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Isolation of *Acetobacter persici* DS1 from over-ripened sapodilla fruit as a potential strain for fruit vinegar production

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ABSTRACT

The objective of this study was to isolate and identify the acetic acid bacteria (AAB) from over-ripened fruit Sapodilla fruit. The isolated strain was identified by using 16S rRNA sequencing, and strain was designated as *Acetobacter persici* DS1. The selected strains were utilized to formulate fruit vinegar using two-stage fermentation process using locally obtainable raw fruit processing waste. The strain was found to be a potent strain which can withstand high initial ethanol concentrations and accumulating acetic acid. It is observed that the increase in optical density during the ethanol tolerance or resistance up to 5.5 % (v/v) initial ethanol concentrations and accumulating acetic acid in GYC broth was observed as ethanol and aceto-tolerant strains. The strain *A. persici* DS1 have substantial growth up to 5.5% (v/v). The strain's maximum enzyme activity was seen at a 10% acetic acid concentration. ADH and ALDH activity of *A. persici* DS1 were 2.93U mg^{-1} and 3.52U mg^{-1}, respectively. According to the final data the acetic acid producing strain *A. persici* DS1 isolated from sapodilla indicated that the highest acetic acid production 4.63 g/100ml with absolute ethanol and 3.82 g/100ml with distilled ethanol. Therefore, the obtained potential AAB strain can be used for fruit vinegar fermentation at high titration. It can be concluded that the studied strain is well suited to be used as a parental strain to prepare a starter for fruit vinegar production.

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Introduction

Louis Pasteur first described AAB as "vinegar bacteria" over 150 years ago. AAB is best known for producing acetic acid, also known as ethanolic acid, or CH_3COOH, during vinegar production (Pasteur 1864).

AAB are various set of microorganisms found in plant assets like fruits and flowers and used in production of alcoholic and non-alcoholic beverages (Montet & Ray 2016). They have the capacity to make use of a variety of carbon substrate as energy sources including sugar and alcohol. AAB are recognized for their capacity to directly oxidize alcohols, sugars and carbs, collecting vast quantities of the resulting oxidation products while obtaining metabolic energy. The "oxidative fermentation" is a crucial aspect of AAB metabolism (Matsushita & Matsutani 2016).

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AAB strains from diverse ecosystems or niches have varying nutritional needs, the nutrient medium for the isolation and development of AAB might differ, primarily in the forms of carbon sources used. Fruit juices have historically been utilized in a two-step process for both domestic and industrial manufacturing of vinegar with fermentation of alcohol from the fruit sugars by yeasts, generally *Saccharomyces cerevisiae* as the first step and formation of acetic acid by AAB from this ethanol as the second step (Subramanian & Suresh 2025 a,b)

AAB are important bacteria in vinegar fermentation as they can oxidize ethanol quickly and partly to acetic acid. Vinegar is not only used to flavor fruit, but it is also used for its neutraceuticals properties. This beverage contains anti-obesity, antibacterial, antihypertensive, anti-oxidative and anti-diabetic properties. Bioactive substances such as polyphenols and micronutrients are responsible for these beneficial qualities (Es-sbata et al., 2021).

The work and applications of AAB have a prolonged past in fermentation industry and the fermentation process. The manufacturing of vinegar, which is made by converting ethanol to acetic acid, is the standard application of AAB. However, AAB are used industrially to create other important substances, principally ascorbic acid (vitamin C), miglitol (Shinjoh & Toyama 2016) and partially oxidized a range of alcohols and carbohydrates to harvest organic acids as the end product (González & Mas 2011). *Acetobacter* creates a cellulose-based film or pellicle in liquid medium. The cellulose pellicle entangles the acetic acid bacteria and yeasts in the fermentation broth, forming the "mother of vinegar," a mat-like structure (Mathew et al., 2019). Bacterial cellulose and acetan are the most lucrative exo-polysaccharides (EPS) produced by several AAB species. Bacterial cellulose has various benefits over plant-derived cellulose, including the absence of hemicellulose and lignin present in cellulose. They are also crucial to cocoa fermentation (Pothakos et al., 2016). *Acetobacter* species are significant acetic acid bacteria that are frequently used in industrialized operations, including the manufacturing of vinegar (Ndoye et al., 2007). On the basis of recent developments and taxonomic revisions, which include the gradual emergence of new species and the characterization of new genera from a taxonomic perspective, 19 genera of AAB have been identified as of 2021 (Garcia et al., 2023).

Sapodilla, often referred to as *Manilkara zapota*, is the Sapotaceae family's most popular and widely cultivated fruit. Sapodilla has a variety of bioactive compounds, the bulk of which consist of depsides, phenolic acid, ellagitannins, gallotannins, and flavonoids (anthocyanins and flavanols). It is an abundant supply of minerals (potassium, calcium and iron), carbohydrates, proteins, and amino acids, among other nutrients (Bangar et al., 2022). Sapodilla fruit is rich in nutrients because water makes approximately 73% of its weight. Additionally present are bioactive compounds (antioxidants and polyphenols), minerals (copper, potassium, and iron), vitamins (Retinol, ascorbic acid, folate, niacin, and pantothenic acid), sugars, ascorbic acid, and fat (Kaur et al., 2020). It is believed that simple sugars including glucose, fructose and sucrose are thought to be responsible for the fruit's sweetness (Tulloch et al., 2020).

AAB are selective organisms, and strains have been observed to exhibit a number of characteristics during sub-culturing, including the capacity to generate greater amounts of acetic acid (Mas et al., 2014). Under aerobic conditions, several other bacterial species can also oxidize ethanol but, in highly acidic conditions, they cannot. Two consecutive catalytic processes are used by AAB strains to oxidize ethanol to acetic acid in highly acidic atmosphere.

First, membrane-bound alcohol dehydrogenase (ADH) relies on pyrroloquinoline quinone (PQQ) catalyses the oxidation of ethanol to acetaldehyde. Then, (ALDH) Aldehyde dehydrogenasebound to membrane, it is located near ADH, quickly converts the produced acetaldehyde to acetate. Since no aldehyde is released during alcohol oxidation process, the bacterial membrane contains a multi-enzyme complex made up of ADH and ALDH that operates in succession to create acetic acid from ethanol (Adachi et al., 2007). The producing acetic acid is discharged into the growing media, where it collects up in *Acetobacter* species to 5% to 10% (Andres et al., 2016).

