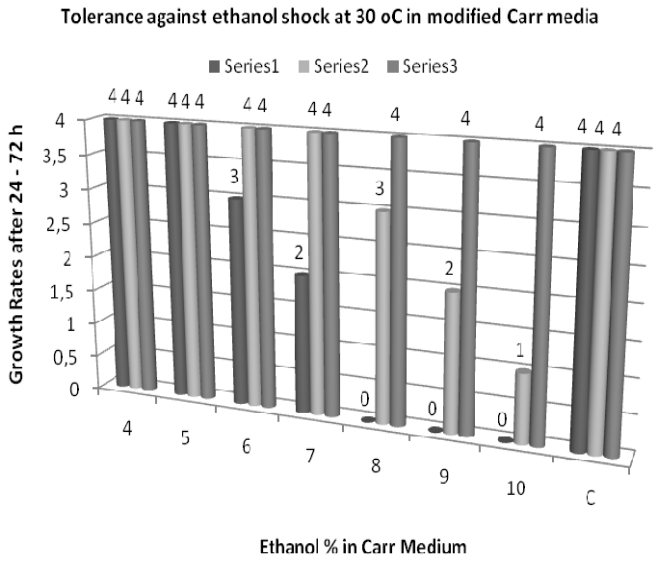

Fig. 2 The tolerance of *Acetobacter* strain isolated from Iranian white red cherry against increasing ethanol concentrations in modified Carr mediums at 30 oC. Series 1: after 24 hours, Series 2: after 48 hours and series 3: after 72 hours, C: control

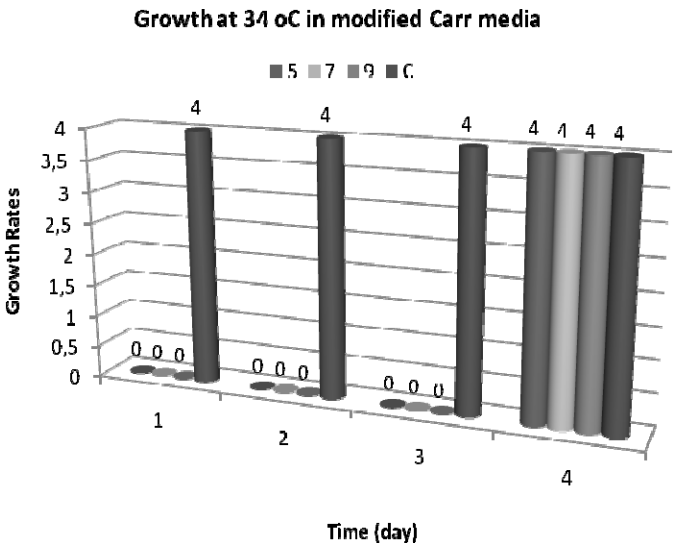

Fig. 3 The interaction of tolerance to ethanol shock and critical temperature after 24, 48, 72 and 96 hours incubation at 34 oC in *Acetobacter* strain isolated from Iranian white red cherry. 5: ethanol 5%, 7: ethanol 7%, 9: ethanol 9% and C: control

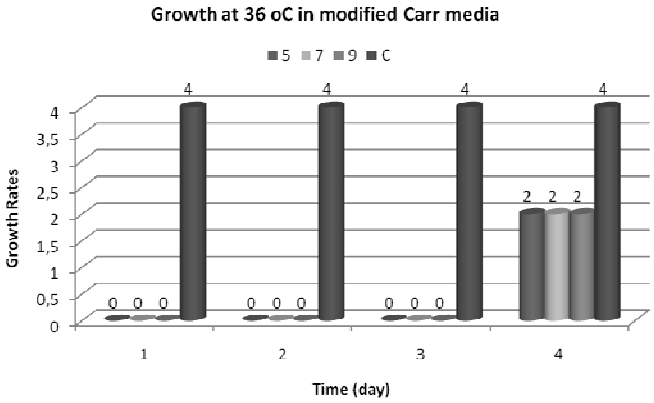

Fig. 4 The interaction of tolerance to ethanol shock and critical temperature after 24, 48, 72 and 96 hours incubation at 36 oC in *Acetobacter* strain isolated from Iranian white red cherry. 5: ethanol 5%, 7: ethanol 7%, 9: ethanol 9% and C: control

The growth at Frateur medium at 30° C was occurred after 96 hours so that around the colonies had been transparent confirming that the isolated strain has dissolved the $CaCO_3$ and was belonged to acetic acid bacteria.

