Ann Microbiol (2015) 65:2293–2299
DOI 10.1007/s13213-015-1071-8

ORIGINAL ARTICLE

Manufacture of a beverage from cactus pear juice using "tea fungus" fermentation

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Received: 20 August 2014 / Accepted: 2 March 2015 / Published online: 27 March 2015

Abstract Kombucha is a beverage that is prepared by fermenting sweetened black tea using a tea fungus, which is a symbiotic culture of acetic acid bacteria and yeasts. In the present study, cactus pear juice was used as a substrate for kombucha fermentation in order to develop a new beverage with enhanced nutritional properties. Changes in chemical and microbiological parameters of the fermented juice were determined during fermentation. The growth of microflora induced a reduction in pH from 5.1 to 3.5 and a 23 % increase in total phenol content after 6 days of fermentation. The antioxidant potential of the beverage was also improved, reaching 81 % and 65 % as determined, respectively, by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) radical scavenging assays. The improved antioxidant capacity was attributed to betalains and to certain metabolic products formed during the fermentation process. Furthermore, the fermented juice showed antimicrobial activity against the tested Gram-positive (*Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 11778, *Staphylococcus epidermidis* ATCC 12228 and *Enterococcus faecalis* ATCC 10541) and Gram-negative bacteria (*Escherichia coli* ATCC 10536, *Klebsiella pneumoniae* ATCC 10031, and *Pseudomonas aeruginosa* ATCC 9027), which was attributed to its acetic acid content. Sensory evaluation of fresh juice and juice after 6 and 12 days of fermentation by a taste panel showed high acceptability of the juice after the first 6 days of fermentation, as the cactus pear juice taste qualities were still present, without the higher acidity that some panelists found unacceptable after longer fermentation.

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Keywords Cactus pear juice · Tea fungus · Antioxidant activity · Antimicrobial activity · Sensory evaluation

Introduction

The cactus pear "*Opuntia ficus-indica*" is a member of the plant family Cactaceae. A native of Mexico, the plant grows well in Mediterranean regions. The cactus pear fruit has garnered increasing attention within the research community in recent years, which can be explained by a variety of factors. First, the fruit is rich in important nutrients such as vitamins, amino acids, minerals, polyphenols, betalains, and indicaxanthin (Castellar et al. 2003; Piga 2004; Zafra-Rojas et al. 2013). Second, studies have reported various associated health benefits, including anti-ulcerogenic (Galati et al. 2003), antioxidant (Dehbi et al. 2013), anti-cancer (Zou et al. 2005), and anti- inflammatory effects (Park et al. 2001).

Unfortunately, the cactus pear has a high pH level and very low acidity, limiting long-term storage and worldwide distribution. The fruits' short shelf-life requires processing techniques capable of providing products of high nutritional as well as sensorial properties (Sepúlveda 1998; Sáenz 2000). Consequently, numerous strategies have been suggested, but these have not yet been incorporated into industrial production (Feugang et al. 2006). Fermentation is known to be an effective method for ensuring the stability and safety of food products. Moreover, fermented foods are high in sensory and nutritive properties. Fermentation of cactus pear juice using a "tea fungus" can be regarded as an opportunity to produce a new beverage and to increase its health benefits. The tea fungus represents a symbiotic relationship between acetic acid bacteria (Greenwalt et al. 2000) and yeasts (Mayser et al. 1995; Liu et al. 1996), which can ferment a mixture of black tea and sucrose into a refreshing sour and slightly effervescent beverage known as kombucha. Several medical studies have

reported beneficial health effects associated with kombucha (Dufresne and Farnworth 2000).

The objective of this study was to explore the possibility of making a fermented beverage using cactus pear juice with a tea culture used as inoculum, and to elucidate the relationship between the fermentation time and beverage composition.

Material and methods

The cactus pear juice preparation

Cactus pear fruit from purple Tunisian cultivars (Table 1) was obtained from the western region of Tunisia (Kasserine). The fruits were gently washed manually and peeled. To extract the juice, the pulp was homogenized using a blender (Moulinex LM9001B1) and then passed through a strainer to remove the seeds.

Tea fungus inoculum

The tea fungus was purchased from a supplier of traditionally prepared Russian black tea. Four hundred milliliters of water was boiled with 40 g saccharose, and 4.8 g black tea was added and left to draw for 5 min. After cooling, the sweet tea was filtered, transferred into a sterile glass vessel (1 l), and then inoculated with the kombucha culture (3 % of cellulose pellicles and 10 % of kombucha beverage from a previous fermentation). The glass vessel was covered with a cotton cloth and then incubated for 15 days at 30 °C in the dark to avoid oxidation of the phenolic compounds.