Most typically, submerged bioreactors are used to manufacture alcohol and wine vinegars. These reactors provide the bacteria with a steady supply of oxygen, enabling an effective manufacturing process (Callejon et al., 2009). The "mother of vinegar," which is made from remaining vinegar, is added to the newly fermented mixture to initiate the oxidation process. There is variance in the quality of the products produced because this culture is not well defined microbiologically and there are not many comprehensive research’s about the acetic acid bacteria and other microorganisms in such cell cultures. Furthermore, the majority of procedures take at least three weeks to yield an acetic acid concentration that is sufficient. Therefore, the necessity for readily available "starter cultures" is critical to maintaining product quality and reducing fermentation time (Stornik et al., 2016).

Comprehensive research on the use of omic tools for the identification of vinegar microbiota, primarily acetic acid bacteria, is reviewed. They were making it possible to overcome many of the conventional challenges for the isolation and characterization of microorganisms that live in these media in the case of vinegar (Román et

al., 2024). In order to produce a process including efficient starter culture for fruit vinegar fermentation which meets technological traits, it was essential to select endogenous strains from over ripened fruits habitats. The key objective of the present study was to select potent AAB strains from environmental samples to exploit the fruit waste generated from agro-industries processing and to obtain endogenous strains that can be utilized to the fermentation of local substrates for vinegar fermentation.

The study aims to fill the knowledge gap regarding the selective optimization of high-yield AAB strains for industrial use. Although it is known that a number of AAB strains can produce acetic acid, little is known about how to isolate and characterize hyper-producing strains that are more resilient to cultural stress like high acidity, temperature changes, and ethanol concentrations.

Materials and Methods

Sample collection and transportation

Over ripened Sapodilla fruits were randomly collected from local market of Jaipur (27.1330° N, 75.9567° E), Rajasthan, INDIA. The samples were transported in pre-sterilized zip bags to the Amity Institute of Microbial Technology, Amity University, Rajasthan, Jaipur, under cold chain were kept at 4°C and stored there until they were essential for analysis. All the samples were collected during the study complied with local or national guidelines.

Isolation of Acetic Acid Bacteria

In order to isolate *Acetobacter* strain from the sample of overripe sapodilla fruit were crushed and homogenized with the enrichment medium composed of (1% peptone (w/v), 1% Yeast extract (w/v), 1% Glucose (w/v) with 1% fruit wine 9 (w/v)) and incubated for 24 h at 28 °C. After incubation 0.9% of fruit wine was again supplemented. Furthermore, the sample was diluted serially with saline solution and cultured on two different media Glucose-yeast extract- calcium carbonate agar (GYC) media (5% Glucose(w/v), 1% yeast extract (w/v), 0.5% $CaCO_3$ (w/v), 2% agar (w/v)) and Carr media (yeast extract 3% (w/v), ethanol 2% (w/v), bromocresol green 0.002% (w/v) and agar 2% (w/v)). All the chemicals and constituents were used are of analytical reagent grade and procured from Himedia, India Further, culture plates were aerobically incubated for 72 h at 30 °C. The colonies which produced the zone of clearance on GYC agar media plates were selected and further purified on GYC agar plates. The isolated pure strain were preserved using glycerol stock (30% v/v) at -80 °C and GYC agar slants at 5 °C till further use.

Identification of AAB Isolates

Several physiochemical and biochemical analyses, including gram staining, oxidase, catalase, nitrate reduction, and motility testing, were used to identify the isolated AAB strain. Briefly, the oxidase test was performed using Kovacs oxidase reagent applied for oxidase test (Aryal 2022). Briefly, the catalase test was completed using 3% solution of Hydrogen Peroxide (Aryal 2018). Concisely, the motility test was completed using GYC agar medium and observed for a diffuse zone of growth splaying out from the line of inoculation to determine whether an organism is motile or non-motile. Nitrate reduction test was carried out using nitrate broth (0.5% peptone (w/v), 0.3% meat extract (w/v) and 0.1% potassium nitrate (w/v) at pH-7). The strain was inoculated into the media and incubated at 28 °C - 30 °C for 24 h. Further the tubes were mixed with 6 to 8 drop of reagent-A (sulfanilic acid) and reagent-B (alphd-napthylamine) and observe for color development. If color development is not occur then added reagent-C (zinc powder) and observe for 3 min minimum. This method is mentioned by (Buxton 2011).

Molecular Identification

The genomic DNA was extracted and amplified for molecular identification by using 16SrRNA sequencing. The 16SrRNA universal primer was used i.e., forward primer 5′AGAGTTTGATCCTGGCTCAG 3′ and reverse primer 5′ACGGCTACCTTGTTACGAC3′. Sequences were submitted into GenBank and given an accession number.

Phylogenetic tree construction

ClustalW was used to align the sequences, and de-fault settings were applied. Using the MEGA analytic program version 11.5 to generate 1000 boot-strap repeats, UPGMA clustering based on the Dayhoff PAM250 matrix was used to infer phylogenetic relationship with statistical support (Tamura et al., 2021).

Growth analysis under different culture conditions

Growth profile of selected bacterial strain

The bacterial strain were inoculated in GYC broth medium and set the pH at 5.5. For culture medium conditions, three variables were taken into consideration: temperature, shaking conditions, and composition of the culture media. Calculate the absorbance of culture medium at different time intervals in the terms of optical density at 600nm.

Ethanol tolerance

The ethanol tolerance of isolated strain was evaluated as described by (Yuan et al., 2013). The method was

adopted and slightly modified for the determination of ethanol tolerance. The GYC broth media was inoculated with 10% (v/v) inoculum supplemented with different concentrations of ethanol ranging between 0.5% (v/v) - 10% (v/v) and incubated for 24 h at 30 °C. After incubation, all the test tubes were observed and optical density (OD) at 600nm was recorded from all different concentrations.

Tolerance of acetic acid

The acetic acid tolerance of isolated strain was evaluated as described by (Kourouma et al., 2022). The method was adopted and slightly modified for the determination of acetic acid tolerance. 10% (v/v) inoculum was mixed with GYC broth in the presence of diverse acetic acid concentrations extending between 0.5% (v/v) to 5.5% (v/v), after that, incubated for 24 h at 30 °C. Subsequently, all the test tubes were observed and optical density (OD) at 600nm was recorded from all different concentrations.

Determination of ADH and ALDH enzyme activity

The isolate was inoculated with the growth medium known as YG2 medium. The composition of YG2 medium is (0.51% Glucose (w/v), 0.05% KH_2PO_4 (w/v), 2.62% yeast extract (w/v), 0.05% NaH_2PO_4 (w/v), 0.05% $MgSO_4$ (w/v) and 5% Edible alcohol (w/v) in 1L Distilled water) suitable for the growth of isolate. The experiment involved different ethanol concentration (2%, 4%, 6%, 8% and 10% (v/v)) in the YG2 media.

