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Isolation and Identification of Acetic Acid Bacteria

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λAbstract. The aim of this study was to isolate and identify the acetic acid bacteria strains.The acetic acid bacteria were isolatedfrom the natural fermented broth of apricot dreg, Chinesedate vinegar and solid fermentation substrate of vinegar by the dilution plate method. The acid production was measured and the experiments ofbiochemical identification were conducted with each selected strain. The strains, which can produce highest acid content, were identified by 16Sr DNA sequence and by the phylogenetic tree. The results showed that 17strains of acetic acid bacteria were isolated. The acetic acid yield of the Ac11strain from the natural fermented broth of apricot dregcan reach 5.92 g/100mlwhenthe initialethanol content was 6%(v/v). The degree of similarity of 16SrDNA sequencewas more than 99% between Ac11 and *Acetobacterpomorum*strain LMG 18by NCBI (NationalCenter for Biotechnology Information).The Ac11strain was *Acetobacterpomorum* strain LMG 18848.The Ac11strain is a potential acetic acid bacteriafor application in apricot dregfruit vinegar production

Keywords: Acetic acid bacteria, identification, isolation

1. Introduction

Acetic acid bacteria is a large group of Gram-negative bacteria and strict aerobe,which has a marked feature thatwhen ethanol is exposed to air and under the function of vinegar bacteria, it can be converted into acetic acid and water(except *Asaia*). *Acetobacteraceae* belongs to *Proteobacteria, Alphaproteobacteria, Rhodospirillales*, which include *Acetobacter* and *Gluconobacterasia*. The oxidation of glucose is strongerfor *Gluconobacterasia*. Therefore, most acetic acid is produced by *Acetobacter*. Acetic acid bacteria are the main strains which can determine the yield and quality of vinegar [1]. *AS1.41(A.rancensL)* is a main strain in fruit vinegar production in China, but both itsability of acid production and itstolerance of alcohol are not strong. Besides, the fruit vinegar by *AS1.41* fermentation does nothave agood flavour. So it is important to isolate good strains for fruit vinegar.

The good acetic acid bacteria which can produce acid more than 20% are successfully isolated by multi-layer agar. The strains isolated from vinegar have been identifiedinto the genus *Acetobacter*, e.g. *Acetobacteraceti, Acetobacterhansenii, Acetobacterpasteurianus* and *Acetobacterxylinus*. In addition, Sievers et al. described *Acetobactereuropaeus* as a new species which represents the main flora component in industrial vinegar fermentation in Central Europe.The acetic acid bacteria isolated from vinegar fermented grains have tolerance under high temperature and high acid yield. Chenwei, Xiqing, et al. researchers conducted a pilot study of the separation of acetic acid bacteria, and obtained some better acetic acid bacteria. But it is not reported that the acetic acid bacteria fit for the apricot dreg vinegar. The aim of this study was to find better acetic acid bacteria especially forthe fermentation of apricot dreg vinegar.

2. Materials and Methods

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2.1. Materials

Source of acetic acid bacteria: natural fermented broth of apricot dreg, Chinese date vinegar and solid fermentation substrate of vinegar.Proliferation broth: 1% glucose, 1% yeast extract, 0.5% of 0.02% crystal violet solution, 5mg/ml Nysfungin (5×10^5 u), pH5.5, autoclaveat 0.1M Pafor 30min, cooling to 70℃, then 3%(v/v) ethanol was added. Calcium carbonate agar: 1% glucose, 1% yeast extract, 2% calcium carbonate, 3% enthanol, 1.8%agar powder, nature pH. Bromcresol purple agar: 1% glucose, 1% yeast extract,5% of 0.04% bromcresol purple, 2% agar, 100mldistilled water, autoclave at 0.1Mpa for 20min, cooling to 70℃, then 4%(v/v) ethanol was added. Fermentation broth:1% glucose, 1% yeast extract, 0.05%KH_2PO_4,0.05%$MgSO_4$,6% (v/v)ethanol.

