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Isolation of Acetic Acid Bacteria and Preparation of Starter Culture for Apple Cider Vinegar Fermentation

Bernadette Mathew, Shaily Agrawal, Nandita Nashikkar, Sunita Bundale*, Avinash Upadhyay

Hislop School of Biotechnology, Hislop College, Nagpur, Maharashtra, India
Email: *sbundale@gmail.com

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Abstract

Vinegars are commonly used as food condiments and preservatives. Apple cider vinegar (ACV) is also used in the Ayurvedic pharmaceutical industry because of its medicinal properties. Since specifically selected starter cultures for commercial vinegar production are not readily available, apple juice supplemented with sugar is commonly inoculated with a microbiologically undefined culture obtained from the previous batch of ACV. The present work focuses on the isolation of yeasts and acetic acid bacteria from ACV and the preparation of a starter culture. ACV was produced in a bench scale bioreactor using a traditional fermentation process wherein an acetic acid concentration of 3.8% was obtained after three weeks. Several acetic acid bacteria (AAB) were isolated from ACV using selective media. Microscopy revealed the cultures to be gram negative to gram variable short rods. The growth pattern of the isolates on differential media and biochemical tests suggested the presence of *Acetobacter* and *Gluconobacter* species. Ten potent isolates were selected for starter culture preparation. Two consortia were formulated with five AAB isolates in each along with a yeast isolate and used for ACV production, wherein an acetic acid concentration of 4.2% - 4.9% was obtained in 10 - 12 days. Thus, these two starter cultures with locally isolated AAB can be used for the commercial production of apple cider vinegar.

Keywords

Acetobacter, *Gluconobacter*, Apple Cider Vinegar, Starter Culture, Acetic Acid

1. Introduction

Vinegar, from the French *vin aigre*, meaning "sour wine," can be made from

almost any fermentable carbohydrate source, including wines and ciders, molasses, dates, grains, honey, maple syrup, starchy vegetables, whey and fruits [1]. It is a widely used food preservative and condiment. According to FDA (Food and Drug Administration of the United States), it contains 4% acetic acid that is produced from sugary or alcoholic materials through fermentation along with varying amounts of fixed fruit acids, salts, coloring materials and some volatile products such as esters and phenolics which impart characteristic aroma and flavor [2]. Apple cider vinegar (ACV), one of the most popular vinegars, is well known for its medicinal properties and general health benefits. It contains trace minerals and vitamins-A, C, E and several different forms of vitamin-B including beta carotene and bioflavonoid that are needed for cell function. It's antioxidant, anti glycemic, anti hypertensive, antibacterial, antifungal and anti tumor properties that are also well established [3]. Further, ACV is extensively used as the base in Ayurvedic preparations termed "asavas".

Traditionally, fruit juices have been used for domestic as well as industrial production of vinegar in a two-step process—the fermentative production of alcohol from the fruit sugars by yeasts, mainly *Saccharomyces ellipsoideus* and *Saccharomyces cerevisiae* followed by the oxidation of the ethanol and residual or added sugars to acetic acid by AAB [4]. These are obligate aerobic Gram negative or Gram variable, ellipsoidal to rod-shaped, straight or slightly curved, 0.6 - 0.8 μm × 1.0 - 0.4 μm, occurring singly, in pairs or chains. Pleomorphic forms occur which may be spherical, elongated, swollen, club shaped, curved or filamentous. They are able to oxidize substrates such as glucose, ethanol, lactate or glycerol to acetic acid. On the basis of their abilities to over oxidize acetate or lactate and the positions of their flagella, these bacteria are conventionally categorized into two major genera—*Acetobacter* and *Gluconobacter.* In liquid media, *Acetobacter* forms a film or pellicle made of cellulose. The AAB and yeasts present in the fermentation broth get entangled in the cellulosic pellicle to form a mat-like structure called the "mother of vinegar" [5].

Alcohol and wine vinegars including apple cider vinegar are most often produced in submerged bioreactors, which supply the bacteria with a constant inflow of oxygen and enable an efficient production process [6]. The oxidation is started by adding the "mother of vinegar" obtained from previous vinegar to the fresh fermentation. This is a microbiologically undefined culture and very few in-depth studies are available about the AAB and other microbes in such seed cultures, which leads to variation in the product quality. Moreover, most processes require at least three weeks to produce an adequate concentration of acetic acid. Hence, there is a pressing need for easily available "starter cultures" so that product quality is maintained and fermentation time is cut short [7].

