Research Article

Received: 5 May 2022 Revised: 11 July 2022 Accepted article published: 22 July 2022 Published online in Wiley Online Library: 5 August 2022

(wileyonlinelibrary.com) DOI 10.1002/jsfa.12138

Effect of the type of acetic fermentation process on the chemical composition of prickly pear vinegar (*Opuntia ficus-indica*)

Ikram Es-sbata,[a,b] Remedios Castro,[a] Rachid Zouhair[b] and Enrique Durán-Guerrero[a*]

Abstract

BACKGROUND: In several countries, the cactus plant (*Opuntia ficus-indica* (L). Mill.) has received renewed attention because of its ecological, socio-economic and environmental role. In this study, prickly pear vinegar was produced employing two types of acetification processes: surface and submerged culture. Both acetification processes were performed at different temperatures (30, 37, 40 °C) by using two different species of thermotolerant acetic acid bacteria (*Acetobacter malorum* and *Gluconobacter oxydans*). Polyphenols and volatile compounds analyzed by ultra-performance liquid chromatography with diode array detection and stir bar sorptive extraction–gas chromatography–mass spectrometry, respectively, were considered as the main variables to determine the effect of the acetification process on the quality of the vinegar.

RESULTS: As a result, 15 polyphenols and 70 volatile compounds were identified and quantified in the vinegar samples produced by both acetification processes. The results showed that the surface acetification method led to an increase in the concentration of phenolic components, which was higher than that in the submerged process. However, a significant increase in volatile compounds predominated by esters and acids was observed when submerged culture acetification was employed, whereas alcohols were predominant in surface culture vinegars. Moreover, multivariate statistical analysis showed that the components that mostly contributed to the differentiation between all vinegar samples were the volatile compounds.

CONCLUSION: It has been proved that prickly pear vinegar could be successfully produced at higher temperatures than usual, by employing thermotolerant bacteria, and that the type of acetification method significantly affects the final quality of the vinegar produced.

Supporting information may be found in the online version of this article.

Keywords: prickly pear; vinegar; thermotolerant bacteria; volatile compounds; polyphenolic compounds; acetification process

INTRODUCTION

Currently, due to environmental motivations, many countries from the Mediterranean basin are turning their agricultural policies into new strategies with a more ecological impact and that imply less water consumption. In this sense, cactus plant (*Opuntia ficus-indica* (L). Mill.) is perfectly appropriated for the development of arid and semiarid areas.[1] Its fruit is seasonal and its production and harvest take place over a short period of time (from June to September). Cactus pear fruits, or prickly pears, are highly appreciated by consumers because of their flavor and excellent nutritional properties, which give them a good commercial value.[2] These fruits are used for the production of different food products such as jams,[3] alcoholic beverages[4] and juices.[5] Cactus pear juice can also be used for the production of vinegar owing to its richness in fermentable sugars. This production of a new type of vinegar from prickly pear juice opens an alternative method for a new product in the market and can add value through a simple process that can be applied at different industrial scales.[6] *Opuntia* plants have been used as a good source of antioxidants due to their phenolic acids and flavonoids. Polyphenolic components have a major effect on the organoleptic properties of beverages and plant-derived foods, especially color and taste. In addition,

* *Correspondence to: E Durán-Guerrero, Analytical Chemistry Department, Faculty of Sciences – IVAGRO, University of Cadiz, Agrifood Campus of International Excellence (CeiA3), Polígono Río San Pedro, s/n, Puerto Real 11510, Cadiz, Spain. E-mail: enrique.duranguerrero@uca.es*

a *Analytical Chemistry Department, Faculty of Sciences – IVAGRO, University of Cadiz, Agrifood Campus of International Excellence (CeiA3), Cadiz, Spain*

b *Laboratory of Plant Biotechnology and Molecular Biology, Department of Biology, Faculty of Sciences, Moulay Ismail University, Meknes, Morocco*

numerous studies have noted that their daily consumption impacts positively on health because of their biological and pharmacological properties such as reducing the risk of neurodegenerative disorders,[7] cardiovascular diseases and specific types of cancer.[8] The major phenolic acids identified in this plant as antioxidants are vanillic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, protocatechuic acid, caffeic acid, salicylic acid, gallic acid and sinapic acid, among others. Concerning flavonoids, rutin, isoquercitrin and kaempferol are found as the main flavonoids identified in these plants.[9,10] The presence of polyphenols in cactus pear fruits relies on various factors such as growing region, maturity stage and post-harvest.[11] On the other hand, the aroma profile is considered to be the main criterion for product acceptance and varies with different fruit varieties; however, the flavor property of some fruits can be changed by food processing. The aroma profile of vinegars is formed both by the compounds of the substrate and by those generated during fermentation, so the final volatile composition of vinegar is clearly influenced by the acetification conditions. In cactus pear fruits, alcohols and esters have been identified as the dominant volatile compounds.[12] Various studies have investigated the volatile composition of different cultivars of prickly pear fruits and their juice, but only a few investigations of the aroma profile of prickly pear vinegar have been conducted. A previous study on the chemical characterization of prickly pear vinegar conducted by our research group considered the volatile and polyphenolic composition of this product.[13]