2.2. Methods

2.2.1. The Proliferation of acetic acid bacteria

10g natural fermentation broth of apricot dreg was put in a triangle flask (250 ml) with 90 ml proliferation broth and was incubated for 3d at 30 ℃and 110revolutions per minute (r/min). The samples of Chinese date vinegar and solid fermentation substrate of vinegar were the same treated as above. The proliferation culture solution was obtained.

2.2.2. The qualitative test of acetic acid bacteria

10ml proliferation culture solution was centrifuged at 3000r/min for 5 min, 5mlsupernatant was obtained and adjusted to pH7.0 by drop wise adding 4% Na OH solution, and boiled. Then, 5-7 droplets of 5% ferric chloride solution was put into the supernatant. It would prove acetic acid bacteria existing if red brown appeared.

2.2.3. The isolation of acetic acid bacteria

The proliferation culture solution wasserially dilutedin 0.85% saline. 250uL of both $10^{-6}$and 10^{-7}dilution were taken to spread on calcium carbonate agar and bromcresol purple agar. The single colonies which produced big transparent circle or color-changed circlewere selected to conduct further purification after incubation for 48hat 30℃.

3. The determination of acetic acid yield

Every purified stain of acetic acid bacteria was inoculated in 500ml triangle flask (250ml) with fermentation broth(200 ml) at 30℃ and 110r/min for 30d. Each of the acid yields was determined per 2d, and the acid yield curve was conducted. Titration acid yield was determined as indicator as follows: two or three droplets 1% phenolphthalein reagent was put into a triangle flask (100 ml) with both 2ml culture solution and 50ml distilled water. Pink appeared when 0.1 mol/l NaOH solution was added, then the acid yield representedby acetic acid.

The formula of acetic acid yield (g/L) = $\frac{(V - V_0) \times C_{NaOH}}{V\text{sample}} \times 60$

V represented the consumption of 0.1 mol/lNaOH solution for fermented broth sample.

V_0 represented the consumption of 0.1 mol/lNaOH solution for distilled water.

V_{sample} represented the volume of fermented broth sample.

4. Theresults of the physiological and biochemical identification

According to Bergey's Manual of Determinative Bacteriology (Edit 8^{th}) and Common Bacteria Identification System, the experiments which are contact enzyme test, glycerin ketone test, 5-ketone glucose acid salt form test, glucose acid salt test, ethanol oxidation test, cellulose production test, water-soluble pigments test, acetic acid calcium oxide test, starch hydrolysis test and calcium lactate test were conducted with 7 strainsacetic acid bacteria which can produce higher acid content.

5. The DNA extract and 16S r DNA PCR enlargement

The acetic acid bacteria which had higher acid yield ability were chosen.The test followed the protocol with Bacteria Genomic DNA Kit(Beijing Zoman Biotechnology Co:LTD).

The sequence of prime 27F was 5′-AGAGTTTGATCCTGGCTCAG-3′. The sequence of prime 1492 was 5′-GGTTACCTTGTTACGACTT-3. 25uL PCR enlargement system was as follow, 2.5uL10×buffer (include Mg^{2+}), 2uL 2.5mmol/L dNTP, 1uL Template, 0.5uL 10mmol/L prime 27F and prime 1492R, 1uL 5U/uLTaq DNA polymerase, 17.5uLdouble distilled water. PCR amplification program was as follow, degeneration 5 min at 94 ℃, degeneration 30 s at 94 ℃, annealing 30 s at 55 ℃, extensions 1 min at 72 ℃, it was 30 circulation from degeneration to extend, at last extensions 7 min at 72 ℃, heat preservation for 4 min. The PCR product went through electrophoresis with 1% agarose. After EB dyeing it would test by fluorescence-visible gel imaging analysis system.

6. The building of the phylogenetic tree

16S rDNA amplification was entrusted to Beijing Huada Gene Product Research Centre to sequencing, after the sequence was put into NCBI.