The present work focuses on the bench scale production of ACV using a traditional apple recipe for isolation of AAB and yeast strains and formulation and assessment of mixed "starter cultures" made up of yeast and high acetic acid producing isolates in view of rapid and maximum production of acetic acid.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and solvents were of analytical grade and purchased from Merck, Germany.

2.2. Culture Media

Culture media were purchased from Hi-media, Mumbai, India. A few media were modified as per the requirements. Several selective and differential media such as Carr medium [8] with bromothymol blue as indicator, Frateur medium [9], GYC medium containing glucose 4%, yeast extract 1%, $CaCO_3$ 1%, agar 1.5%, DSM agar [10] containing dextrose sorbitol and mannitol and MS agar, a mannitol salts medium with phenol red as the indicator were used for the enrichment, isolation and differentiation of AAB. Potato dextrose agar (PDA) was used to isolate yeasts. A glucose (10%) yeast extract (5%) peptone (3%) medium (GYP) was used for the production of ACV using the selected isolates and the formulated starter cultures.

2.3. Production of ACV for Isolation of AAB

ACV was prepared using suitably modified reagent bottles as small bioreactors. Holes were punched in the lids to allow the CO_2 produced during fermentation to escape. Seven experimental bioreactors were organized each containing 50 g crushed apples and 2 g glucose in 60 ml water. Commercially available dried active yeast powder containing lyophilized *Saccharomyces cerevisiae* or innocula from different old unpasteurized wines were added to hasten alcoholic fermentation. After 72 h the contents of the bottles were filtered through muslin cloth and transferred to conical flasks. The flasks were thereafter incubated aerobically on a shaker incubator or statically at 30˚C for four weeks.

2.4. Estimation of Acetic Acid

Aliquots were withdrawn regularly and acetic acid was estimated titrimerically [11]. The acetic acid in vinegar represents 98% of acids [12]; the total acidity is therefore also a measure for acetic acid concentration.

2.5. Isolation of Yeasts and AAB from ACV Bioreactors

Appropriately diluted samples and pellicle material from the flasks showing the highest concentration of acetic acid were plated on Frateur medium containing ethanol and $CaCO_3$ for initial isolation of AAB. Isolated pure cultures were maintained on GYP agar slants containing $CaCO_3$ and sub cultured regularly.

PDA was used for the isolation of yeasts from the bioreactors at 48 h. A loopful of the fermenting liquid was streaked on to PDA plates and incubated at 30˚C. Isolated pure yeast cultures were maintained on PDA slants and sub cultured every three weeks.

2.6. Microscopic and Biochemical Characterization of Yeast and AAB Isolates

The selected AAB isolates were gram stained and the morphology observed. Motility was observed by the hanging drop method. Standard biochemical tests generally used to identify AAB such as production of catalase and oxidase, nitrate reduction, and Voges Proskauer's test was performed [13]. The utilization of various carbohydrates such as glucose, trehalose, malonate, arabinose, mannitol, citrate, sucrose, and arginine was also studied. The yeast cultures were simple stained and the morphology observed.

2.7. Differentiation between *Acetobacter* and *Gluconobacter*

Twelve selected AAB pure cultures were streaked onto differential media such as DSM, MS and Carr medium to differentiate between *Acetobacter* and *Gluconobacter*. Biochemical characterization, including carbohydrate utilization, along with differences in growth pattern on the differential media was considered to categorize the isolates as species of *Acetobacter* and *Gluconobacter.*

2.8. Preparation of Mixed Starter Cultures

Two sets of starter cultures were prepared, designated SC-I and SC-II with five AAB isolates comprising of both *Acetobacter* and *Gluconobacter* cultures in each group.

The selected AAB isolates were inoculated in 5 ml each of GYP medium in test tubes and incubated for 48 h at room temperature. SC-I consisted of one ml of 48 h old broth cultures each of isolates A1, A5, G3, G8, A9 and SC-II consisted of one ml of 48 h old broth cultures each of isolates A2, A4, A6, G7, G10. The yeast isolate was cultivated in potato dextrose broth and 1ml of a 72 h yeast culture was included in the starter cultures SC-I and SC-II.