Many factors affect the quality and organoleptic properties of vinegar, such as the raw material (substrate), microbial diversity (especially acetic acid bacteria) and the technological process used for its production.[14,15] There are two main biotechnological processes implicated in the production of vinegar; the first one is the fermentation of sugars to alcohols (alcoholic fermentation) by yeasts – generally *Saccharomyces* species – and the second process, called acetification, which is the oxidation of alcohols to acetic acid by using acetic bacteria (especially *Acetobacter* species,[16] although recent studies on vinegar production indicate that the most important genus, in quantitative terms, is *Komagataeibacter*).[17] In general, the methods for vinegar production range from surface culture (traditional/slow method) to submerged culture (industrial/quick method).[18] In addition to the existence of different methods, there are also various raw materials for vinegar production. Substrates normally used for acetification can be wine, cider, beer or another alcoholic substrate derived from the fermentation of cereals, fruits, honey and molasses,[19] fruits being one of the most important raw materials for the production of vinegars.[20] Thus the final quality of vinegars depends on the production method and the raw material.[21] Vinegars produced by surface culture usually have good sensory quality, while vinegars elaborated by the submerged process are faster and cheaper. However, this latter method is commercially preferred by producers because it is more economical and has a higher yield.[22,23] On the other hand, vinegars produced by the traditional method (surface culture) are generally more expensive because of their better sensory quality, which is more recognized by the consumer.[18]

In this study, we examined the influence of the acetification process on the chemical composition of prickly pear vinegar. To do so, the acetic fermentation was conducted by submerged and surface cultures at different temperatures (30, 37 and 40 °C) by using two different pure thermotolerant acetic acid bacteria (*Acetobacter malorum* and *Gluconobacter oxydans*). Multivariate statistical analysis of the identified volatile and phenolic compounds was conducted to determine the main factors that contributed to the final quality of the vinegars.

MATERIALS AND METHODS

Wine making

Prickly pear (*Opuntia ficus-indica*) juice (14.24 °Brix) prepared as described in the previous study[13] was submitted to alcoholic fermentation. A defined concentration (0.20 g L^{-1}) of commercial *Saccharomyces cerevisiae* strain (Enartis Ferm SB, Trecate, Italy) was activated into the prickly pear juice (at 35 °C for 20 min) and used as a starter culture for the alcoholic fermentation. Fifty liters of fresh prickly pear juice was alimented by adding 60 mg L^{-1} total sulfur dioxide (potassium metabisulphite, Agrovin, Alcázar de San Juan, Spain) to avoid the development of undesirable microorganisms and 0.35 g L^{-1} diammonium phosphate (Actimax Plus, Agrovin) as a nutrient. To reach the maximum concentration of ethanol, the fermentation temperature, sugar content and pH of the prickly pear juice were controlled. Fermentation was conducted under anaerobic conditions at 20 °C, in duplicate, and employing covered stainless-steel tanks. In order to increase the final alcoholic degree, the sugar content was increased until 14 °Brix before the fermentation finished, by adding commercial white refined beet sugar (AB Azucarera Iberia, Madrid, Spain). The final alcohol value reached in the prickly pear wine was 8.7% (v/v).

Vinegar processing

The acetification process was conducted using surface and submerged cultures. The obtained wine was inoculated by a pure culture of thermotolerant acetic acid bacteria (AAB) previously identified as *Acetobacter malorum* and *Gluconobacter oxydans*. In order to proliferate and obtain a fresh bacterium, the selected AAB were suspended in a liquid medium and submitted to a vigorous agitation (at 30 °C during one night). When the bacterial charge was in the exponential phase (OD 600 nm = 1.2) the cells were collected to perform the acetic fermentation.

Surface culture fermentation

The surface culture fermentation method was realized in sterilized Erlenmeyer flasks (500 mL). These flasks were filled to 50% capacity (250 mL) with prickly pear wine that was inoculated separately with 10% (v/v) of precultured inoculum of *Acetobacter malorum* and *Gluconobacter oxydans*. During the acetic acid fermentation, the flasks were incubated at 30 and 37 °C in duplicate and in a static condition, enabling atmospheric oxygen to slowly penetrate into the flasks. Acetic acid content produced by AAB was measured in triplicate every 3 weeks by titration with NaOH and the acidity was expressed as grams of acetic acid per 100 mL vinegar. When the acidity stopped increasing, the fermentation finished and the resulting vinegar was stored for chemical analysis. The processes at 30 and 37 °C took 2 and 3 months, respectively.

Submerged culture fermentation

The submerged culture was performed at different temperatures (30, 37 and 40 °C) in a Frings Acetator (Heinrich Frings, Bonn, Germany) of 8 L capacity using a semi-continuous mode, and the maximum volume of medium employed was 3 L. The prepared fermentation medium enriched previously with 0.35 g L^{-1} diammonium phosphate (Agrovin) was inoculated separately with 10% (v/v) inoculum of each AAB. This started culture was

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previously activated in a mixture of prickly pear wine and water. When acetic acid content was around 1 g acetic acid per 100 mL solution, the starting culture was accomplished, and 1 L wine was added to start the acetification process. The loading/unloading steps during the process were defined by the measurement of alcohol content using a calibrated alcohol sensor (Alkosens, Heinrich Frings). A fixed volume of vinegar was unloaded when the alcohol content decreased to 0.3% v/v, and then the loading phase initiated until reaching a volume of 3 L with a low speed, at around 0.25 L h^{-1} to prevent any sharp changes in the broth medium. This step is called a cycle. In the following cycles, 1 L vinegar was employed in the unloading step and 1 L wine in the loading step. An air flow rate of 7.5 L h^{-1} was used during the process and all the parameters were controlled by a computer program (Acetomat S7, Siemens AG, Munich, Germany). Acetic acid content of the final vinegar was also measured in triplicate by titration with NaOH.