The phylogenetic tree of the acetic acid bacteria sequence was established with 16S rDNA which represented the related species in Gene bank by the software DNAMAN and DNA Star. The position of the isolated strains in the development process was more accurately judged through this way.

7. Results and Discussion

7.1. The results of qualitative test of acetic acid bacteria and the isolated acetic acid bacteria

The test proved that the acetic acid bacteria existed because red-brown precipitation appeared.

17strains which were bacillus or elliptic bacteria were isolated.2 strains from the natural fermented broth of apricot dregnamed Ax1 and Ac11. 4 strains from Chinese date vinegar named Az1, Az2, Az3 and Az4. 11 strains from the solid fermentation substrate of vinegar named Ac1, Ac2, Ac3, Ac4, Ac5, Ac6, Ac7, Ac8, Ac9, Ac10 and Ac12. They could produce transparent circle on calcium carbonate plate and make bromcresol purple plate yellow. The morphology of colonies was as follows: tidy edge, smooth surface, milky white or yellow colour, and raised round colonies with 0.5-1.0mm diameter.

It was known that the bacteria which produced transparent circle were Gram-negative bacteria. Individual characteristics of isolated bacteria were as follows: long rod shape, middle rodshape, short rodshape and elliptical shape. They presented as single, a couple, chain or alignment.

7.2. The determination results of acid content

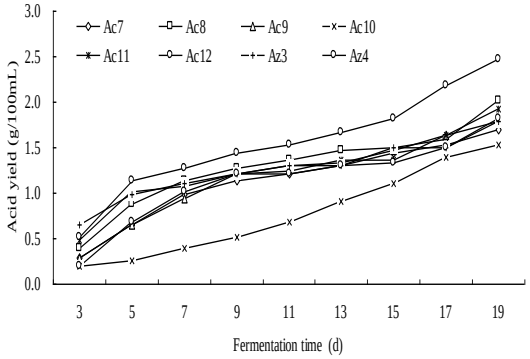


Fig. 1: Acid yield curve of eight strains of acetic acidbacteria

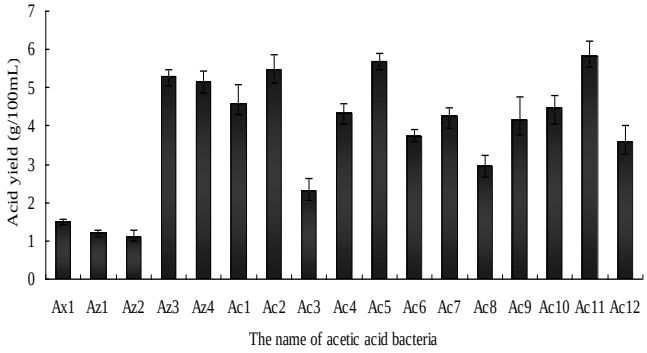


Fig. 2: Acid yields of seventeen strains of acetic acid bacteria

The acid yield of eight strains of acetic acid bacteria increased with the extension of fermentation time from the Fig1. The acid yield curve of 7 strains of acetic acid bacteria was similar except Ac10.The acid yield of Ac10 increased with the extension of fermentation time, but the acid yield was lower than the others. The acid yield of Az3, Az4, Ac2, Ac5 and Ac11 was more than 5g/100mlfrom the Fig2, and the acid yield of Ac11 was 5.92g/100ml. Its acid yield was highest among the other strains.