2.9. ACV Production Using Prepared Starter Cultures

The pre-formulated starter cultures SC-I and SC-II were added to two different 250 ml Erlenmeyer flasks containing 100 ml sterile GYP medium containing 50 g crushed apples. The inoculated flasks were stoppered with a rubber cork and incubated statically for 48 h for ethanolic fermentation. The side arm tube facilitated the dispersal of liberated CO_2. Thereafter, the flasks were placed on a rotary shaker at 80 rpm at 28˚C to facilitate the aerobic acetification. After six days some of the flasks were incubated statically to observe pellicle formation. All the flasks were monitored for two weeks. Aliquots were withdrawn at 48hr intervals and acetic acid was estimated.

3. Results

3.1. Production of ACV for Isolation of AAB

All the seven ACV bioreactors showed active bubbling of CO_2 within 24 h indicating a robust ethanolic fermentation (Figure 1). This fermented dry apple cider after 48 h had a sweetish taste and an alcoholic odor.

Figure 1. Production of ACV for isolation of AAB; robust ethanolic fermentation of apple juice using various innocula.

An acetic acid concentration above the required 4% was observed only after six weeks and only in ACV bioreactors B and D. The acetic acid concentration in the other bioreactors was found to be less than 1%. Hence bioreactors B and D were used for the isolation of AAB. The ACV flask B incubated statically developed a typical pellicle produced by *Acetobacter spp* (**Figure 2**).

3.2. Isolation of Yeast from ACV Bioreactors

The fermented apple cider at 72 h was used for the isolation of yeast. The yeast cultures were identified microscopically. Since commercial active dry yeast powder containing *S. cerevisiae* had been added to hasten ethanolic fermentation, the same yeast was isolated and pure cultures were maintained on PDA slants.

3.3. Isolation of AAB from ACV Bioreactors

The supernatant and pellicle in the ACV flasks B and D which showed maximum acetic acid production was used for isolation of AAB. Initial screening was done on GYC and Frateur medium containing 2% ethanol. Pin point colonies with relatively large zones of clearance as well as medium sized colonies with $CaCO_3$ clearance zones were preliminarily selected as AAB (**Figure 3**).

The selected isolates were streaked on to Carr medium and acid production was confirmed by the appearance of yellow zones. A total of 12 isolates selected as hyper producing AAB were obtained as pure cultures. These isolates which appeared to be distinctly different from each other based on their colony morphology were used for further characterization (**Figure 4**).

3.4. Cultural & Microscopic Characterization

The 12 selected isolates were all aerobic, producing pin point to small colonies which were moist, translucent, white, beige or yellowish in color. These were found to be gram negative or gram variable (isolates G3 and A5) small rods, straight or slightly curved, coccoid or club shaped. All the isolates except A6 and G7 were found to be motile.

Figure 2. Production of ACV for isolation of AAB; Acetification with pellicle formation.

Figure 3. Screening for acetic acid bacteria on Frateur medium containing ethanol. The colonies showing $CaCO_3$ clearance zones were selected as AAB.

Figure 4. Pure cultures of AAB isolates.

3.5. Biochemical Characterization

Ten isolates showing significant zones of clearance on Frateur agar and which were found to be catalase positive and oxidase negative were preliminarily identified as AAB according to the standard guidelines of Bergey's manual of Determinative Bacteriology [14].

The results of the biochemical characteristics are presented in Table 1.

Table 2 depicts the utilization of some selected carbohydrates by the ten isolates putatively identified as AAB. All the isolates were able to ferment glucose with acid production as expected whereas sucrose was not utilized except for a variable +/− result shown by isolate G10. Mannitol was utilized by all the isolates.

Table 1. Biochemical characterization of AAB isolates.

Isolate	Gram stain	Catalase	Oxidase	Nitrate reduction	Motility	VP
A1	−ve	+	−	−	+	−
A2	−ve	+	−	+	+	+
G3	var	+	−	+	+	−
A4	−ve	+	−	−	+	−
A5	var	+	−	+	+	−
A6	−ve	+	−	−	−	−
G7	−ve	+	−	+	−	+
G8	−ve	+	−	+	+	−
A9	−ve	+	−	+	+	+
G10	−ve	+	−	+	+	+

−ve indicates gram negative, var. indicates gram variable; +indicates positive reaction, −indicates negative reaction.

Table 2. Utilization of carbohydrates by AAB isolates.