Analysis of phenolic compounds

All the vinegars obtained by two different acetification processes and under different parameters were subjected to analysis using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with diode array detection (DAD) and with BEH C18 column (100 mm length × 2.1 mm ID, with 1.7 µm particle size). The samples were previously filtered through a combination of nylon filters of 0.45 and 0.22 µm diameter (Scharlab, Barcelona, Spain). The identification of phenolic compounds was performed using the chromatograms obtained at 280 nm (for gallic acid, hydroxytyrosol, epigallocatechin, catechin, tyrosol, vanillic acid, syringic acid, ethyl gallate, *m*-coumaric acid, hesperidin and naringenin), 320 nm (for protocatechualdehyde, *p*-coumaric acid, ferulic acid, quercetin and cinnamic acid) and 255 nm (for *p*-hydroxybenzoic acid) by comparing retention times and UV–visible spectra with those provided from commercial standards (Fluka, Buchs, Switzerland; Sigma, Steinheim, Germany; and Eastman Kodak, Rochester, NY, USA). For quantification, the calibration curves were obtained with the corresponding standards at seven levels of concentration, except for hydroxytyrosol, which was quantified as tyrosol. All analyses were carried out in duplicate.

Analysis of volatile compounds

Volatile compounds of prickly pear vinegars were analyzed according to the method previously proposed by Guerrero *et al.*[24] The analysis was conducted using stir bar sorptive extraction–gas chromatography–mass spectrometry (SBSE-GC-MS). Polydimethylsiloxane commercial stir bars of 10 mm length × 0.5 mm film thickness (Gerstel, Mülheim a/d Ruhr, Germany) were used to extract the volatile compounds from the samples. For identification, for all the detected compounds, the retention indices were determined (on a DB-Wax polar column) and compared with those from the literature, and the spectra analogy was confirmed using the Wiley 7 N Edition Library (Wiley Registry of Mass Spectral Data, 7th Edition, 2000). Semi-quantitative data were obtained by measuring the base ion peak relative area in relation to the internal standard, 4-methyl-2-pentanol. All analyses were realized in duplicate.

Statistical study

Statistical analysis for all the obtained data was carried out using Statistica 12.5 software (StatSoft, Inc., Tulsa, OK, USA). Analysis of variance (ANOVA, $P < 0.05$) with Tukey's test was used to determine significant differences between the compounds of vinegars, followed by principal component analysis (PCA) and cluster analysis (CA).

RESULTS AND DISCUSSION

Acidity

Acetic acid is the principal acid of interest in vinegar production. The concentration of acetic acid is highly related to the dominance of acetic acid bacteria (AAB) present in the vinegar. As can be seen in Table 1, a higher acidity was produced when the submerged culture method was employed, compared to the surface culture method. These results are in agreement with those obtained in previous studies, in which orange vinegar made by submerged culture attained a higher concentration of acetic acid if it was compared to that obtained by surface culture.[25] On the other hand, as can be seen in Table 1, when the fermentation temperature increased, the acetic acid content of the final vinegars decreased. Concretely, when surface culture method was employed, a drastic decrease in the acidity (around 5 points) was observed when passing from 30 to 37 °C, and the acetic fermentation did not even start at 40 °C (Table 1). When the submerged culture method was employed, the effect of temperature on acidity was less important, perhaps due to the enhanced aeration of the process that favored a higher yield.[20] In addition, the type of bacteria employed for the production of vinegar seemed to be less influential and only in the case of submerged culture were slight differences appreciated when the two genera were compared, *A. malorum* being more productive in

Table 1. Mean acidity measurements and standard deviations (SD) of vinegars samples obtained by surface and submerged cultures with two bacteria and at different temperatures (surface culture: $N = 6$; submerged culture: $N = 3$)

	Surface culture		Submerged culture	
		Acidity (g 100 mL^{-1})		Acidity (g 100 mL^{-1})
Bacteria	Temperature	Mean ± SD	Temperature	Mean ± SD
Acetobacter malorum	30 °C	7.01 ± 0.35	30 °C	8.78 ± 0.04
	37 °C	2.19 ± 0.30	37 °C	7.82 ± 0.12
	40 °C	—	40 °C	7.84 ± 0.09
Gluconobacter oxydans	30 °C	7.56 ± 0.44	30 °C	8.32 ± 0.09
	37 °C	1.97 ± 0.03	37 °C	7.60 ± 0.17
	40 °C	—	40 °C	7.06 ± 0.15

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Table 2. Mean concentrations (mg L^{-1}) and standard deviations (SD) of phenolic compounds identified by UPLC-DAD in different vinegar samples produced by surface and submerged cultures with two bacteria and at different temperatures

Compound	Surface culture Mean ± SD	Submerged culture Mean ± SD	ANOVA *F*-ratio	ANOVA *P*-value
Gallic acid	1.59 ± 0.50	1.61 ± 0.46	0.01	0.9259
Hydroxytyrosol	1.24 ± 1.80b	NDa	17.59	0.0001*
Epigallocatechin	7.86 ± 1.24b	5.19 ± 3.45a	8.98	0.0042*
Catechin	NDa	4.49 ± 2.80b	40.80	0.0000*
Tyrosol	53.1 ± 7.9	51.8 ± 14.6	0.13	0.7239
Vanillic acid	0.346 ± 0.620a	1.09 ± 0.37b	29.24	0.0000*
Syringic acid	2.24 ± 0.32b	1.80 ± 0.54a	9.07	0.0041*
Hesperidin	8.72 ± 1.69	7.62 ± 2.31	2.92	0.0936
Naringenin	3.55 ± 0.80	3.62 ± 0.77	0.11	0.7463
Protocatechualdehyde	1.34 ± 0.11	1.36 ± 0.22	0.08	0.7728
p-Coumaric acid	1.04 ± 0.08b	0.682 ± 0.673a	4.50	0.0388*
Ferulic acid	1.29 ± 0.17	1.39 ± 0.33	1.24	0.2711
Quercetin	1.32 ± 0.35b	1.08 ± 0.31a	6.27	0.0156*
Cinnamic acid	0.12 ± 0.08	0.11 ± 0.09	0.22	0.6429
p-Hydroxybenzoic acid	1.03 ± 0.29a	1.64 ± 0.56b	16.84	0.0001*