Culture conditions

Erlenmeyer flasks with 300 ml of cactus pear juice were inoculated with tea fungus inoculum (3 % of cellulose pellicles and 10 % of kombucha beverage). The mixture was then incubated in darkness for 12 days at 30 °C.

Table 1 Physicochemical characteristics of fresh cactus pear juice and juice fermented using a fungus tea

Parameter	Fresh juice	Final product
pH	5.1±0.21	3.5±0.38
Degrees Brix	13.03±0.9	7.4±0.61
Titratable acidity (g/l)	1.53±0.21	6.91±0.13
Betacyanin (mg/l)	33.1±1.34	34.65±2.34
Betaxanthin (mg/l)	14.9±1.11	16.05±0.03
Total phenols (mg GAE/l)	660±14.54	857±17.24

Analytical methods

pH and titratable acidity

The pH of fermented juice was determined with a pH meter (Mettler-Toledo EL20). Titratable acidity was assessed by titrating 10 ml of diluted juice (1:5 juice-to-water, by volume) with 0.1 M NaOH using phenolphthalein as the visual endpoint indicator. The total acidity was calculated (as gluconic acid) by multiplying the volume of NaOH needed to titrate the sample by 1.96 (Greenwalt et al. 1998). Brix degrees was generally used as an indicator of soluble solid content (%). The total soluble solids of the juice samples were evaluated using a handheld refractometer (RF.5532 Euromex Brix hand refractometer).

Determination of total phenol content

The phenol content of the juice was determined according to the method described by Stintzing et al. (2005). One hundred microliters of diluted sample (1:5) was supplemented with 500 μl of 10 % Folin–Ciocalteu reagent and 400 μl of sodium carbonate solution (7.5 %), and was then incubated at room temperature for 30 min.

The optical density was measured at 765 nm using a spectrophotometer (Jenway 63200 UV/Vis). The concentration of total phenol was expressed as mg of gallic acid equivalents/l (mg GAE/l).

Determination of betalains

Betalains were extracted with methanol – water (80–20 v/v) (Fernandez and Almela 2001). Betacyanin and betaxanthin content was determined according to Cay and Corke (1999). Betacyanin was detected at 538 nm and betaxanthin at 480 nm. For betacyanin, the extinction coefficient was 60, 000 l/(mol·cm) and MW=550 g/mol, and for betaxanthin it was 48,000 l/(mol·cm) and MW=308 g /mol. The following equation was used:

$$\text{Betacyanin or betaxanthin content}(\text{mg/l}) = [(A \times MW \times 1000)/\varepsilon \times l]$$

where A = absorbance at 535 or 480 nm, MW is molecular weight, ε is the extinction coefficient, and l is the width of the spectrophotometer cell (1 cm).

Total antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The method used was described by Zafra-Rojas et al. (2013); this assay is based on the measurement of the reducing ability

of antioxidants toward $DPPH^+$. The sample was diluted in deionized water (1:50). An ethanolic solution (0.074 mg/ml) of the stable DPPH radical was prepared. One hundred microliters of diluted sample was added to 500 μl of the DPPH solution, and the mixture was incubated at room temperature for 1 h. The solution was stirred and centrifuged at 3,000 rpm for 10 min, and absorbance was measured at 520 nm.

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assay

The method used was based on Delgado-Andrade et al. (2005). The radical cation $ABTS^+$ was produced by the reaction of 7 mmol/l ABTS stock solution with 2.45 mmol/l potassium persulfate under conditions of darkness and room temperature for 16 h before use. The $ABTS^+$ solution was diluted with deionized water to an absorbance of 0.70±0.10 at 734 nm. Fifty microliters of juice samples diluted tenfold were mixed with 950 μl $ABTS^+$ solution; the concentration of antioxidant achieving the same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was calculated in terms of Trolox equivalent antioxidant capacity (TEAC) at 6-min contact.

The equation used for calculating the scavenging capacity of the juice was:

$$\text{DPPH or ABTS radical scavenging activity \%} = \left[\left(A_{control} - A_{sample}\right)/A_{control}\right] \times 100$$

where $A_{control}$ is the absorbance of the blank control (containing all reagents except the sample), and A_{sample} is the absorbance of the test sample.