In addition to varying ethanol concentrations, the experiment also tested different acetic acid concentration ranging from (2%, 4%, 6%, 8% and 10% v/v) for 36 h at 32 °C and 150 rpm. To harvest cells, cultures were centrifuged at 4 °C for 10 min at 8000×g. The sample was re-suspended in the same buffer after being twice washed with Phosphate buffer saline 0.1M, pH 7.0 (3 ml of buffer is needed for 1g of wet bacteria) under the similar centrifugal conditions. The cell was lysed under ice-water bath for 10 min. To obtain the supernatant, the fluid was centrifuged again at 4 °C for 30 minute at 10,000 ×g (Wu et al., 2018).

Using an enzyme-catalyzed substrate dehydrogenase and potassium ferricyanide as an electron acceptor, the quantities of ADH and ALDH were determined (Adachi et al., 1978). One unit of enzyme activity was defined as the quantity of enzyme needed to catalyze the oxidation of 1 μmol of substrate per minute. ADH and ALDH activity assay used the supernatant fraction rather than pure enzyme. Every enzyme activity determination was carried out at 25 °C. Using bovine serum albumin as a reference protein, the modified Lowry technique was used to define the concentration of protein (Wu et al., 2018).

Acetic acid production

Peptone water was used to dilute the chosen colonies from the GYC broth until an OD600 of 0.5 was reached. To produce acetic acid, 4% of the inoculum size from the previous GYC broth was cultivated in YGEA medium. The medium at rotating shaker incubated and sample was taken at 48 h intervals (Sharafi et al., 2010).

Acetic acid Estimation

AAB culture (5 ml), distilled water (20 ml) and three to five drops of phenolphthalein indicator were combined. A 0.5N NaOH titration was performed on the solution. The following formula was used to determine how much acetic acid was formed in 100 ml of the medium (Maal & Shafiee 2009).

Acetic acid (g/100ml) = Volume of NaOH (ml) used in titration × 0.06 / Volume of sample (ml).

Whole genome analysis

The isolate was deposited for whole genome sequencing and the draft genome was subjected for gene prediction using RAST (Rapid Annotation using Subsystem Technology) server. The RAST server is a bioinformatics tool for gene annotation and prediction, particularly in microbial genomes. The web-based service RAST automates the process of annotating the genomes of viruses, bacteria, and archaea by predicting genes and assigning functional annotations based on predefined functional systems (Aziz et al., 2008).

Result and discussion

Identification of the isolated strains

Thirty bacterial isolates were obtained from ripened sapodilla, collected from local market of Jaipur (27.1330° N, 75.9567° E), Rajasthan, INDIA. For further analysis, every isolates that indicate a transparent region on GYC agar was chosen. Primary screening was carried out by inoculating the isolates on GYC agar supplemented with CaCO3 as a selection factor in order to determine the acetic acid production of each isolate (Fig.1). The diameter of the clear zones on the GYC agar is associated with each isolate's rate of acidity. The phenotypic and biochemical trait of the selected isolate (DS1) was determined according to the Bergy's Manual (Ley 1984). The isolate was found to be non-motile, oxidase negative, rod-shaped, gram-negative, and catalase positive (Table 1).

It does have been evident that various types of fruits were processed for the isolation of acetic acid bacteria such as apple, mango, pineapple, banana, and grapes *etc.* (Buddhika et al., 2021). In their findings, 43 different bacterial strains from 43 different samples were isolated.

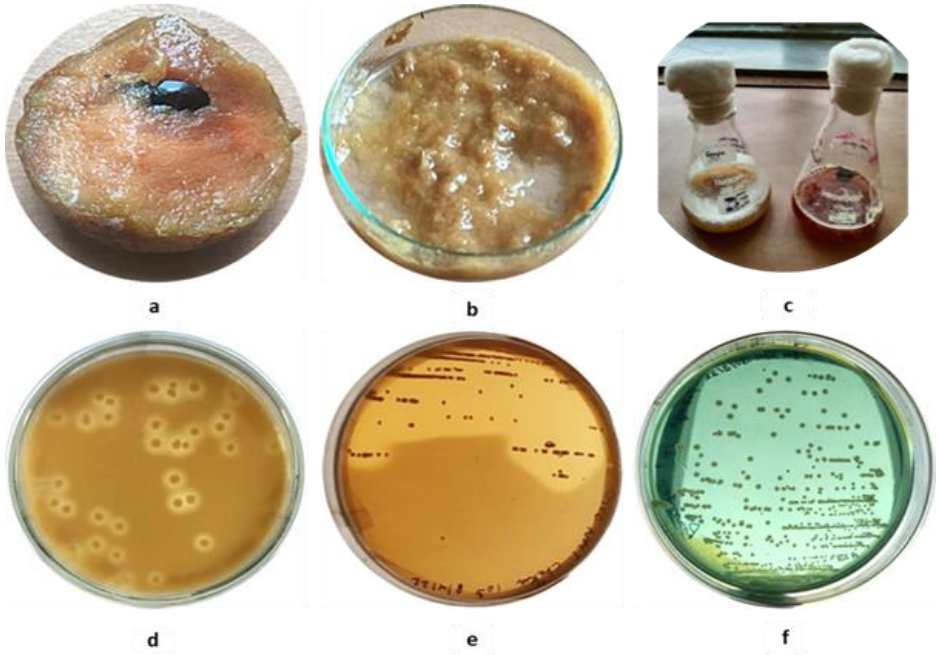

Fig 1. (a) Sapodilla fruit, (b) Chopped sapodilla, (c) Mixed with enrichment medium and fruit wine, (d) Isolates showing colonies on GYC agar medium by spreading method, (e) Streaking on GYC medium and (f) Screening on Carr medium.

Table 1 Morphological, physiological, and biochemical characteristics of bacterial isolates obtained in this study for probable genus identification.

