LWT - Food Science and Technology 50 (2013) 349–352

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Research note

Fermentation conditions of walnut milk beverage inoculated with kefir grains

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ARTICLE INFO

Article history:
Received 31 October 2011
Received in revised form
22 July 2012
Accepted 27 July 2012

Keywords:
Beverage
Fermentation
Kefir grains
Walnut

ABSTRACT

The aim of the present study was to evaluate the use of kefir grains as inoculum for the preparation of walnut milk beverage. The results showed that the single factor effect of fermentation time, fermentation temperature and sucrose concentration on walnut milk beverage fermentation was very significant ($P = 0.01$), and the single factor effect of inoculum size was significant ($P = 0.05$). The suggested optimum fermentation conditions are the following: fermentation temperature of 30 °C, fermentation time of 12 h, inoculum size of 3 g of kefir grains (wet weight) and sucrose concentration of 8 g/100 mL. Under the optimum fermentation conditions, the sensory evaluation score of the beverage reached its maximum value of 88, and the pH and titratable acidity of the beverage were respectively 4.16 and 72 °T. The viable cell counts of lactococci, lactobacilli and yeast surviving in the beverage were 8.2×10^7, 1.1×10^8 and 1.0×10^6 CFU/mL respectively.

1. Introduction

Walnut (*Juglans regia* L.) is a crop of high economic interest to the food industry: the edible part of the fruit (the seed or kernel) is consumed, fresh or toasted, alone or in other edible products. It is globally popular and valued for its nutritional, health and sensory attributes. The fresh natural kernels are consumed mainly as whole nuts or used in various confectioneries. In recent decades, several researchers have tried to ferment walnut milk using various species of lactic acid bacteria (LAB) in order to produce walnut-milk-based probiotic beverages (Hou, Xing, & Liu, 2008; Jing, 2006; Wang, 2010).

Kefir is a type of sour fermented milk in which kefir grains are employed as a starter culture (Bosch et al., 2006). The kefir grains have a specified structure and behave as biologically vital organisms. Various LAB present in kefir grains or kefir products were isolated and identified by physiological and biochemical tests (Chen, Wang, & Chen, 2008; Witthuhn, Schoeman, & Britz, 2005). Beside LAB, yeasts and acetic acid bacteria have also been shown to be present in kefir grains. Kefir culture has been used as a starter culture in cheese production (Dimitrellou, Kourkoutas, Banat, Marchant, & Koutinas, 2007; Kourkoutas et al., 2006) and has also been used for the production of bread with improved quality and shelf life (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005) and for the production of a new whey beverage with good organoleptic properties (Athanasiadis, Paraskevopoulou, Blekas, & Kiosseoglou, 2004).

The question arose whether the walnut milk could be fermented using kefir grains as a starter culture (i.e. as inoculum) and be processed into a probiotic beverage. Therefore, the aim of this study was to determine the effect of fermentation temperature, fermentation time, inoculum size and sucrose concentration on walnut milk fermentation, as well as, to optimize the fermentation conditions of the walnut milk beverage, and investigate the chemical, microbial and sensory characteristics of the walnut milk beverage.

2. Materials and methods

2.1. Kefir grains and inoculum preparation

The kefir grains used in this study were collected from Tibet, China and were preserved by the Food Chemistry Laboratory, Shanxi University. Frozen kefir grains were activated by placing 18 g of the grains in 500 mL of sterile cow milk, followed by incubation at 25 °C. After 24 h, the grains were sieved out and washed with cooled sterile distilled water. This step was repeated for three consecutive days (Witthuhn et al., 2005). The inoculum was prepared by transferring kefir grains (small grains of approximately 1–2 mm diameter) to a 250 mL Erlenmeyer flask containing 100 mL of sterile cow milk. Kefir grains cultivation was carried out statically in an incubator set at 25 °C for 24 h, renewed daily, for a duration of 7 days. Kefir grains as inoculum were stored at 4 °C prior to use.

2.2. Preparation of walnut milk

The methodologies proposed by Su, Chen, Zhang, Heng, and Liu (2008) were used for walnut milk preparation. Walnut fruits (*J. regia* L.) were collected from commercial plantations at Fenyang

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http://dx.doi.org/10.1016/j.lwt.2012.07.043

City, Shanxi Province, China. After cleaning, the fruits were dried at 30 ± 2 °C for 24 h and were then cracked and shelled manually. The skin of the nuts was separated by soaking the nuts in water overnight and removed by hand. After removing the skin, the nuts were mixed with 4.5 times their weight of distilled water (50 °C) and then ground in a blender for 5 min. The resultant slurry was filtered through a double-layered cheesecloth to yield walnut milk. Walnut milk was dispensed into containers and was autoclaved at 121 °C for 15 min.