Microbiological analysis

The growth rates of yeasts and acetic acid bacteria were determined using potato dextrose agar (PDA, Merck, Germany) and Kneifel medium, respectively. The Kneifel medium (OIV 2010) consisted of 30 g/l of yeast extracts, 1 ml/l of bromocresol green (2.2 %), and 20 g/l of agar. The culture medium was also supplemented with 20 ml/l ethanol 95 %, 100 mg/l of natamycin to inhibit the growth of yeasts, and 12.5 mg/l of penicillin to eradicate the growth of lactic acid bacteria after autoclaving. Blue colonies represented *Acetobacter*, and green colonies represented *Gluconobacter*.

Antimicrobial activity

The antimicrobial activity was determined using the well diffusion method and was tested with the following bacteria: *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 10541, *Bacillus cereus* ATCC 11778, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 10536.

The strains were grown on Mueller-Hinton slants at 37 °C for 24. After incubation, the cells were washed from the agar surface and suspended in sterile physiological solution. The bacterial suspensions were adjusted to a concentration of 10^7 CFU/ml. A volume of 1 ml of this suspension was homogenized with 19 ml of melted Mueller-Hinton agar and poured into petri dishes. Three wells (9 mm in diameter) were cut into the agar media, and 100 μl of juice was then poured into the wells. Inoculated plates were incubated at 37 °C for 24 h, and zones of inhibition were measured in cm. Chloramphenicol (30 μg) was used as a reference control to evaluate the susceptibility of the tested strains.

Descriptive analysis

Sensory evaluation tests were performed to determine the quality and acceptance of the fermented juice. Descriptive analysis was conducted on three samples: fresh juice and juice fermented for 6 and 12 days.

The sensory panel comprised ten students who had previously participated in descriptive panels. The panelists participated in four 1-h training sessions, where descriptors were developed. The final descriptors were chosen by the panelists after discussions during training. After the training period, samples were evaluated in duplicate.

In all cases, samples were presented in random order, and were served at an ambient temperature in coded clear plastic glasses. Potable water was available for rinsing the mouth between test samples. A scoring range of 1 to 9 (lowest to highest) was used for odor, sweetness, acidity, taste, color, and overall acceptability.

Statistical calculations

All data presented are the average of triplicate measurements± SD. Analysis of variance (ANOVA) testing was conducted using SPSS Statistics for Windows version 16.0 software.

Results and discussion

Cactus pear juice is consumed primarily for its nutritional characteristics. In order to increase its health benefits and stability, it was fermented using a tea fungus. The relationships between fermentation time and the composition of fermented cactus pear juice by the fungus tea were elucidated.

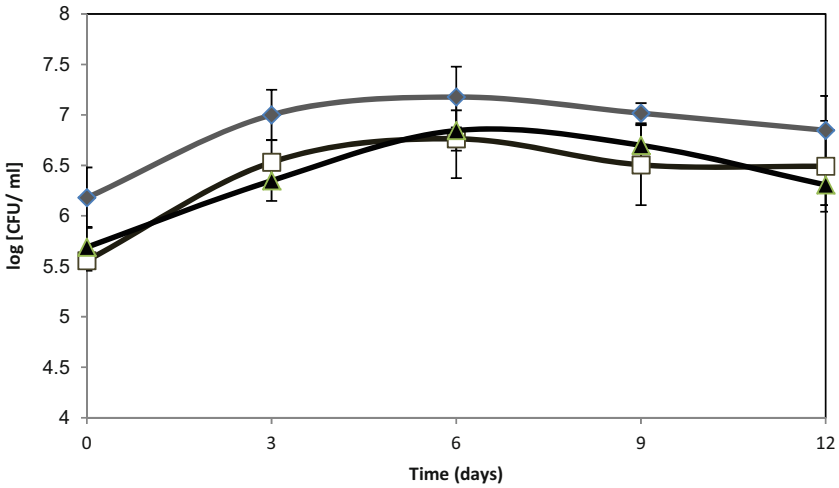

Fig. 1 Changes in the yeasts (♦), *Gluconobacter* (□), and *Acetobacter* (▲) number during the fermentation of cactus pear juice using a tea fungus

Changes in chemical and microbiological parameters during fermentation

The number of yeast cells (Fig. 1) increased until the sixth day of the process, and then stabilized as a consequence of nutrient limitations. Similar changes were observed for acetic acid bacteria. However, yeast cell concentrations were higher than those of acetic acid bacteria, given their faster rate of growth. Frank (1995) reported that the metabolic activity of acetic acid bacteria was lower than that of yeasts, as bacteria rely on yeasts as a source of nutrients. Teoh et al. (2004) noted that the yeast species present in the fermented tea reached maximum growth between the sixth and eighth days of fermentation, in contrast to Goh et al. (2012), who reported maximum yeast growth at the fourth day of black tea fermentation.