Isolate	Glucose	Sucrose	Arabinose	Trehalose	Mannitol	Arginine	Citrate	Lactate
A1	+	−	+/−	+	+	−	−	+
A2	+	−	−	+	+/−	+	+	+
G3	+	−	+	−	+	+	−	−
A4	+	−	+/−	+/−	+/−	−	+/−	+
A5	+	−	+	+	+	−	+/−	+
A6	+	−	+	−	+	−	+/−	+
G7	+	−	+	−	+/−	−	−	+/−
G8	+	−	+/−	−	+/−	−	−	−
A9	+	−	+/−	+/−	+	+	+/−	+
G10	+/−	+/−	+	−	+	+	−	−

+indicates acid production, −indicates no acid production and +/−indicates a variable reaction.

3.6. Identification of the Genera *Acetobacter* Isolated from ACV

Six isolates were able to utilize glucose, arabinose, mannitol (variable) and trehalose but were unable to utilize sucrose (Table 2). *Acetobacter spp.* are distinguished by their capability to over oxidize alcohols to acetic acid and then to CO_2 and H_2O [15]. When these six isolates were grown on MS agar, a change of color from red (neutral pH) to yellow (acidic) was observed within 48 h which started reverting back to red after 96 h, typical of the over oxidizing *Acetobacter spp* (Figure 5).

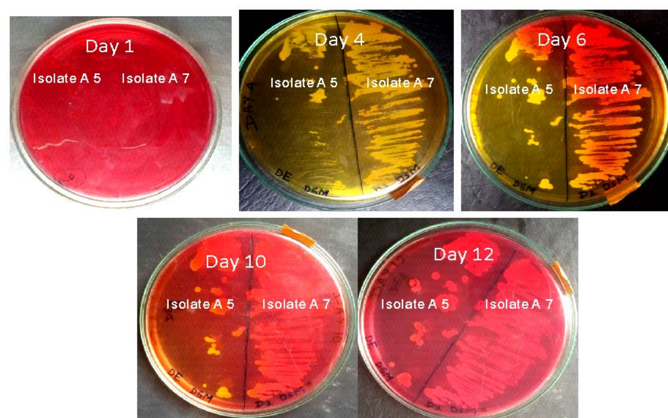

Figure 5. *Acetobacter* isolates A5 and A7 showing overoxidation on MS medium containing phenol red as the pH indicator.

These strains registered positive growth on lactate. Growth on DSM agar resulted in a color change of the medium to purple indicative of preferential lactate utilization which is characteristic of *Acetobacter spp*. (**Figure 6(B)**).

When grown on Carr medium, these isolates produced greenish colonies (**Figure 6(A)**).

Thus, based on the morphological, microscopic and biochemical characterization, these isolates were identified as *Acetobacter* species and designated as A1, A2, A4, A5, A6 and A9.

3.7. Identification of the Genera *Gluconobacter* Isolated from ACV

Four isolates were able to utilize glucose, mannitol (variable reaction) and arabinose but were unable to use trehalose and sucrose. When grown on MS agar containing phenol red these isolates showed change of color from red to yellow indicating acid production within 48 h but the color did not revert back to red, typical of the under oxidizing *Gluconobacter spp*. (**Figure 7**).

The yellow color of the DSM medium was maintained indicating an inability to use lactate, which is characteristic of *Gluconobacter* species. These isolates grew as white colonies on Carr medium (**Figure 6(A)**).

Thus, based on the morphological, microscopic and biochemical characterization, these isolates were identified as *Gluconobacter* species and designated as G3, G7, G8, G10.

3.8. ACV Production Using Prepared Starter Cultures

SC-I and SC-II described above were used for the production of ACV and an acetic acid concentration of 4%, which is prescribed for vinegar was attained within 12 days with both the mixed starter cultures as depicted in **Figure 8**. However, the acetic acid concentration in the fermentation flask with SC-I continued to increase, reaching 5% on the 14th day. On the contrary, an acetic acid concentration of 3.8% was obtained in the control flask with no added inoculum after four weeks. Thus, the fermentation time for ACV production using the

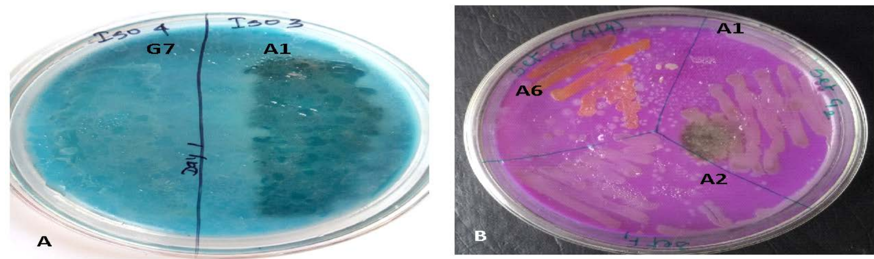

Figure 6. (A) Colony morphology of isolates G7 and A1 on Carr agar supplemented with bromothymol blue (B) Colony morphology of isolates A1, A2 and A6 on DSM agar supplemented with bromocresol purple.