2.3. Effect of fermentation temperature, time, inoculum size and sucrose concentration on walnut milk beverage fermentation

Autoclaved walnut milk (100 mL) and sucrose (8 g) were introduced into an Erlenmeyer flask. The effect of inoculum size on the fermented walnut milk beverage was studied by a series of fermentations conducted using 3, 5, 7 and 9 g of kefir grains (wet weight, containing 89 g/100 g moisture) as the inoculum per flask, carried out at 25 °C for 18 h. Furthermore, the effect of fermentation temperature was studied by a series of fermentations conducted using 5 g of kefir grains (wet weight) as the inoculum per flask, carried out at 25, 30, 35 and 40 °C respectively for 18 h. The effect of fermentation time was studied by a series of fermentations conducted for 9, 12, 15 and 18 h, carried out at 25 °C using 5 g of kefir grains (wet weight) as the inoculum per flask. To study the effect of sucrose concentration, 6, 8, 10 and 12 g of sucrose were added to 100 mL of the walnut milk, resulting in sucrose concentrations of 6, 8, 10 and 12 g/100 mL, respectively, and the fermentations were carried out at 25 °C for 18 h using 5 g of kefir grains (wet weight) as the inoculum per flask.

Fermentations were carried out statically in an incubator set for different times at different temperatures of the experimental design. At the end of the fermentation, the flasks were transferred to a 1–5 °C incubator for ripening for 12 h. Viable counts, pH, acidity and sensory analyses were determined at the end of each process.

2.4. Optimization of fermentation

The optimum fermentation conditions were studied through an orthogonal experimental design, where the initial inoculum size, fermentation temperature, fermentation time and sucrose concentrations were changed from 3 to 7 g of kefir grains (wet weight), 25–35 °C, 9–15 h, and 6–10 g sucrose/100 mL (Table 1).

2.5. Chemical analysis of the fermented walnut milk beverage

The pH of the fermented walnut milk beverage was measured using a pH meter (model 868; Orion, Beverly, MA, USA) fitted with a glass electrode. The titratable acidity (TA) was determined according to the Association of Official Analytical Chemists (AOAC, 1997) Method No. 947.05 and expressed as °T. One acidity unit (°T) means the amount of 1 mL 0.1 mol equiv/L NaOH used for the titration of 10 mL of beverage.

Initial pH and TA of the walnut milk were about 6.68 and 13.7 °T, respectively.

2.6. Viable cell counts determination

Representative 10 mL portions of duplicate beverage samples were blended with 90 mL of sterilized trisodium citrate (2 g/100 mL) solution and subjected to serial dilutions. The following tests on viable cell counts determination were performed (spread plate method): (i) lactococci counts on M17 agar (Difco, Detroit, MI, USA) at 37 °C (thermophilic strains) or 30 °C (mesophilic strains) for 72 h, (ii) lactobacilli counts on acidified MRS agar (Difco, Detroit, MI, USA) at 37 °C for 72 h anaerobically (Anaerobic jar, Anerocult C, Merck, Darmstadt, Germany), (iii) yeast counts on Yeast extract–Peptone–Dextrose agar (YPD, Difco, Detroit, MI, USA) at 30 °C for 72 h. The colonies that appeared on the plates were counted and recorded as colony forming units (CFU) per mL of beverage.

2.7. Sensory evaluation

The sensory properties of the walnut milk beverage were evaluated by a 5-member trained panel (2 men, 3 women; age range 21–50) from the School of Life Science, Shanxi University. The samples were served at 7–10 °C in plastic cups and were coded with three-digit numbers. Order of presentation of samples was randomized. The panel was asked to give scores on a 0–20 scale for color (milky white, light yellow and dark), a 0–30 scale for flavor (walnut fragrance, light smell and bad smell), a 0–30 scale for taste (walnut taste, slightly walnut taste and bitter taste) and a 0–20 scale for texture (uniformity, some sediment and sediment) (Jing, 2006). The beverages were evaluated in five replicates in each session and the mean score of the beverages for each quality attribute was calculated.

2.8. Experimentation and analysis

All experiments were replicated in three flasks and the data are presented as the mean and standard error of three independent experiments. Duncan's multiple range test (Du, 1985) was used to determine the significant differences among mean values at the $P = 0.05$ level. Analysis of variance of the orthogonal experimental results was carried out using the Statistical Analysis System software (SAS version 9.00, SAS Institute, Inc., 2000), the sources of variance being inoculum size, fermentation temperature, fermentation time and sucrose concentration.

Table 1
Sensory evaluation result of orthogonal experiment to assess optimal fermentation conditions.

Experimental no.	Inoculum size (g/100 mL)	Fermentation temperature (°C)	Time (h)	Sucrose concentration (g/100 mL)	Titratable acidity (°T)	Sensory evaluation scores
1	3	25	9	6	54 ± 1.2	71 ± 3.2^{c}
2	3	30	12	8	72 ± 2.0	88 ± 3.6^{a}
3	3	35	15	10	80 ± 2.1	79 ± 3.5^{b}
4	5	25	12	10	60 ± 1.5	80 ± 3.5^{b}
5	5	30	15	6	74 ± 1.9	82 ± 3.4^{b}
6	5	35	9	8	67 ± 1.5	74 ± 3.1^{bc}
7	7	25	15	8	62 ± 1.4	80 ± 3.6^{b}
8	7	30	9	10	68 ± 1.8	77 ± 3.4^{bc}
9	7	35	12	6	79 ± 2.1	74 ± 3.2^{bc}

Data represent means ± standard deviation ($n = 15$).
Values in the same column with different superscripts are significantly different ($P = 0.05$).