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ISOLATION AND IDENTIFICATION OF AN ACETOBACTER ISOLATE FROM CASHEW APPLE AS A POTENTIAL STRAIN FOR VINEGAR PRODUCTION

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ABSTRACT

Vinegar is defined as a sour liquid containing 4% solution of acetic acid 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.* 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 over oxidizer 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 cashew apple .We used two specific culture media include Carr medium and Frateur 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 °C, this strain was adapted for growth in 34 - 36°C after 96 hours incubation period. These dramatic characteristics suggest a potential strain in production of cashew vinegar with a sweet smell and different nutritional properties in comparison to other vinegar types. The lack of growth after 24, 48 and 72 hours incubation at 34 - 36 °C 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.

Key words: **Keywords:** *Acetobacter,* acetic acid bacteria, cashew apple juice, industrial fermentation, vinegar.

1. INTRODUCTION

Vinegar is defined as a 4% acetic acid solution that is originated from an alcoholic fermentation processing using sugary substances [Joyeux *etet al.*, 1984; Holt *etet al.*,1994]. Recently the vinegar industry has been developed to produce several vinegar types using various qualified native or engineered acetic acid bacteria [Drydale and Fleet 1985, Kocher *etet al.*, 2006]. 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 [Sokollek *et al.*, 1998, Brock *et al.*, 1994].

There are several genus in AAB group but *Gluconobacter sp.* and *Acetobacter sp.* are more discussed as bacteria that can produce acetic acid industrially.

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/1 is resulted in significantly increased growth rate (particularly at low ethanol concentration) above 20 g/1 acetic acid, however growth is severely restricted and virtually inhibited at an acetic acid concentration in the region of 50 g/1, whatever the amount of ethanol present [Nanda *etet al.*,2001]. There is no doubt that the presence or absence of oxygen greatly impacts the growth of acetic acid bacteria and in industrial vinegar fermentation. Most of acetic acid bacteria are mesophilic but recently a novel strain has been isolated which can tolerate up to 40°C; 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

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Acetobacter are mainly involved in vinegar production [Sokollek *etet al.,* 1998], [Kadere *etet al.,* 2008]. 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 [Joyeux *etet al.,* 1984 Drydale and Fleet 1985], [Kadere *etet al.,* 2008, Du Toit and Lambrechts, 2002]. *Acetobacter* strains have been isolated from several natural origins such as grape, date and palm resources and coconut [Kadere *etet al.,*2008] and have been applied for production of several vinegar types from various substrates e.g. sugarcane [Kocher *etet al.,*2006], rice [Nanda *etet al.,*2001] and balsam [Giudici and Rinaldi 2007,Falcone and Giudici 2008]. 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.

2. MATERIALS AND METHODS

2.1 Preparation of cashew apple juice extract

The spoilages of cashew apple juice were collected from several areas in Pandruti, Neyveli. The samples were placed in a safe cabinet with good ventilation and at room temperature for 10 days, 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 30° C for 4 days. The bottle was being examined every 24 hours according to alcoholic fermentation and sour smelling appearance.

2.2. *Isolation of bacterial strain*

For isolating bacterial strains that were responsible for vinegar smelling, 20 ml of cashew apple juice extract were transferred to sterile tubes aseptically and centrifuged at 3000 rpm for 5 minutes. The pellets were injected into culture mediums and incubated at 30° 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.

2.3. Culture media and ingredients

The culture mediums 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/1; $CaC0_3$, 20 g/1; ethanol, 20 g/1; agar, 20 g/1 and distilled water, 1000 ml] and modified Carr media with 4%, 5%, 6%, 7%, 8%, 9% and 10% ethanol were used [Holt *etet al.,*1994].

2.4. Characterization and biochemical tests

After isolation of acetic acid bacteria from cashew apple juice, macroscopic examinations, microscopic and morphological properties, gram reaction, oxidase and catalase tests, over oxidation and lactate utilization capability on Carr medium were investigated.

2.5. 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.

2.6. Bacterial growth

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

2.7. *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 30° C and after 24 and 48 hours incubation periods.

2.8. *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 °C and 36 °C, simultaneously.

3. RESULTS

The culture media after 24, 48, 72, 96, 120, 144 and 168 hours incubation at 30° 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 2.0%, 2.4%, 3.3%, 5.2%, 6.4%, 7.2%, 9.4% respectively as are shown in Fig. 1.

The bacteria were cultured from culture media to Carr medium and after 24 hours incubation at 30°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

Table.1 Growth and acetic acid production in differential ethanol concentration growth and acetic acid production in different ethanol concentration at 30° C after 24 hours

Time	Ethanol (%)	Growth	Acid production
24 hours	4	+4	+4
	5	+4	+4
	6	+3	+3
	7	+2	+2
	8	-	-
	9	-	-
	10	-	-

Table. 2 Growth and acetic acid production in different ethanol concentration at 30° C after 48 hours

Time	Ethanol (%)	Growth	Acid production
48 hours	4	+4	+4
	5	+4	+4
	6	+4	+4
	7	+4	+4
	8	+3	+3
	9	+2	+2
	10	+1	+1

Table. 3 Growth and acetic acid production of isolated bacteria at 34°C after 96 hours

Temp	Ethanol (%)	Growth	Acid production
34ºC	2	+2	+4
	6	+2	+4
	9	+2	+4

Table .4 Growths and Acetic acid Production of Isolated Bacteria at 36°C after 96 hours

Temp	Ethanol (%)	Growth	Acid production
36ºC	5	+1	+2
	6	+1	+2
	9	+1	+2

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). 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, Also this strain grew on modified Carr media with 8%, 9% and 10% ethanol after 48 hours incubation period with the growth rate of 3+, 2+ and 1+ respectively. 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 °C (Table III) and 36 °C (Table IV) temperatures showed that in all ethanol concentrations after 24, 48 and 72 hours incubation at 34°C 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°C 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 cashew apple juice are indicated in Figs. 3 and 4.