Tested parameters	Bacterial isolate
Shape	Rod
Gram reaction	-
Spore	-
Motility	-
Growth on two different media	
GYC media plates	+
Carr media plates	+
Growth at pH 5.5	+
Growth at different temperature	
25°C	+
30°C	+
Oxidase reaction	-
Catalase reaction	+
Nitrate reduction	-
Alcohol tolerance (v/v)	
2%	+
4%	+
6%	+
8%	-
10%	-
Acetic Acid tolerance test (v/v)	
2%	+
4%	+
5.5%	+
6%	-
8%	-
10%	-
***Probable genus**	*Acetobacter*

It was discovered that every bacterial strain was rod-shaped, catalase positive and gram negative. Other studies also isolated AAB from fruit, fermented fruit juice, red grape, cantaloupe, longan, longkong, papaya, and strawberry. All of the isolates belonged to the AAB family and were all rod-shaped, gram negative and catalase positive (Klawpiyapamornkun et al., 2015). It has been observed that over ripened fruits are harboring various acid producing bacteria (Es-sbata et al., 2021). Isolation of AAB and their potential to produce the organic acids were determine using solidified GYC agar plates and can be confirmed on the basis of clear zone against $CaCO_3$ opaque milky background. Similar type of methodology was adopted by (Kim et al., 2023) for the isolation of competent organic acid producing bacteria results in formation of clear zone surrounding all the colonies. On GYC agar medium, potential acetic acid bacteria were examined from several sources. The GYC agar was used for the isolation of different strain of AAB and *Gluconobacter* sp., for example *Acetobacter aceti*, *Acetobacter pasteurianus*, *Acetobacter orleansis*, *Acetobacter cibinongensis*, and *Gluconobacter* sp. (Arifuzzaman et al., 2014). All the biochemical identification indicated that the isolated strain is probably AAB strain as submitted previously by (Gullo et al., 2006). According to other studies the genus *Acetobacter* showed oxidase negative, catalase positive, motile or non-motile with peritrichous flagella (Maal & Shafiee 2010). This is proved that the outcomes in the current investigation the bacterial isolates could be *Acetobacter* sp.

The colonies with significant transparent zone for acetic acid production were chosen for molecular identification. Isolate DS1 is most closely related to *Acetobacter persici* according to molecular identification based on 16SrRNA sequencing, and a phylogenetic tree was created (Fig. 2). The consensus gene sequence that was obtained was added to GenBank under accession number ON724153. MEGA version 6.0 software was used to generate phylogenetic trees and multiple sequence alignments. According to their evolutionary divergence, the nominated strains similarity to other AAB species was examined (Es-sbata et al., 2021). Some other research, based on 16SrRNA gene sequencing analysis, the chosen strains had over 99% genotypic relationship. The samples' classifications and the relative relationships within the *Acetobacteraceae* family were made evident by the phylogenetic tree that was created by using the sequences.

Vinegar manufacturing makes extensive use of the two species *A. pasteurianus* and *A. cerevisiae* that are found here. *A. persici* has potential uses in specialized markets linked to fruit-based fermentation, despite not being as widely used in industry as *A. aceti* or *A. pasteurianus*. Additionally, it can be used as a starter culture to produce some vinegars and fruit-based fermented products, as well as to ferment fruit juices (Yuan et al., 2024).

AAB identification with 16S rRNA using the NCBI database's with basic local alignment search tool (LAS) (Kim et al., 2023). Furthermore, the acetic acid bacteria identified by PCR-RFLP of the 16S rRNA also PCR-RFLP of the 16S-23SrRNA intergenic spacer permitted species-level categorization. 16S rRNA PCR-RFLP was validated by 16S-23S rRNA PCR-RFLP (Gerard et al., 2020).

Growth profile of selected bacterial strain

The result of bacterial growth profile in terms of optical density or percentage absorbance at 600nm showed a characteristic growth curve for bacteria contains 4 distinct stages: the lag phase, the exponential phase, the stationary phase, and the death phase. The culture was incubated at 30 °C in rotating incubator. In this study, lag phase began on day 2, at 48 hour of incubation time, than log phase began on day 3 at 60 to 72 h of incubation period. Therefore, the log phase shifted to stationary phase which can be seen on day 4 to day 5, between 72 to 92 h of incubation, death phase occurs on day 5, after 98 h of incubation period (Fig. 3). It is recognized that the majority of AAB are mesophilic, as they exhibit their best growth at 30 °C. But some *Acetobacter* strains and *Gluconobacter* strains can mature at temperatures as high as 40 °C. These strains are recognized as thermo-tolerant AAB strains helpful for oxidative fermentation in tropical environments as stated by (Saeki et al., 1997).

Tolerance with Ethanol Concentration

The ethanol tolerance ability of the strain was investigated on GYC broth media supplemented with different ethanol concentrations ranging between 1% (v/v) to 10% (v/v), and the mixture was then incubated for 48 hours at 30 °C. As seen by figure 4(a), the strain significantly increased its alcohol content up to 5.5% (v/v). The ethanol resistivity dropped when the alcohol content increased from 6% to 10%. The strain was considered as alcohol-tolerant. It seen observed that the increase in optical density during the ethanol tolerance or resistance up to 5.5 % (v/v) initial ethanol concentrations in GYC broth was observed as ethanol-tolerant strains (Fig. 4a). This suggested that an initial ethanol concentration that is gradually increased (from 1% to 10%, (v/v)) might be used as a useful isolating stress to screen for AAB strains that are ethanol-tolerant.

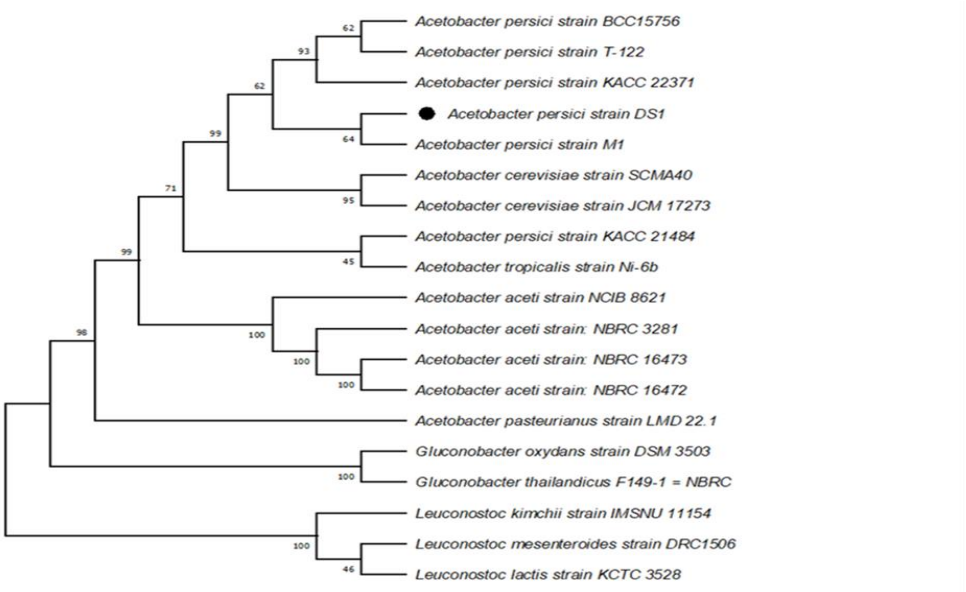

Fig 2. Phylogenetic tree using Mega (11.5v) software of the bacterial strain *Acetobacter persici* DS1.