7.3. The results of physiological and biochemical identification

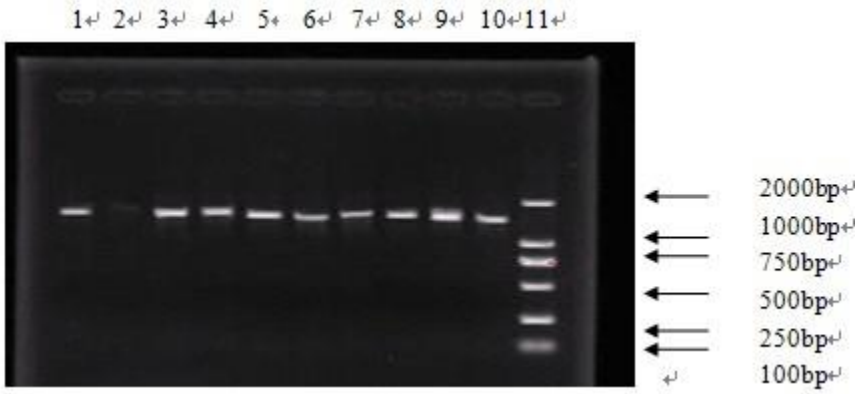
The results of physiological and biochemical identification were known as Table 1. According to the common bacteria system identification manual, Ac1, Ac2, Ac7, Ac9, Ac10, Ac11 and Az3 belonged to *Acetobacter*. According to Bergey's Manual of Determinative Bacteriology, initially determined bacteria Ac2, Ac7 and Ac9 could belong to *Acetobacteraceti*. But Ac1, Ac10, Ac11 and Az3 could not be determined. These 7 strains need to be further identified.

Table 1: Results of physiological and biochemical identification

Name	contact enzyme	glycerin ketone	5-ketone glucose acid salt form	glucose acid salt	ethanol oxidation	cellulose production	water-soluble pigments	acetic acid calcium oxide	starch hydrolysis	calcium lactate
Ac1	+	+	+	-	+	-	-	+	-	+
Ac2	+	+	+	+	+	+	-	+	-	-
Ac7	+	+	+	+	+	+	-	+	-	+
Ac9	+	+	+	+	+	+	-	+	-	+
Ac10	+	+	+	+	+	-	-	+	-	+
Ac11	+	+	-	+	+	-	-	+	-	+
Az3	+	-	+	+	+	-	-	+	-	+

7.4. 16 S rDNA analysis results

8. The Agarose Gel Electrophoresis Figure.



(Note: In this figure, 1 and 8 was Ac1, 2 was Ac2, 3 was Ac4, 4 and 9 was Ac5, 5 was Ac10, 6 and 10 was Ac11, 7 was Az3, and 11 was Mark.)

Fig. 3: Agarose gel electrophoresis of seven strains of acetic acid bacteria

It is known that the length of acetic acid bacteria 16S rDNA was about 1600bp-1800bp fromthe Fig 3.

9. The Sequence Results of 16S rDNA

The sequence of 16S rDNAstrainscompared with the 16S rDNA sequences of NCBI database,the higher similarity strainsof Ac10 and Ac11was selected. The evolutionary tree of both Ac10 and Ac11 was found by adjacency method of DNAstar and DNAMAN.