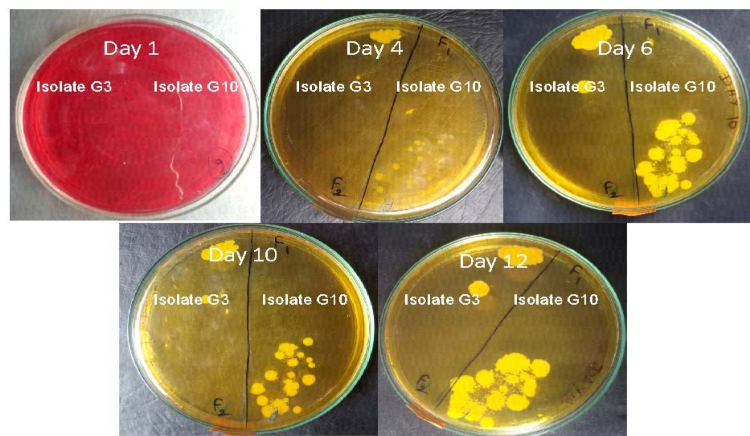

Figure 7. *Gluconobacter* isolates G3 and G10 showing underoxidation on MS medium containing phenol red as the pH indicator.

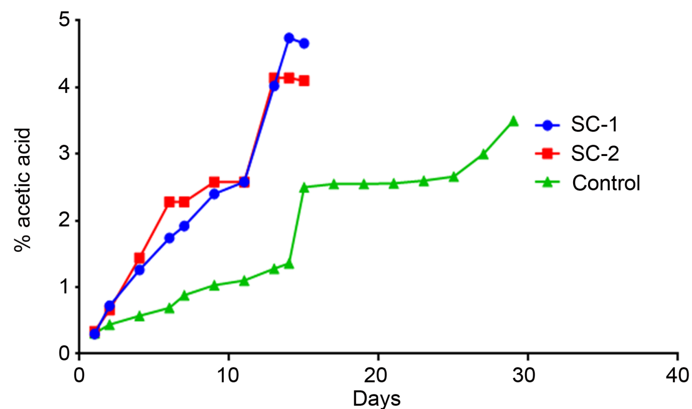

Figure 8. ACV production using prepared starter cultures. SC-1: Starter Culture 1; SC-2: Starter Culture 2; Control: No added inoculum.

starter cultures was reduced considerably as compared to the time required to obtain an acetic acid concentration of 4% using the traditional process for ACV production. The acetic acid concentration obtained upon using the AAB isolates in monoculture fermentation ranged from 0.4% to 1.2%. Thus, ACV production using a pre-formulated mixed starter culture was found to be more effective as compared to both-monoculture fermentation as well as traditional natural fer-

mentation with no added inoculum.

4. Discussion

Several authors have worked on the isolation and characterization of acetic acid bacteria from sugary and starchy substrates and oriental fermented foods [16] [17]. However, there are relatively few reports on the laboratory production of ACV specifically for the isolation of yeasts and AAB to be used for the development of a starter culture. The present work began with the bench scale production of ACV using a traditional recipe with apples. The initial ethanolic fermentation is carried out by several yeasts found naturally on fruits. These wild yeasts mainly belong to the species *Saccharomyces*, *Kloeckera*, *Candida* and *Pichia* [18]. The yeast culture is not removed from the bioreactor after the ethanolic fermentation and ethanol and acetic acid production may go on simultaneously [19]. Hence samples were withdrawn throughout the course of the fermentation in an effort to isolate high acid producing and ethanol tolerant AAB strains. These bacteria are difficult to isolate and culture and Frateur medium, which supports the growth of all strains of AAB. [20] was used in the initial isolation of high acid producing strains. This medium contained ethanol as the major carbon source hence the clearance of $CaCO_3$ was due to its solubilization by the acetic acid produced by the AAB colonies. Twelve hyper acid producing isolates were identified by taking into consideration the clearance zone: colony size ratio.