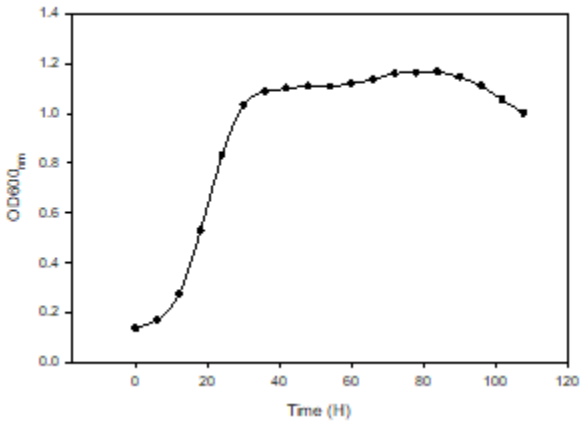

Fig 3. The growth profile of *Acetobacter persici* DS1 by percentage absorbance method (OD 600nm)**.**

According to few reports, the AAB strains have potential ethanol-tolerant ability. Initially, the AAB strains were cultured on GYEC agar plates with 4% ethanol at 40 °C added as a selective stressor. Separate colonies showing distinct clear zones appeared. On GYEC agar plates using 10% ethanol, the strains that showed prominent clear zones were further chosen and classified as ethanol-tolerant strains. Therefore, 10% ethanol could be tolerated by the seven isolate strains (Chen et al., 2016). Some researchers pointed out that the selected isolates showed 7% to 8% ethanol tolerance as well as distinct clear zone. Other studies specify that, AAB with 4% - 13% ethanol for 8 days to determine the characteristics of AAB at various ethanol concentrations. They measured the growth and acid yield (Yuan et al., 2013).

Ethanol tolerance isolates were tested at 4 different ethanol concentrations (4%, 6%, 8% and 10%). *G. cerinus* isolates produced from apricot are immune to 4% to 6% ethanol. This is likely due to the higher ethanol content in vinegar compared to fruit (Buyukduman et al., 2022). In comparison to *Acetobacter*, according to another study, *Gluconobacter* species on Carr medium have great ethanol tolerance ability up to 15% (v/v) (Kourouma et al., 2022).

Tolerance with Acetic acid concentration

GYC broth medium was used to test the strain's resistance to acetic acid at concentrations ranging from 0.5% (v/v) to 10% (v/v). The mixture was then incubated at 30 °C. Result of figure (4b) showed that the strain *A. persici* DS1 have substantial growth up to 5.5% (v/v).

When the concentration of acetic acid exceeded from 6%-10%, the viability of the strain towards acetic acid stress decreased.

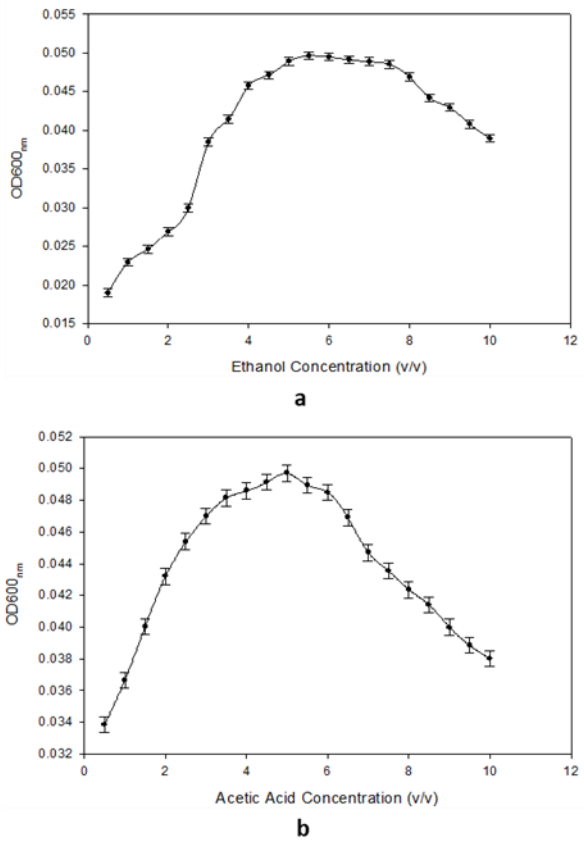

Fig 4. (a) Tolerance of ethanol concentration on growth, (b) Tolerance of Acetic acid concentrations on growth.

All the results were expressed in terms of the optical density ($OD600_{nm}$). The strain was observed as acetic acid tolerant. According to figure 4(b), strains that exhibited an increase in optical density up to 5.5% (v/v) of primary acetic acid concentration in GYC broth were identified as acetic acid-tolerant. Thus, the strain might be able to tolerate a concentration of acetic acid at 5.5% (v/v). This showed that an initial acetic acid concentration that is gradually increased (from 0.5% to 10%, v/v) might be used as a useful isolating strain to show that the AAB strains are acetic acid-tolerant (Yang et al., 2019).

Furthermore, the isolate capacity to resist acetic acid was observed using YPG broth added with 5% (v/v) alcohol and the concentrations of acetic acid extending from 0.5% (v/v) - 6% (v/v), all while being incubated for five days at 37 °C. All strains were capable of growing an acetic acid concentration of up to 4.5% (Kourouma et al., 2022). *A. pasteurianus* developed slowly and eventually stopped growing when the acidity reached 5.5%, in contrast to other *Acetobacter* sp. that had the acetic acid tolerance at greater concentrations of acetic acid. Even though *A. pasteurianus* overoxidized acetic acid at lower acetic acid concentrations, it is incapable of overoxidizing (Trcek et al., 2006). In our current study, also the strain *A. presici* DS1 showed similar pattern and can be considered as moderately tolerant to accumulated acetic acid and could moderately oxidized ethanol.

Assays of ADH and ALDH enzyme activity

To simplify the special properties of acetic acid assimilation on *A. persici* DS1, the actions of these crucial enzymes were consistent. As showed in figure 5(a & b), the strain's maximum enzyme activity was seen at a 10% acetic acid concentration. ADH and ALDH activity of *A. persici* were 3.52U mg^{-1} and 2.93U mg^{-1}, respectively.