Fermentation resulted in a reduction in pH due to the formation of organic acids due to the physiological activity of yeasts and acetic acid bacteria (Fig. 2). The pH dropped gradually during fermentation, reaching a final value of 3.5. The concentration of total titratable acidity increased during the first 9 days, and remained relative constant thereafter, showing only a slight increase at the end of the fermentation process. These observations were largely in agreement with the results of other studies (Jayabalan et al. 2007; Malbaša et al. 2011). In addition, the pH reduction was correlated with the increase in acidity.

The cactus pear fruit is naturally rich in polyphenols, flavonoids. and betalains (Sawaya et al. 1983; Fernández et al. 2010). Total phenol content increased with fermentation time (Table 2), and was highest at day 9. These results are consistent with the findings of other studies (Chu and Chen 2006; Jayabalan et al. 2007). The increase can be explained by the occurrence of acid hydrolysis and bioconversion of condensed phenolic compounds.

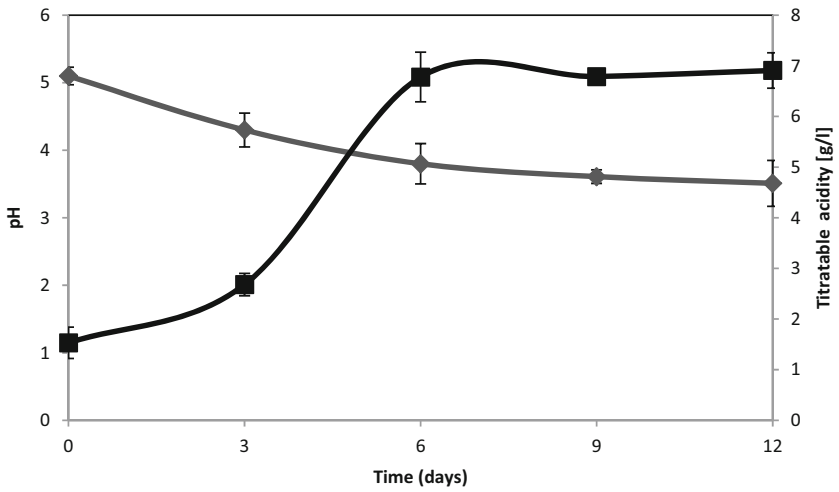

Fig. 2 Changes in pH values (♦) and titratable acidity (■) during the fermentation of cactus pear juice by tea fungus

Table 2 Changes in total phenols, betacyanins, betaxantins, and antioxidant activity during the fermentation of cactus pear juice by tea fungus

Fermentation time (days)	Total phenols (mg GAE/l)	Betacyanin (mg/l)	Betaxanthin (mg/l)	Betacyanin/ betaxanthin	Antioxidant activity	
					DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)
0	660±14.54	33.1±1.34	14.9±1.11	2.22	63±4.35	45.19±7.15
3	755±25.73	29.75±3.26	15.04±1.72	1.97	78.8±9.37	58±6.22
6	840±24.68	31.03±1.54	13.57±3.33	2.28	80.7±6.85	64,74±8.14
9	858±33.79	34.1±1.12	15.86±1.09	2.15	81.1±8.14	67.9±7.57
12	857±17.24	34.65±2.34	16.05±0.03	2.15	81±6.09	67.51±3.13
P	<0.01	0.018	0.525		0.02	<0.01

Two betalain derivatives were present in cactus pear juice: betacyanin, responsible for their red-purple color, and betaxanthin, for their yellow-orange color (Castellar et al. 2003; Livrea and Tesoriere 2009). An improvement was observed in betacyanin concentrations, which increased throughout the fermentation process, but a similar increase was not found for betaxanthin levels (Table 2). The color of the fermented juice was not modified, however, as the betaxanthin/betacyanin ratio remained constant. The acidity produced in the medium appears to preserve the quantity of pigments present. Jackman and Smith (1996) demonstrated that these pigments are relatively stable, in a pH range of 3 to 7.

In this study, two methods (ABTS, DPPH) were used to determinate the antioxidant activity in the fermented juice. The results obtained are shown in Table 2. The DPPH scavenging abilities of the fermented juice increased significantly until the sixth day of fermentation, and then stabilized. The ABTS radical scavenging capacity of cactus pear juice during fermentation also increased with culture time, and reached 1.5 times that of the control after 12 days. These results confirm that the antioxidant activity of fermented juice increased significantly as a result of fermentation. Scavenging effects were attributed to the sum of the antioxidant capabilities of many of the compounds present in the fruit, and to the synergistic effects of the interaction among certain metabolic products formed during the fermentation process.