Microscopic and biochemical characterization indicated the isolates to be AAB. These were differentiated into *Acetobacter* and *Gluconobacter* by traditional methods, mainly based on their ability/inability to over-oxidize ethanol to carbon dioxide and water, their ability/inability to oxidize lactate and their comparative ability to utilize selected sugars. Differential media such as DSM agar, MS agar and Carr medium were used to differentiate *Acetobacter* and *Gluconobacter.* DSM agar contains lactate as the major carbon source and bromocresol purple as the pH indicator. Isolates belonging to the genus *Acetobacter* were able to utilize lactate resulting in an increase of pH causing a color change of the medium from yellow to purple whereas isolates belonging to the genus *Gluconobacter* were unable to oxidize lactate and the yellow color of the medium was maintained as there was no increase of the pH [21].

MS agar contains mannitol as the major carbon source and phenol red as the pH indicator. All the isolates grew well, produced acid and turned the medium yellow. However, *Acetobacter* species possess a functional tricarboxylic acid cycle and can further oxidize the acetic acid to CO_2 and H_2O when there is a high level of dissolved oxygen and no ethanol in the medium. This acetate over-oxidation resulted in a reversal of the color back to red. *Gluconobacter* species on the other hands, are underoxidizers and hence no color reversal is observed [22]. The growth pattern on Carr's medium further confirmed the differentiation of *Acetobacter* and *Gluconobacter*.

The main species responsible for the production of vinegar belong to the ge-

nera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* because of their superior capacity to oxidize ethanol and resistance to acetic acid released into the fermentative medium. AAB are fastidious organisms and strains are known to lose several features, including their ability to produce higher concentrations of acetic acid upon subculturing [23]. Thus maintenance of AAB isolated from bioreactors too is not easy since these are known to quickly pass into the viable but not culturable state (VNBC). This is mainly due to the lower oxygen availability and the considerable drop in pH because of the continuous acetic acid production which is an energy related or primary metabolite for AAB [24]. In the present work, the isolates were maintained on GYP agar slants containing $CaCO_3$. The inclusion of $CaCO_3$ ameliorates this problem to a great extent by neutralizing the acetic acid produced and relieving the physiological stress which drives the cells into the VBNC state [25].

In the present study, the selected AAB isolates were used in mono culture as well as mixed culture fermentations to assess their hyper acid production capacity in the shortest time. The yield of acetic acid in the single culture fermentations was very poor even after four weeks. Sossou *et al.* [26] have also reported a similar fermentation time of 23 - 25 days for production of vinegar with *Acetobacter* strains isolated from pineapple juice. But Nanda *et al.* [27] isolated hyper producing *Acetobacter* strains from various fruits and have reported the production of around 3% - 4% acetic acid within 4 days in GYP medium containing 4% ethanol using their isolates in monoculture.

In the mixed culture fermentations carried out in this study using SC-I and SC-II, an acetic acid concentration of 4% which is prescribed by FAO for vinegar was attained within 12 days. The starter cultures formulated in this study possibly exhibit some synergistic enhancement of acetic acid production leading to a 50% reduction in the fermentation period as compared to the natural vinegar fermentation period of over four weeks. In the natural fermentation of sugary or alcoholic substrates, there is a succession of microbes beginning with high sugar tolerant yeast strains and moving on to AAB [28]. During acetification, in the early stage, low acid tolerant AAB such as *Acetobacter pasteuranus* predominate and in the later stages, high acid tolerant AAB such as *Komagataeibacter europaeus* or *Gluconacetobacter intermedius* are predominantly present. Although a few reviews and studies [29] [30] using known AAB strains for acetic acid production exist, no preformulated mixed culture studies with yeast and AAB isolates are documented to the best of our knowledge. Moreover, our starter cultures are composed of locally isolated strains which may be better adapted to the prevailing conditions. The importance of locally isolated AAB has been highlighted by other researchers too [31]. Thus the consortia developed in this study may be successfully used for the commercial production of ACV. The main factors deterring the availability of starter cultures are difficulties in culturing the AAB and preserving their acid forming potential in the laboratory. Also, the costs for production of the starter cultures may be higher compared with using seed cultures from previous production batches. The advantage of the

starter culture is that it facilitates a more controlled process that is easier to reproduce and control and gives a standardized product [32]. A comprehensive optimization programme of the various process parameters affecting acetic acid production, studying the acid tolerance profile of the isolates and a systematic trial of permutations and combinations of our isolates in the formulation of the starter cultures will lead to further increase in acetic acid concentration within a shorter fermentation period.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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