4. DISCUSSION

According to Kocher *etet al.*,vinegar can be produced using sugarcane juice and *Acetobacter aceti* at usual temperature, 30^{o}C [Kocher *etet al.*,2006]. [Gullo *etet al.*,Giudici *etet al.*,and Falcone *et al.*,2008] have I showed that balsamic materials can be applied to produce traditional balsamic vinegar using modern fermentation methods with high quality and sensorial properties [Gullo and Giudici 2008],

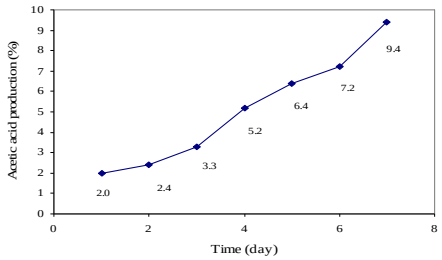

Fig. 1 The high acetic acid productivity of *Acetobacter* strain isolated from cashew apple juice after 24, 48, 72, 96,120,144 and 168 hours at 30° C and 120 RPM aeration speed

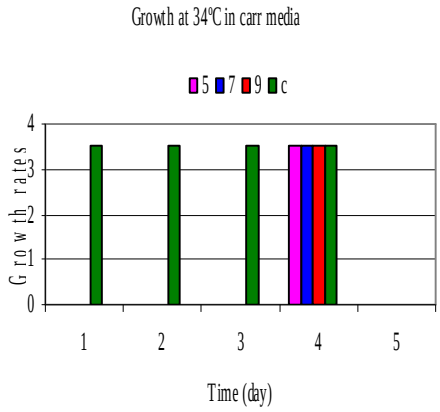

Fig. 3. The interaction of tolerance to ethanol shock and critical temperatures after 24, 48, 72 and 96 hours incubation at 34ºC in *Acetobacter* strain isolated from cashew apple juice. 5: ethanol 5%, 7: ethanol 7%, 9:

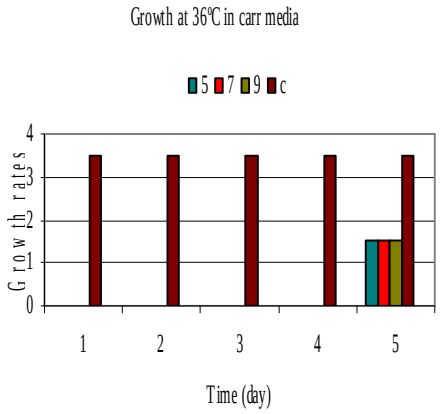

Fig. 4. The interaction of tolerance to ethanol shock and critical temperatures after 24, 48, 72 and 96 hours incubation at 36ºC in *Acetobacter* strain isolated from cashew apple juice. 5: ethanol 5%, 7: ethanol 7%, 9: ethanol 9% and C:

[Giudici and Rinaldi 2007, Falcone and Giudici 2008]. [Nanda *etet al.*,2001] 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 [Nanda *etet al.*,2001]. Recently [Kadere *etet al.*, 2008] have isolated, and identified *Acetobacter* and *Gluconobacter* genera from coconut. The isolated *Acetobacter* strain in that research has been cultured at 40 °C but they have not reported Potentials of mentioned strain against ethanol stress [Kadere *etet al.*,2008].

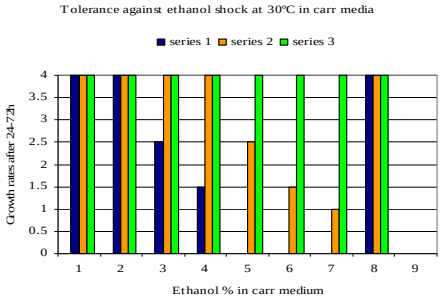

Fig. 2 The tolerance of *Acetobacter* strain isolated from cashew apple juice against increasing ethanol concentrations in modified Carr medium at 30ºC. Series1: after 24 hours, Series 2: after 48 hours and series 3: after 72 hours, C:

In this research study, we isolated an *Acetobacter* native strain with high acetic acid productivity from cashew apple juice, a delicious and favourable summer fruit that is very sensitive to decay by microorganisms. This strain showed 9.4% 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 culture medium made delicious cashew 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 °C simultaneously, that suggests a proper strain in the field of industrial microbiology and microbial biotechnology. The experiments showed that, the speed and type of aeration are so important factors in growth and then acetic acid production by isolated *Acetobacter* strain from cashew apple juice. 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 certain, cashew apple juice. This strain could be a potential strain for production of new vinegar type with a new and desirable taste, vinegar, and could use the spoilage of this fruit as substrate to preserve the bioenvironmental from food spoilage contaminations.

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