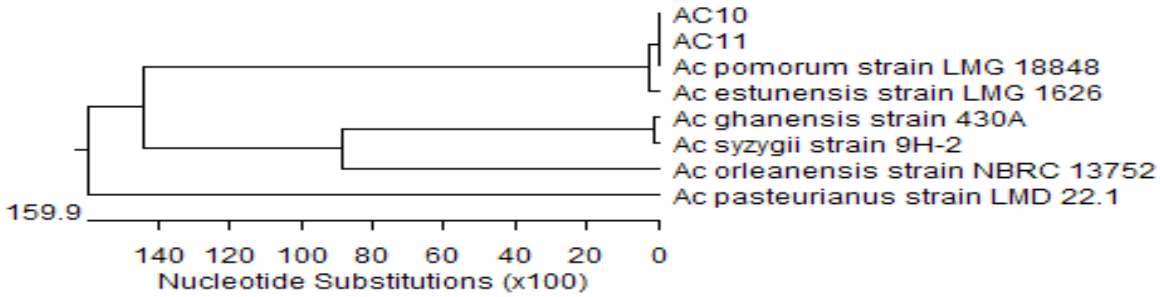
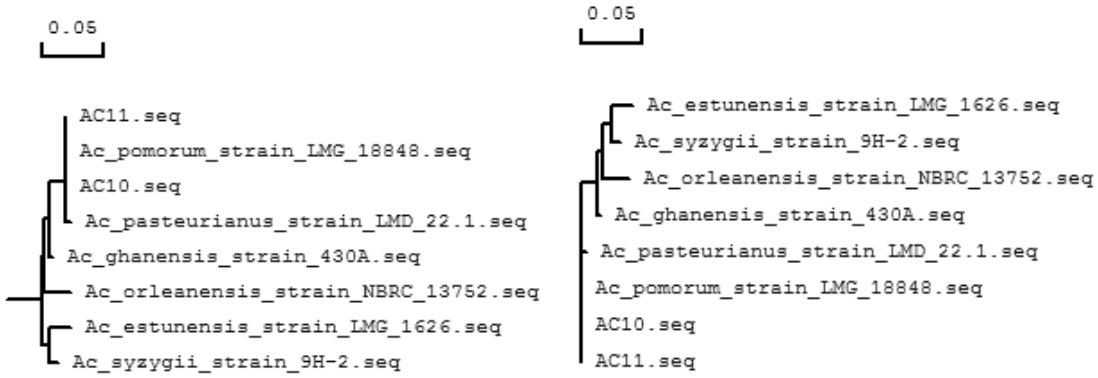


Fig. 4: Phylogenetic trees of acetic acid bacteria Ac11 and Ac10by DNAstar



The rooted tree Theunrooted tree

Fig. 5: Evolutionary tree of Ac10 and Ac11 by DNAMAN

The similarity degree of both Ac11 and Ac10reached more than 99% compared with *Acetobacterpomorum* strain LMG 18848 from Fig 6. The evolution position of both Ac11 and Ac10 was the same compared with *Acetobacterpomorum*strain LMG 18848. So Ac11 belong to *Acetobacterpomorum* strain LMG 18848 [2], [3]. This strain was reported by Cleenwerck I in 2002 [4] and Wong CN in 2011[5].

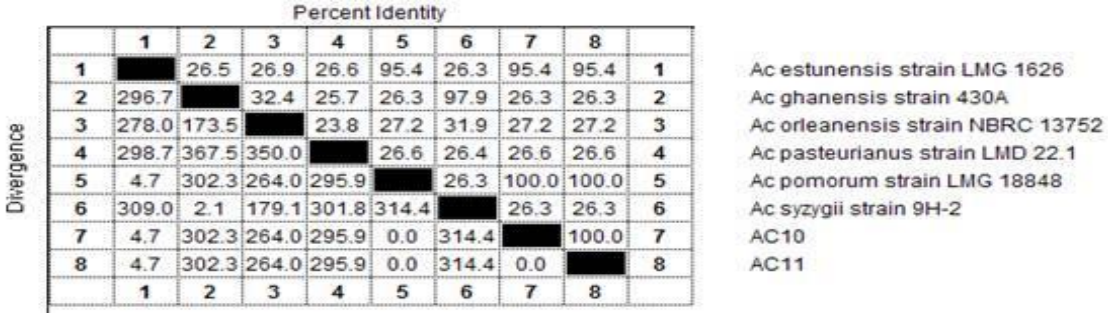


Fig. 6: 16S rDNA of Ac11 andAc10 homology analysis of acetic acid bacteria

10.Conclusion

17 strains acetic acid bacteria were isolated from natural fermented broth of apricot dreg, Chinese date vinegar and solid fermentation substrate of vinegar. The acetic acid yield ofAc11straincan reach 5.92g/100mlwhen initial alcohol content was 6% (v/v). Ac11 strain belongs to *Acetobacterpomorum* strain LMG 18848. The strain of Ac11 is one of the potential acetic acid bacteria in apricot dreg vinegar.

11.Acknowledgements

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12.References

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