Prior research has demonstrated that the higher the vinegar yield, the more constant the ADH and ALDH enzymes are under high acidity (Andrés et al., 2012). As shown in figure 5(c, d), the strain showed the highest enzyme activity at 10% ethanol concentration, ADH and ALDH activity of the strain were 3.62U mg^{-1} and 3.43U mg^{-1} respectively. Other research showed that ALDH activity was more complex than ADH activity. The activity of ADH was increased with 11% acetic acid to 8.9U mg^{-1} whereas ALDH activity was decreased with 1% to 7.1U mg^{-1}. Under every situation ALDH activity is less than ADH activity (Xia et al., 2015). A high activity of ADH is an essential component for AAB to establish and maintain metabolic activity at high acetic acid concentrations. The ADH and ALDH activities of original strain rose when the ethanol concentration increased in the medium (Zheng et al., 2015). Furthermore, other scientists worked on the ADH and ALDH activity, enhancing the enzyme's effectiveness and acidity by the addition of ferrous ions, and PFE was studied (Yin et al., 2018).

Estimation of acetic acid production

Yeast glucose ethanol acetic acid (YGEA) medium with composition (2% glucose, 2% yeast extract, 3% ethanol and 3% acetic acid) was used to culture isolated strain whose catalase, oxidase, and gram stain agreed with Bergey's manual of determinative bacteriology. The amount of acetic acid formed by isolate was determined through titration describe previously by (Sharafi et al., 2010). According to the final data the acetic acid producing strain *A. persici* DS1 isolated from sapodilla indicated that the highest acetic acid production 4.63 g/100ml with absolute ethanol and 3.82 g/100ml with distilled ethanol. Other research showed the strain

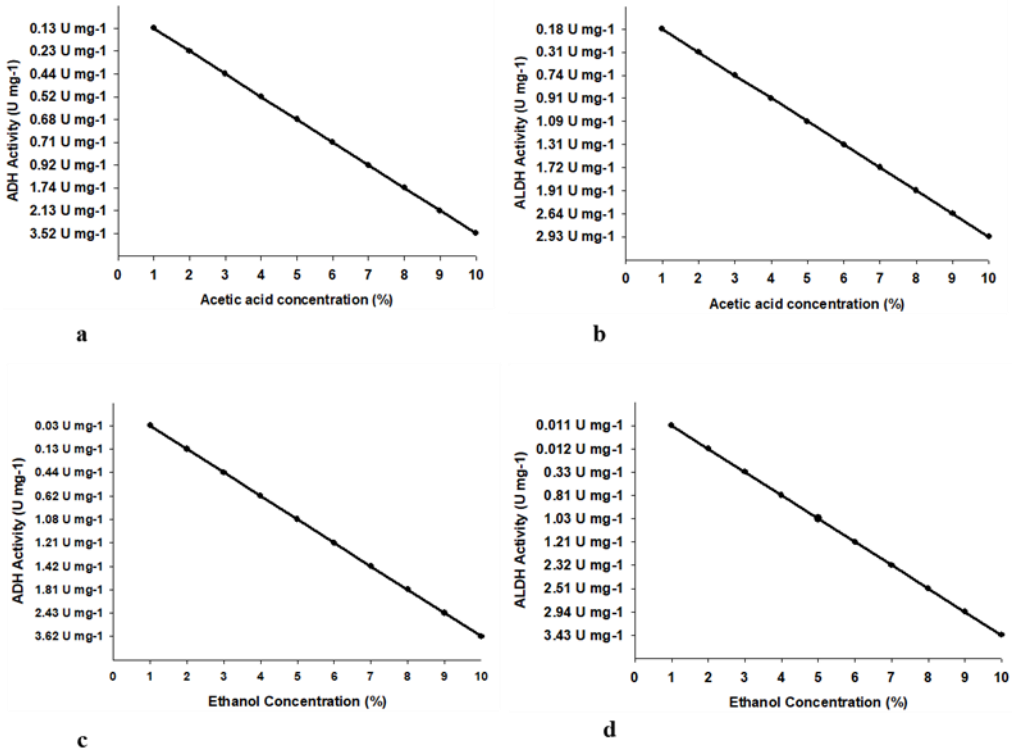

Fig 5. Combined graph of Effect of different concentration of Acetic acid (0.5% to 10% v/v) on **(a)** ADH and **(b)** ALDH Enzyme Activity, effect of different Ethanol concentration (1% to 10% v/v) on **(c)** ADH and **(d)** ALDH.

Acetobacter isolated from Iranian white-red cherry, produced maximum volume of acetic acid, respectively this is similar to the productivity of acetic acid with decomposed fruits by (Diba et al., 2015) but conflicting of research is completed by some different food products (Alisigwe et al., 2022). Even other study presented that, the high yield of acetic acid per 100 ml is 4.88 g/100 ml for the bacterial isolate, extracted from apple waste (Upadhyay et al., 2023). As compared to *A. persici*, the strain *A. malorum* and *A. tropicalis* also produced the highest concentration of acetic acid was 4.67 % (Al-Kharousi et al., 2024). The process of batch fermentation and experimental setup was having similarity as described earlier by Sharma (Singh et al., 2020).

Role of genes for acetic acid production and tolerance

Gene prediction during the whole genome sequencing (accession number JBHIKZ000000000) reveled that many different types of genes such as, Alcohol dehydrogenase (ADH) genes (EC 1.1.1.1, Iron-containing, Zinc-containing, Zinc type ADH like protein and Quinohemo protein ADH, Type III), Aldehyde dehydrogenase (ALDH) genes (EC 1.2.1.3, A EC 1.2.1.22, EC 1.2.1.3 in 4-hydroxyproline) and terminal oxidase gene (Terminal oxidase biogenesis protein CtaM) were identified and responsible for the production of acetic acid. Citrate synthase gene (Citrate synthase EC 2.3.3.1) and Acetyl-CoA hydrolase (aarc) gene (EC 3.1.2.1 and Acetyl-CoA hydrolase transferase family protein) involved in acetic acid resistance mechanism. Cytochrome oxidase gene (Cytochrome oxidase biogenesis protein and Heme A synthase, cytochrome oxidase biogenesis) present in ethanol respiratory chain and ATP synthase β gene (ATP synthase β chain EC 3.6.3.14) was accountable for ATP generation in acetic acid fermentation process.