For each row, different letters indicate significant differences at $P < 0.05$ (Tukey's test). ND, not detected.

terms of acidity, also at high temperatures such as 40 °C. However, both strains produced concentrations of acetic acid higher than 7 at all employed temperatures when the submerged culture was employed. These strains were isolated from prickly pear in a previous study and their thermotolerant character had already been observed.[26]

Phenolic compounds

Fifteen polyphenols were identified in the studied vinegar samples. In order to be able to compare both acetification processes, the information is presented in Table 2 taking into account this variable. ANOVA showed that among these compounds eight of them were significantly affected by the production method. As illustrated in Table 2, some compounds such as hydroxytyrosol, epigallocatechin, syringic acid, *p*-coumaric acid and quercetin were significantly higher in vinegar produced by surface culture if it was compared to submerged culture, whereas just three compounds (catechin, vanillic acid and *p*-hydroxybenzoic acid) were at a higher concentration in those vinegars obtained by the submerged method. For both acetification processes, a high concentration for tyrosol, hesperidin, naringenin and gallic acid was found. In agreement with our results, other authors showed that a higher concentration of phenolic compounds was observed when the surface culture acetification process was used to produce orange vinegar.[25] The differences observed between both acetification methods might be explained by the possible degradation of phenolic compounds when these are in contact with a high level of oxygenation during the acetic fermentation process, mainly by submerged culture. During submerged fermentation, the use of an excess of oxygen to ensure and accelerate the process could affect polyphenolic compounds, whereas oxygen availability is limited in surface culture because it is continuously consumed by acetic acid bacteria and therefore it does not affect phenolic composition.[14]

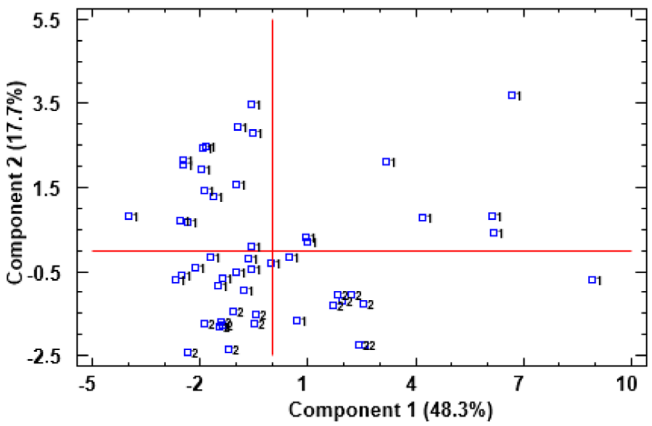

Figure 1. Principal component analysis on polyphenolic compounds. Distribution of all vinegar samples elaborated by submerged and surface cultures with two bacteria at different temperatures onto the plane defined by the first two PCs. 1: submerged culture, 2: surface culture.

Taking into account other variables such as temperature of fermentation, it was confirmed that the highest content of polyphenolic compounds was identified in vinegars produced at 37 °C by surface culture. The same result was found when the acetification process was conducted in submerged culture, which registered significant increases on phenolic compounds when the temperature changed from 30 to 40 °C. Furthermore, for both type of processes, the vinegars produced by *A. malorum* presented a greater concentration of phenolic compounds than those produced by *G. oxydans* (Supporting Information Table S1).

The data obtained were submitted to multivariate statistical study (principal component analysis, PCA) using the identified phenolic compounds as variables. The analysis revealed the existence of three PCs that explain 77.98% of the total variability (eigenvalues > 1). Figure 1 shows the distribution of all vinegar samples produced by submerged and surface cultures onto the plane defined by the first two PCs, which accounted for 66.04%

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Table 3. Retention times (RT, min), mean relative areas and standard deviation (SD) of volatile compounds identified by SBSE-GC-MS in different vinegar samples produced by surface and submerged cultures with two bacteria and at different temperatures