This strain grew on modified Carr media with 4%, 5%, 6% and 7% ethanol after surprisingly unexpected 24 hours incubation period with the growth rate of 4+, 4+, 3+ and 2+ respectively in comparison to control, Carr medium. Also this strain grew on modified Carr media with 8%, 9% and 10% ethanol after 48 hour's incubation period with the growth rate of 3+, 2+ and 1+ respectively.

TABLE III
GROWTH AND ACETIC ACID PRODUCTION OF ISOLATED BACTERIA AT 34°C AFTER 96 HOURS

Temperature	34°C		
Ethanol %	5	6	9
Growth	+2	+2	+2
Acid Production	+4	+4	+4

TABLE IV
GROWTH AND ACETIC ACID PRODUCTION OF ISOLATED BACTERIA AT 36°C AFTER 96 HOURS

Temperature	36°C		
Ethanol %	5	6	9
Growth	+1	+1	+1
Acid Production	+2	+2	+2

The results of the tolerance tests against ethanol shock are shown in Fig. 2. The cultivation of isolated strain in modified Carr media with 5%, 7% and 9% ethanol at unusual 34 ºC (Table III) and 36 ºC (Table IV) temperatures showed that in all ethanol concentrations after 24, 48 and 72 hours incubation at 34 ºC there was no growth observed but after 96 hours a 4+ growth rate was shown. Also in all ethanol concentrations after 24, 48 and 72 hours incubation at 36 ºC there was no growth observed but after 96 hours a +2 growth rate was shown.

The results of interactions between ethanol concentrations and critical temperatures and their relation with the growth rate of isolated *Acetobacter* strain from Iranian white – red cherry are indicated in Figs. 3 and 4.

IV. DISCUSSION

According to Kocher et al, vinegar can be produced using sugarcane juice and *Acetobacter* aceti at usual temperature, 30º C [3]. Gullo et al, Giudici et al and Falcone et al have showed that balsamic materials can be applied to produce traditional balsamic vinegar using modern fermentation methods with high quality and sensorial properties [7], [11] – [12]. Nanda et al have reported that rice vinegar (Komesu) and unpolished rice vinegar (kurosu) could be made using acetic acid bacteria and have isolated and characterized responsible strains [8]. Recently Kadere et al have isolated and identified *Acetobacter* and *Gluconobacter* genera from coconut. The isolated *Acetobacter* strain in that research has been cultured at 40 ºC but they have not reported pothentials of mentioned strain against ethanol stress [9].

In this research study, we isolated an *Acetobacter* native strain with high acetic acid productivity from Iranian white – red cherry, a delicious and favourable summer fruit that is very sensitive to decay by microorganisms. This strain showed 9.5% acetic acid production after one week incubation that is a very good characteristic in producing vinegar in a short period of time in comparison to vinegar manufacturing time of acetic acid bacteria that is 14 – 30 days routinely [ ]. Passage of the pure strains to industrial culture medium made a delicious cherry vinegar with a sweet smell and different nutritional properties nearby recent vinegar types. In addition to high acetic acid productivity, this *Acetobacter* strain was capable of tolerating 5% - 9% ethanol concentration shocks and high temperatures of 34 – 36 ºC simultaneously, that suggests a proper strain in the field of industrial microbiology and microbial biotechnology. The experiments showedthat, the speed and type of aeration are so important factors in growth and then acetic acid production by isolated *Acetobacter* strain from Iranian white – red cherry. The interaction effects of ethanol concentrations and temperature on growth and acetic acid production of this strain suggests that the concentration of ethanol influences the temperature tolerance of this isolate so that with the increase of ethanol concentration, the sensitivity of strain to high temperature is increased and the bacterium needs more time to adapt to new stress conditions i.e. the lag period of growth curve is increased.

In conclusion, this is the first report of *Acetobacter* isolation from a certain cherry, Iranian white – red cherry. This strain could be a potential strain for production of new vinegar type with a new and desirable taste, cherry vinegar, and could use the spoilage of this fruit as substrate to preserve the bioenvironment from food spoilage contaminations.

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