Conclusion

One of the greatest well-known uses of acetic acid bacteria in industry is ethanol oxidation to acetic acid to generate vinegar. However, a number of factors, including temperature, ethanol concentration, and acidity buildup, have an impact on AAB during the acetification process. During the current study, based on their growth characteristics, tolerance to ethanol and ability to tolerate acetic acid, *A. persici* DS1 was discovered to be a promising candidate for the production of vinegar at 30 °C temperatures. It was discovered that the AAB species isolated from ripe fruit sapodilla belonged to the genus *Acetobacter*. These results further suggested that *A. persici* DS1 is a suitable strain for producing fruit vinegar with higher levels of acetic acid. The strains that were

produced can exhibit ethanol concentrations of ≥6% (v/v) and can also produce acetic acid. Additionally, they exhibit tolerance to acetic acid concentrations of 5.5% (v/v).

Conflicts of Interest

We all declare no conflict of interest among all the authors and co-authors.

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References

Aziz R K, Bartels D, Best A A, DeJongh M, Disz T, Edwards R A, Zagnitko O. (2008). The RAST Server: rapid annotations using subsystems technology. BMC genomics, 9, 1-15.

Adachi O, Ano Y, Toyama H, Matsushita K. (2007). Biooxidation with PQQ-and FAD-Dependent Dehydrogenases. Modern biooxidation: Enzymes, reactions and applications, pp.1-41.

Adachi O, Tayama K, Shinagawa E, Matsushita K, Ameyama M. (1978). Purification and characterization of particulate alcohol dehydrogenase from Gluconobacter suboxydans. Agricultural and Biological Chemistry, 42(11), pp.2045-2056.

Alisigwe C V, Njoku G C, Iloanusi D U, Uchendu J C, Aririguzo P C. (2022). Study about Acetic Acid Bacteria Isolated from Some Selected Waste Protein Source. Journal of Advances in Microbiology 22 (5):22–28. https://doi.org/10.9734/jamb/2022/v22i530460.

Al-Kharousi Z S, Al-Ramadhani Z, Al-Malki F A, Al-Habsi N. (2024). Date Vinegar: First Isolation of *Acetobacter* and Formulation of a Starter Culture. Foods, 13(9), 1389.

Andrés-Barrao C, Saad M M, Chappuis M L, Boffa M, Perret X, Pérez R O, Barja F. (2012). Proteome analysis of *Acetobacter pasteurianus* during acetic acid fermentation. Journal of proteomics, 75(6), pp.1701-1717.

Andrés-Barrao C, Saad M M, Ferrete E C , Bravo D, Chappuis M L, Pérez R O, Junier P, Perret X, Barja F. (2016). Metaproteomics and ultrastructure characterization of *Komagataeibacter* spp. involved in high-acid spirit vinegar production. Food microbiology, 55, pp.112-122.

Arifuzzaman M, Hasan M Z, Rahman S B, Pramanik M K. (2014). Isolation and characterization of *Acetobacter* and *Gluconobacter* spp from sugarcane and rotten fruits. Res. Rev. Biosci, 8(9), pp.359-365.

Aryal S. (2021). Catalase test-principle, uses, procedure, result interpretation with precautions. (https://microbenotes.com/catalase-test-principle-procedure-and-result-interpretation/). Accessed on 12/12/2024.

Aryal S. (2022). Oxidase Test-Principle, Uses, Procedure, Types, Result Interpretation, Examples and Limitations. (https://microbiologyinfo.com/oxidase-test-principle-uses-procedure-types-result-interpretation-examples-and-limitations/). Accessed on 12/12/2024.

Bangar S P, Sharma N, Kaur H, Kaur M, Sandhu K S, Maqsood S, Ozogul F. (2022). A review of sapodilla (Manilkara zapota) in human nutrition, health, and industrial applications. Trends in Food Science Technology, 127, pp.319-334.

Buddhika M A A, Perumpuli P A B N, Kaumal M N. (2021). Identification and characterization of acetic acid bacteria species isolated from various sources in Sri Lanka. Ceylon Journal of Science 50(4) 2021: 521-532.

Buxton R. (2011). Nitrate and nitrite reduction test protocols. American Society for Microbiology, pp.1-20.

Büyükduman E, Kırtıl H E, Metin B. (2022). Molecular identification and technological properties of acetic acid bacteria isolated from Malatya apricot and home-made fruit vinegars. Microbiology and Biotechnology Letters, 50(1): 81-88.

Callejón R M, Tesfaye W, Torija M J, Mas A, Troncoso A M, Morales M L. (2009). Volatile compounds in red wine vinegars obtained by submerged and surface acetification in different woods. Food Chemistry, 113(4), pp.1252-1259.

Chen Y, Bai Y, Li D, Wang C, Xu N, Hu Y. (2016). Screening and characterization of ethanol-tolerant and thermotolerant acetic acid bacteria from Chinese vinegar Pei. World Journal of Microbiology and Biotechnology, 32, pp.1-9.

De Ley J. (1984). Genus *Acetobacter*. Bergey's manual of systematic bacteriology, 1, 268-274.

Diba F, Alam F, Talukder A A. (2015). Screening of acetic acid producing microorganisms from decomposed fruits for vinegar production. Advances in Microbiology, 5(5), pp.291-297.

Es-sbata I., Lakhlifi T, Yatim M, El-Abid H, Belhaj A, Hafidi M, Zouhair R. (2021). Screening and molecular characterization of new thermo-and ethanol-tolerant *Acetobacter malorum* strains isolated from two biomes Moroccan cactus fruits. Biotechnology and Applied Biochemistry, 68(3), pp.476-485.

Gerard L M, Davies CV, Soldá C A, Corrado M B, Fernández M V. (2020). Application of molecular methods for the identification of acetic acid bacteria isolated from blueberries and citrus fruits. Microbiol Biotechnol Lett, 48, pp.193-204.

González Á, Mas A. (2011). Differentiation of acetic acid bacteria based on sequence analysis of 16S–23S rRNA gene internal transcribed spacer sequences. International journal of food microbiology, 147(3), pp.217-222.

Gullo M, Caggia C, De Vero L, Giudici P. (2006). Characterization of acetic acid bacteria in "traditional balsamic vinegar". International journal of food microbiology, 106(2), pp.209-212.

Kaur J, Singh A, Singh B, Sharma S. (2020). Sapota. Antioxidants in fruits: Properties and health benefits, pp.181-199.

Kim S H, Jeong W S, Kim S Y, Yeo S H. (2023). Quality and Functional Characterization of Acetic Acid Bacteria Isolated from Farm-Produced Fruit Vinegars. Fermentation, 9(5), p.447.