Compound	RT (min)	Surface culture Mean ± SD	Submerged culture Mean ± SD	ANOVA *F*-ratio	ANOVA *P*-value
Ethyl acetate	8.89	0.1665 ± 0.1272a	2.2349 ± 1.3488b	18.3657	0.0004*
1,3-Dioxolane, 2,4,5-trimethyl-	11.72	0.0304 ± 0.0488	0.3327 ± 0.4793	3.1051	0.0950
Diacetyl	13.08	0.0076 ± 0.0117	0.0035 ± 0.0083	0.8516	0.3683
Isobutyl acetate	15.00	0.0050 ± 0.0032a	0.1147 ± 0.1215b	6.3981	0.0210*
Hexanal	18.01	0.0028 ± 0.0013	0.0023 ± 0.0012	0.8205	0.3770
2-Methyl-1-propanol	19.04	0.0082 ± 0.0125	0.0146 ± 0.0109	1.4768	0.2400
Isoamyl acetate	19.74	0.0515 ± 0.0336a	0.6198 ± 0.6553b	5.8968	0.0259*
Acetic acid, pentyl ester	21.59	ND	0.0703 ± 0.1238	2.5316	0.1290
2,6-Dimethyl-4-heptanone	21.69	0.0061 ± 0.0051	0.0108 ± 0.0247	0.2756	0.6060
1-Butanol, 2-methyl-	23.11	0.0573 ± 0.0947	0.0788 ± 0.0717	0.3374	0.5686
3-Meth-1-butanol	23.24	0.0645 ± 0.0962	0.1176 ± 0.0599	2.3383	0.1436
Hexanoic acid, ethyl ester	23.85	0.0047 ± 0.0044	0.0069 ± 0.0074	0.5514	0.4673
Styrene	24.50	0.0003 ± 0.0002a	0.0051 ± 0.0024b	32.1178	0.0000*
1-Pentanol	24.58	0.0003 ± 0.0004b	NDa	5.1605	0.0356*
Hexyl acetate	25.50	0.0004 ± 0.0008	0.0487 ± 0.0726	3.4709	0.0789
Acetoin	25.72	0.1164 ± 0.1708a	0.2413 ± 0.0683b	5.2831	0.0337*
Acetol	26.08	0.0112 ± 0.0095b	NDa	17.2485	0.0006*
2-Octanone	26.12	NDa	0.0293 ± 0.0107b	59.1093	0.0000*
(*Z*)-3-Hexen-1-ol acetate	26.90	ND	0.0009 ± 0.0027	0.9516	0.3422
E-3-Hexenyl acetate	26.91	ND	0.0012 ± 0.0029	1.4350	0.2465
Ethyl lactate	27.55	0.4379 ± 0.2315b	0.2208 ± 0.1043a	8.2304	0.0102*
1-Hexanol	28.14	0.0019 ± 0.0035	ND	3.5708	0.0750
(*Z*)-3-Hexen-1-ol	29.20	0.0005 ± 0.0009	ND	3.5773	0.0748
Acetic acid	30.79	0.2435 ± 0.2555	0.4289 ± 0.1972	3.3570	0.0835
Octanoic acid, ethyl ester	31.46	ND	0.0006 ± 0.0015	1.3829	0.2549
trans-Linalool oxide	31.60	0.0080 ± 0.0061b	NDa	20.8195	0.0002*
cis-Linalool oxide	32.61	0.0055 ± 0.0016b	0.0012 ± 0.0010a	53.8457	0.0000*
1-Hexanol, 2-ethyl-	33.05	0.0160 ± 0.0140	0.0120 ± 0.0054	0.8414	0.3711
Benzaldehyde	34.41	0.0110 ± 0.0075b	0.0045 ± 0.0021a	8.2111	0.0103*
2,3-Butanediol	34.67	0.0176 ± 0.0083b	0.0090 ± 0.0040a	9.7750	0.0058*
Linalool	35.02	0.0023 ± 0.0026a	0.0106 ± 0.0068b	10.8851	0.0040*
Isobutyric acid	35.40	0.0163 ± 0.0143	0.0210 ± 0.0111	0.6823	0.4196
1-Octanol	35.53	0.0022 ± 0.0040	ND	3.5870	0.0744
Butanoic acid	37.65	0.0009 ± 0.0016	0.0009 ± 0.0013	0.0040	0.9503
Sulfide, allyl methyl	38.12	0.0044 ± 0.0018	0.0084 ± 0.0077	2.1144	0.1631
Isovaleric acid	39.17	0.0328 ± 0.0433a	0.0713 ± 0.0372b	4.5160	0.0477*
1-Nonanol	39.23	0.0058 ± 0.0048b	NDa	18.4621	0.0004*
Butanedioic acid, diethyl ester	39.62	0.0283 ± 0.0103b	0.0183 ± 0.0038a	9.5380	0.0063*
α-Terpineol	40.76	0.0091 ± 0.0056	0.0117 ± 0.0037	1.5389	0.2307
2-Nonen-1-ol, (*E*)-	41.13	0.0005 ± 0.0009	0.0009 ± 0.0009	1.2137	0.2851
cis-6-Nonenol	41.22	0.0287 ± 0.0379b	0.0012 ± 0.0017a	6.4823	0.0203*
Benzyl acetate	41.59	0.0050 ± 0.0057	0.0151 ± 0.0129	4.2483	0.0540
β-Citronellol	42.96	0.0034 ± 0.0016	0.0041 ± 0.0020	0.8122	0.3794
trans, cis-2,6-Nonadien-1-ol	42.96	ND	0.0001 ± 0.0003	1.2587	0.2766
Citronellol	42.96	0.0033 ± 0.0016	0.0056 ± 0.0028	4.3247	0.0521
Methyl salicylate	43.47	0.0017 ± 0.0015a	0.0046 ± 0.0019b	12.3534	0.0025*
Ethyl phenylacetate	43.60	0.0256 ± 0.0327	0.0153 ± 0.0038	1.1961	0.2885
Phenethyl acetate	44.78	0.2014 ± 0.1795	0.2811 ± 0.2164	0.7406	0.4008
β-Damascenone	45.24	ND	0.0004 ± 0.0007	3.3579	0.0835
Hexanoic acid	45.41	0.0174 ± 0.0146a	0.0285 ± 0.0059b	5.6439	0.0288*
Geraniol	45.74	0.0022 ± 0.0030a	0.0062 ± 0.0030b	8.3967	0.0096*
cis-Geranylacetone	46.28	0.0024 ± 0.0016a	0.0056 ± 0.0031b	7.1769	0.0153*
Benzyl alcohol	46.58	0.0115 ± 0.0018b	0.0061 ± 0.0014a	56.7691	0.0000*
Benzenepropanoic acid, ethyl ester	47.18	0.0074 ± 0.0083	0.0094 ± 0.0037	0.5826	0.4552
Phenylethyl alcohol	47.88	0.3119 ± 0.1463b	0.1640 ± 0.0338a	11.6519	0.0031*