Antimicrobial activity of fermented cactus juice

Fermented juice was inhibitory toward all tested bacteria, with the highest activity against Gram-negative bacteria (Table 3).

No test microorganisms exhibited vulnerability to the unfermented juice component. The observed antimicrobial activity was due to the organic acids, primarily acetic acid, and was eliminated when samples were neutralized. Many studies on kombucha have proven that its antimicrobial activity against pathogenic microorganisms is largely attributable to acetic acid (Steinkraus et al. 1996), which is a known antimicrobial agent (Sreeramulu et al. 2000). Adams (1985) demonstrated that pathogenic and spore-forming bacteria were inhibited at as little as 1 g/l of acetic acid.

Sensory evaluation

The sensory scores were based on the 9-point hedonic scale ratings assigned by the panelists (Fig. 3). The taste of fermented juice varied greatly, depending on the fermentation period. The juice fermented for 6 days was perceived as significantly sweeter by the taste panel than the sample fermented for 12 days. However, the latter had a sour smell, and panelist comments indicated that the juice was too acidic (scores>5). The growth of acetic acid bacteria and yeast during fermentation gave the juice a vinegary taste.

Table 3 Antimicrobial activity of fermented cactus pear juice (diameter, cm) of the halo zone, including the well (9 mm)

Test bacterium	Unfermented juice	Fermented juice	Neutralized fermented juice	Chloramphenicol	P
Escherichia coli ATCC 10536	0	2.9	0	3	<0.01
Pseudomonas aeruginosa ATCC 9027	0	2.6	0	3.2	<0.01
Klebsiella pneumoniae ATCC 10031	0	2.6	0	3.4	<0.01
Staphylococcus aureus ATCC 6538	0	2.2	0	3.1	<0.01
Enterococcus faecalis ATCC 10541	0	2.7	0	3.3	<0.01
Bacillus cereus ATCC 11778	0	2.5	0	2.1	<0.01
Staphylococcus epidermidis ATCC 12228	0	2.2	0	3	<0.01

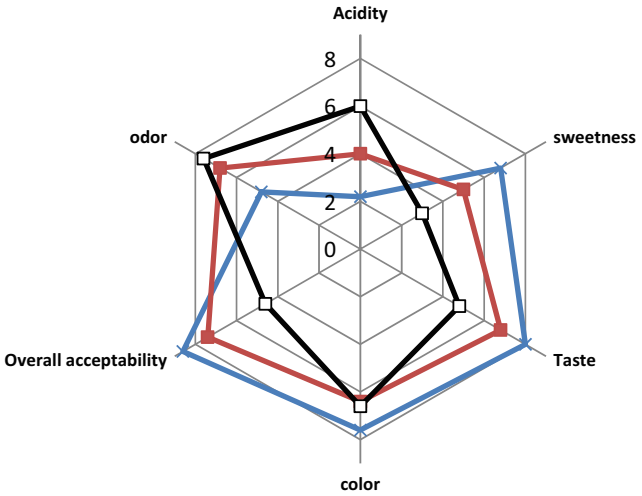

Fig. 3 Sensory evaluation of color, odor, acidity, sweetness, taste, and overall acceptability of fresh and fermented juice (x: fresh juice, ■: juice fermented for 6 days, □: juice fermented for 12 days)

The fermented juice had a bright, vibrant color compared to fresh juice, which was caused by chemical modifications of the phenols and pigments.

With regard to overall acceptance, no significant differences were found between the fresh juice and the sample fermented for 6 days ($P>0.05$). However, there was a tendency toward lower acceptance of the juice fermented for 12 days, with a negative correlation between acceptance and acidity.

Conclusions

The results of the present study confirm that cactus pear juice can be used as a fermentation substrate for the kombucha beverage. Six days of fermentation appeared sufficient for stabilizing and improving the nutritional characteristics of the juice. Indeed, its acidity and polyphenolic content increased, as well as its antioxidant activity. Furthermore, the obtained beverage showed antimicrobial activity against all pathogenic strains tested. In contrast, neutralized fermented juice showed no antimicrobial activity. Prolonged fermentation is not recommended, given that the accumulation of organic acids imparts a vinegary taste to the fermented juice.

Acknowledgments The authors wish to acknowledge the Ministry of Higher Education and Scientific Research for facilitating this work.

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