Klawpiyapamornkun T, Bovonsombut S, Bovonsombut, S. (2015). Isolation and Characterization of Acetic acid Bacteria from Fruits and Fermented fruit juices for Vinegar Production. Food and Applied Bioscience Journal, 3(1), pp.30-38.

Kourouma M C, Mbengue M, Sarr N C D, Sarr K, Kane C T. (2022). Thermoresistant, Ethanol-Resistant and Acid-Resistant Properties of Acetic Acid Bacteria Isolated from Fermented Mango Alcohol. Advances in Microbiology, 12(4), pp.177-191.

Maal K B, Shafiee R. (2009). 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. Word Academy of Science, Engineering and Technology, 3, pp.06-23.

Maal K B, Shafiee R. (2010). Isolation and characterization of an *Acetobacter* strain from Iranian white-red cherry as a potential strain for cherry vinegar production in microbial biotechnology.

Mas A, Torija M J, García-Parrilla M D C, Troncoso A M. (2014). Acetic acid bacteria and the production and quality of wine vinegar. The Scientific World Journal, 2014(1), p.394671.

Mathew B, Agrawal S, Nashikkar N, Bundale S, Upadhyay A. (2019). Isolation of acetic acid bacteria and preparation of starter culture for apple cider vinegar fermentation. Advances in Microbiology, 9(6), pp.556-569.

Matsushita K, Matsutani M. (2016). Distribution, evolution, and physiology of oxidative fermentation. Acetic acid bacteria: Ecology and physiology, pp.159-178.

Montet D, Ray R. C. eds. (2016). Fermented foods, Part I: biochemistry and biotechnology. CRC Press.

Ndoye B, Weekers F., Diawara B, Guiro A T, Thonart P. (2007). Survival and preservation after freeze-drying process of thermoresistant acetic acid bacteria isolated from tropical products of Subsaharan Africa. Journal of food engineering, 79(4), pp.1374-1382.

Pasteur L. (1864). Mémoire sur la fermentation acétique. In Annales scientifiques de l'École Normale Supérieure (Vol. 1, pp. 113-158).

Pothakos V, Illeghems K, Laureys D, Spitaels F, Vandamme P, De Vuyst L. (2016). Acetic acid bacteria in fermented food and beverage ecosystems. Acetic acid bacteria: Ecology and physiology, pp.73-99.

Román-Camacho JJ, Mauricio JC, Santos-Dueñas IM, García-Martínez T, García-García I. (2024). Recent advances in applying omic technologies for studying acetic acid bacteria in industrial vinegar production: A comprehensive review. Biotechnol J., 19(2):e2300566. doi: 10.1002/biot.202300566. PMID: 38403443.

Saeki A, Taniguchi M, Matsushita K, Toyama H, Theeragool G, Lotong N, Adachi O. (1997). Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Bioscience, biotechnology, and biochemistry, 61(2), pp.317-323.

Sharafi S M, Rasooli I, Beheshti-Maal K. (2010). Isolation, characterization and optimization of indigenous acetic acid bacteria and evaluation of their preservation methods. Iranian journal of Microbiology, 2(1), p.38.

Shinjoh M, Toyama H. (2016). Industrial application of acetic acid bacteria (vitamin C and others). Acetic acid bacteria: Ecology and physiology, pp.321-338.

Singh S, Kumar V, Singh S, Dhanjal D S, Datta S, Sharma D, Singh N K, Singh J. (2020). Biosurfactant-based bioremediation. In Bioremediation of pollutants (pp. 333-358). Elsevier.

Štornik A, Skok B, Trček J. (2016). Identifikacija bakterija octenog vrenja izoliranih iz organski i konvencionalno proizvedenog jabučnog octa. Food Technology and Biotechnology, 54(1), pp.113-119.

Subramanian K, Suresh K. (2025a). Optimized fermentation strategies for efficient bioethanol production. Microbial Biosystems, 10(2), 143-151. doi: 10.21608/mb.2025.379468.1299

Subramanian K, Suresh K. (2025b). Strategic metabolic engineering of Escherichia coli for improved ethanol biosynthesis. Microbial Biosystems, 10(2), 259-265. doi: 10.21608/mb.2025.387913.1328

Tamura K, Stecher G, Kumar S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. Molecular biology and evolution, 38(7), pp.3022-3027.

Trcek J, Toyama H, Czuba J, Misiewicz A, Matsushita K. (2006). Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. Applied microbiology and biotechnology, 70, pp.366-373.

Tulloch A, Goldson-Barnaby A, Bailey D, Gupte S. (2020). *Manilkara zapota* (Naseberry): medicinal properties and food applications. International Journal of Fruit Science, 20(sup2), pp.S1-S7.

Upadhyay A, Kovalev A A, Zhuravleva E A, Pareek N, Vivekanand V. (2023). Enhanced production of acetic acid through bioprocess optimization employing response surface methodology and artificial neural network. Bioresource Technology, 376, p.128930.

Wu X, Yao H, Liu Q, Zheng Z, Cao L, Mu D, Wang H, Jiang S, Li X. (2018). Producing acetic acid of *Acetobacter pasteurianus* by fermentation characteristics and metabolic flux analysis. Applied biochemistry and biotechnology, 186, pp.217-232.

Xia X, Zhu X, Yang H, Xin Y, Wang W. (2015). Enhancement of rice vinegar production by modified semi-continuous culture based on analysis of enzymatic kinetic. European Food Research and Technology, 241, pp.479-485.

Yang H, Yu Y, Fu C, Chen F. (2019). Bacterial acid resistance toward organic weak acid revealed by RNA-Seq transcriptomic analysis in *Acetobacter pasteurianus*. Frontiers in Microbiology, 10, p.1616.

Yin XY, Zhong W K, Huo J, Chang X, Yang Z H. (2018). Production of vinegar using edible alcohol as feedstock through high efficient biotransformation by acetic acid bacteria. Food science and biotechnology, 27, 519-524.

Yuan Y H, Mu D D, Guo L, Wu X F, Chen X S, Li X J. (2024). From flavor to function: A review of fermented fruit drinks, their microbial profiles and health benefits. Food Research International, 115095.

Yuan Y, Feng F, Chen L, Yao Q, Chen K. (2013). Directional isolation of ethanol-tolerant acetic acid bacteria from industrial fermented vinegar. European Food Research and Technology, 236, pp.573-578.

Zheng Y, Zhang K, Su G, Han Q, Shen Y, Wang M. (2015). The evolutionary response of alcohol dehydrogenase and aldehyde dehydrogenases of *Acetobacter pasteurianus* CGMCC 3089 to ethanol adaptation. Food Science and Biotechnology, 24, pp.133-140.