Table 3. Continued

Compound	RT (min)	Surface culture Mean ± SD	Submerged culture Mean ± SD	ANOVA *F*-ratio	ANOVA *P*-value
3-Phenyl-1-propanol, acetate	49.09	0.0098 ± 0.0109	0.0107 ± 0.0094	0.0361	0.8515
Phenol	50.73	0.0034 ± 0.0008	0.0038 ± 0.0012	0.5968	0.4498
4-Hydroxynonanoic acid lactone	52.19	0.0513 ± 0.0065b	0.0329 ± 0.0168a	8.6319	0.0088*
Benzenepropanol	52.31	0.0053 ± 0.0021b	0.0027 ± 0.0012a	12.8808	0.0021*
Octanoic acid	52.57	0.0958 ± 0.0746	0.1458 ± 0.0311	4.3455	0.0516
Ethyl cinnamate	55.18	0.0034 ± 0.0023	0.0034 ± 0.0010	0.0001	0.9923
Cinnamyl acetate	55.67	0.0004 ± 0.0004	0.0025 ± 0.0083	0.4770	0.4986
Nonanoic acid	55.92	0.0385 ± 0.0341	0.0628 ± 0.0255	3.3339	0.0845
Thymol	56.40	0.0044 ± 0.0013	0.0051 ± 0.0011	1.6265	0.2184
Decanoic acid	59.15	0.0213 ± 0.0205a	0.0475 ± 0.0271b	5.3788	0.0323*
2-Nonenoic acid	59.62	0.0021 ± 0.0018	0.0015 ± 0.0017	0.5438	0.4704
Dihydromethyl jasmonate	59.95	0.0009 ± 0.0010a	0.0051 ± 0.0028b	16.2356	0.0008*
γ-Dodecalactone	63.08	0.0165 ± 0.0056b	0.0100 ± 0.0055a	6.6906	0.0186*
Dodecanoic acid	66.58	0.0072 ± 0.0070a	0.0626 ± 0.0665b	5.4262	0.0317*
Tetradecanoic acid	78.46	0.0035 ± 0.0032a	0.0090 ± 0.0059b	5.7110	0.0280*

For each row, different letters indicate significant differences at $P < 0.05$ (Tukey's test). ND, not detected.

of the total variability. As can be seen, these two PCs were able to separate the vinegars elaborated by the two acetification processes. A clear separation was visualized for the vinegar samples from the submerged culture, which were separated by PC2 and grouped at the top of the biplot. The compounds that contributed most to PC1 were tyrosol, hesperidin, naringenin, protocatechualdehyde and ferulic acid, whereas the phenolic compounds that contributed with a greater influence on PC2 were catechin, *p*-coumaric acid, ferulic acid and *p*-hydroxybenzoic acid.

Volatile compounds

During acetic fermentation, one of the most significant factors on the physicochemical characteristics of fruit vinegar is the type of acetification process that is employed.[20] To examine the

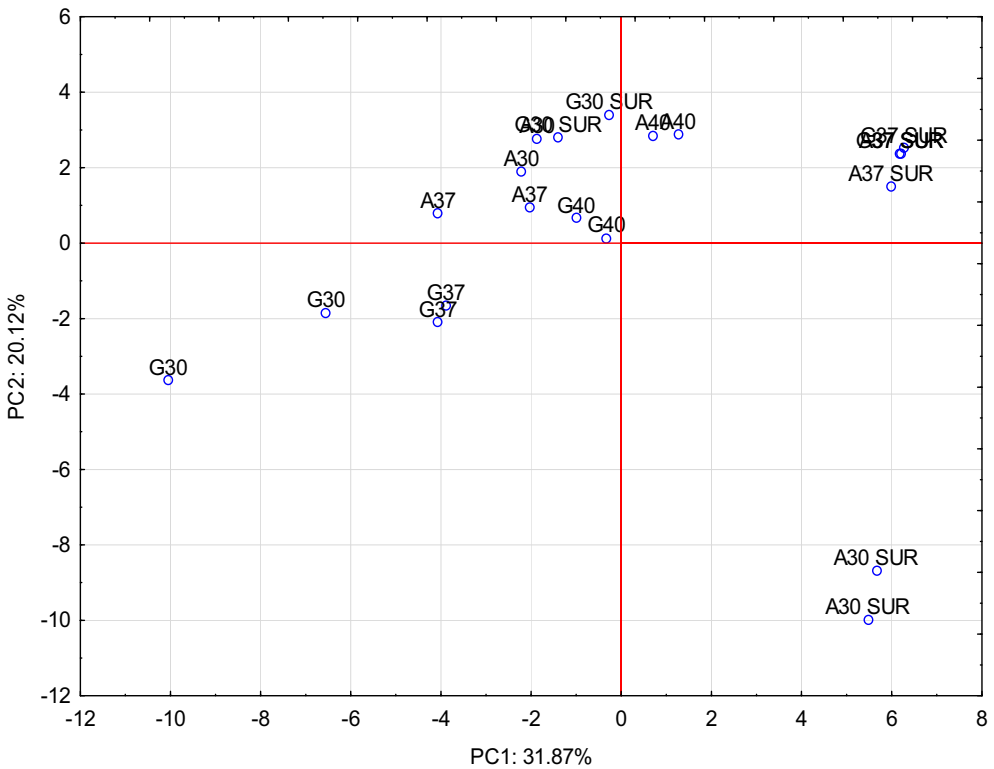

Figure 2. Principal component analysis on volatile compounds. Distribution of all vinegar samples from submerged and surface culture onto the plane defined by the first two PCs. SUR, surface culture; Numbers 30, 37 and 40 represent the temperatures degrees. Letters A and G represent acetic acid bacteria; A: *Acetobacter malorum*; G: *Gluconobacter oxydans*.

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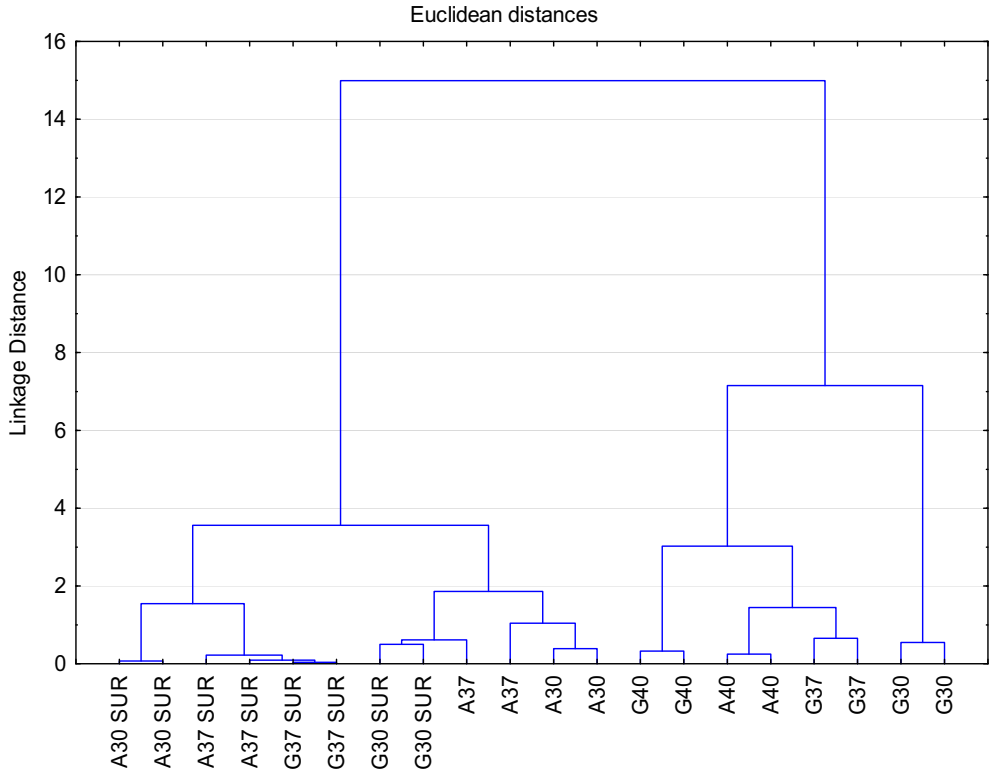

Figure 3. Cluster analysis on volatile compounds of vinegars produced by two acetification processes under different conditions. SUR, surface culture; Numbers 30, 37 and 40 represent temperatures (°C). Letters A and G represent acetic acid bacteria; A: *Acetobacter malorum*; G: *Gluconobacter oxydans*.

differences of the volatile composition in vinegar samples produced by both acetification processes, the obtained data were subjected to ANOVA, taking into account this variable. Table 3 presents a comparative study of the volatile composition between vinegars produced by submerged and surface culture. Seventy compounds were identified and the majority of these compounds presented significant differences, considering the type of acetification. As can be seen, the use of different methods for vinegar production influenced the volatile composition. The abundances of some compounds (mainly esters and acids) in the vinegars obtained by submerged culture were significantly higher than those in the vinegars produced by surface culture such as ethyl acetate, isobutyl acetate, isoamyl acetate, styrene, acetoin, 2-octanone, isovaleric acid, hexanoic acid and others (Table 3). The same observation was mentioned in a previous study, in which the concentration of several volatile compounds was significantly higher in orange vinegar produced by submerged culture compared to surface culture.[25] However, another study of vinegar production from Dimrit grape by submerged and surface methods showed that the use of the surface method for making Dimrit grape vinegar was better in terms of aroma composition than the submerged method.[27] The differences between both studies might be explained by a longer fermentation duration in the latter one. While in our study the submerged culture fermentation finished on the third day, in this other research the acetification period in the fermenter was significantly longer (17–18 days) and therefore a general loss of volatile compounds could have been produced. Another study exhibited an increase in the concentration of volatile compounds in red wine vinegar produced by surface culture acetification, which could be explained by the use of wooden barrels to perform the fermentation process.[22] On the other hand, in our case, some compounds (mainly alcohols) such as 1-pentanol, acetol, ethyl lactate, *trans*-linalool oxide, *cis*-linalool oxide, benzaldehyde, 2,3-butanediol, linalool, *cis*-6-nonenol, benzyl alcohol, phenylethyl alcohol and benzenepropanol presented a higher concentration in prickly pear vinegar produced by the surface culture process. Other authors observed also the dominance of alcohols in the vinegar

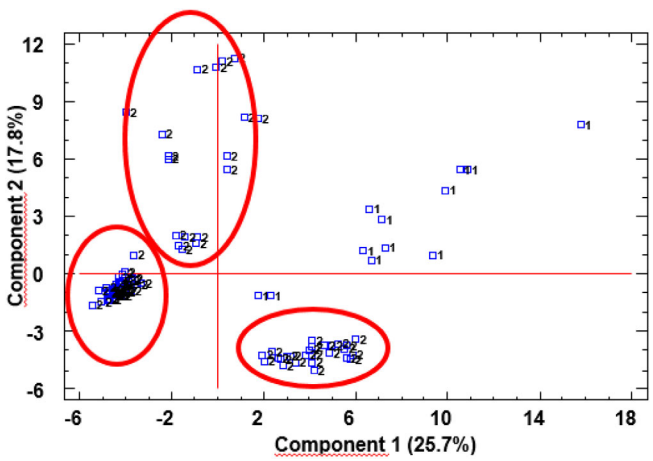

Figure 4. Principal component analysis obtained using data from polyphenolic and volatile composition of all the vinegar samples. Distribution of the samples onto the plane defined by the first two principal components. 1: Submerged culture, 2: Surface culture.

made by surface culture such as methyl alcohol, 1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2,3-butandiol.[27]

The results obtained from these two acetification processes at different temperatures and by different bacteria showed that the abundance of the volatile compounds was significantly affected by fermentation temperature, with clearly lower amounts as temperature increased, whereas few significant differences were observed when comparing the vinegars produced by the two AAB species (Supporting Information Table S2).

The data obtained were also submitted to multivariate statistical study (PCA). This revealed the existence of ten PCs that could explain 94.85% of the variability (eigenvalues > 1). Figure 2, shows the distribution of all vinegar samples produced by submerged and surface culture using different conditions (different temperatures and bacteria) onto the plane defined by the first two PCs, which explained 51.99% of the total variability. According to the biplot in Fig. 2, PC1 was able to separate vinegar samples of surface culture from the submerged culture. Vinegar samples produced at 30 and 37 °C by *Acetobacter malorum* in the surface culture method were located on the right side of the plot. Moreover, PC2 was able to separate vinegar samples from submerged culture produced by *Gluconobacter oxydans* at 30 and 37 °C (G30, G37) and those from surface culture produced by *Acetobacter malorum* at 30 °C (A30SUR), all of them with negative values for this PC, from the rest of samples. Some volatile compounds were strongly related to the first principal component (PC1) such as benzyl alcohol, methyl salicylate, decanoic acid, nonanoic acid, linalool and styrene, whereas the volatile compounds that contributed more to PC2 were ethyl phenylacetate, (*Z*)-3-hexen-1-ol, 1-octanol, 1-hexanol, 1-pentanol and *cis*-6-nonenol.

A cluster analysis was also conducted to look for homogeneous groups among samples. The Euclidean distance as metric and the Ward method as the amalgamation rule were taken into account. The obtained dendrogram illustrated in Fig. 3 showed that there were two main clusters that could be identified. With the exception of a few samples, these two groups corresponded to the type of acetification system. Only vinegars produced by *A. malorum* at 30 and 37 °C by submerged culture were grouped together with the rest of the vinegars obtained from surface culture. Thus the acetification system could differentiate the majority of vinegars in terms of their volatile composition.

Joint study of polyphenolic and volatile compounds

Data obtained from both volatile and polyphenolic compounds analysis were jointly considered and submitted to PCA. In this analysis, 14 PCs (eigenvalue > 1) were obtained to explain 89.60% of the total variability of samples. Figure 4 shows the distribution of all vinegar samples onto the plane defined by the first two PCs, which explained 43.52% of the total variance. As illustrated in Fig. 4, these two PCs were able to differentiate between all the vinegars obtained under different fermentation conditions. Concerning all vinegars produced by surface culture, it appears that vinegar samples produced at 30 °C employing *A. malorum* were grouped in the same quarter of the plot, with negative values for PC1 and positive ones for PC2 (top left corner). On the left downside of the plot, all vinegars produced at 37 °C by *A. malorum* and *G. oxydans* by surface culture were aggregated together. Those produced at 30 °C by surface culture and *G. oxydans* were also placed in negative values of PC2 (bottom right quarter of the biplot). On the other hand, all samples from submerged culture were aggregated together in the same quarter (top right side). This could indicate that neither of the used parameters (temperature and bacteria) could significantly affect the volatile and phenolic compounds of vinegar made by submerged culture. The compounds that showed a greater contribution to PC1 were methyl salicylate, decanoic acid, nonanoic acid, hexanoic acid, benzyl acetate, isovaleric acid and acetoin; therefore, this first PC could be more related to volatile acids. Contrariwise, most of the compounds that contributed more to PC2 were alcohols such as 1-octanol, ethyl phenylacetate, *cis*-6-nonenol, (*Z*)-3-hexen-1-ol, 1-pentanol and 3-methyl-1-butanol. Thus it appears that these two PCs were related to the volatile compounds and not to polyphenols.

CONCLUSIONS

In this study, prickly pear vinegar was produced by two acetification processes and under different conditions (different temperatures and bacteria). High yields of acetic acid were obtained when temperatures higher than usual were employed, such as 37 or 40 °C. It was also shown that acetification conditions affected the chemical characteristics of vinegars. Statistical analysis showed that the amounts of phenolic compounds in vinegar from surface culture acetification were higher and this might affect positively the quality of vinegar by raising its nutritional value. On the other hand, it appeared that the submerged culture was a faster and more efficient acetification method than the surface culture because of the higher concentration of acetic acid in vinegar. It was found that some volatile compounds, especially esters and acids, were significantly higher in vinegars from submerged culture, whereas in the vinegars obtained by the slow surface method alcohols were the most abundant compounds. It has been demonstrated that prickly pear fruit could serve as a new suitable substrate for vinegar production. The application of this xerophytic plant as a substrate on an industrial scale could add value to the bio-economy resources of producing countries.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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