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Assessment of free and immobilized kefir culture in simultaneous alcoholic and malolactic cider fermentations

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Ethanol (PubChem CID: 702)
L-malic acid (PubChem CID: 222656)
Lactic acid (PubChem CID: 612)
3-methyl-1-butanol (isoamyl alcohol) (PubChem CID: 31260)
Acetaldehyde (PubChem CID: 177)
Ethyl acetate (PubChem CID: 8857)
2-methyl-1-propanol (isobutanol) (PubChem CID: 6560)
Acetic acid (PubChem CID: 176)
1-hexanol (PubChem CID: 8103)
Methanol (PubChem CID: 887)

ABSTRACT

The aim of the present study was to assess application of free or immobilized kefir culture on apple pieces and delignified cellulosic material (DCM) in simultaneous alcoholic and malolactic cider fermentations at a wide temperature range (5–45 °C). Repeated batch fermentations were continued for higher than 7 months, showing a high operational stability of the systems and were completed in less than 24 h with immobilized cells on DCM at 37 °C. Malic acid conversion up to 71.5% and ethanol productivity values up to 56.9 g/(Ld) were recorded, which could be adopted by the industrial sector. PCR-DGGE analysis of kefir culture showed no changes in microbial diversity during cell immobilization and fermentations. Analysis of major volatiles documented a significant increase of ethyl acetate content and a decrease of higher alcohols at low temperatures. HS-SPME GC/MS analysis revealed that the highest content of esters and total volatiles was observed in cider fermented by kefir culture immobilized on apple pieces at 20 °C. Principal Component Analysis showed that mainly the immobilization support rather than the temperature had a significant effect on volatile composition. Finally, the sensory evaluation ascertained the high quality of the new products.

1. Introduction

Cider-making is a complex process requiring both alcoholic and malolactic (ML) fermentation. During cider production, reduction of beverage acidity, microbial stability, and organoleptic improvement induced by ML fermentation are generally recognized as important phases for quality development. Therefore, attempts have been focused on simultaneous alcoholic and ML fermentation using mixed cultures consisting of yeasts and ML bacteria (Kourkoutas, Manolović, & Nedović, 2010).

Cell immobilization offers numerous advantages, such as enhanced fermentation productivity, ability for cell recycling, application of continuous configurations, enhanced cell stability and viability, and improvement of quality (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). In this vein, a few attempts

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have been carried out aiming at co-immobilization of *Saccharomyces cerevisiae* and ML bacteria (Nedovic et al., 2000; Servetas et al., 2013). Similarly, apple pieces and delignified cellulosic material (DCM) have been successfully tested as immobilization supports of yeasts and lactic acid bacteria for wine alcoholic and ML fermentations (Agouridis, Kopsahelis, Plessas, Koutinas, & Kanellaki, 2008; Kourkoutas, Komaitis, Koutinas, & Kanellaki, 2001; Koutinas et al., 2012).

Kefir is a consortium of microbes that is mainly used in the production of the low alcoholic, traditional Russian drink "kefir", where milk constitutes the initial fermenting substrate. This mixed culture consists of various yeasts (*Kluyveromyces*, *Candida*, *Saccharomyces*, and *Pichia*), various lactic acid bacteria of the genus *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and acetic acid bacteria (Garofalo et al., 2015; Kesmen & Kacmaz, 2011; Leite et al., 2012). Yeasts and lactic acid bacteria co-exist in a symbiotic association and are responsible for an acid-alcoholic fermentation. Kefir culture has been proposed for fermentation-upgrade of agro-industrial wastes (Kourkoutas et al., 2002; Plessas et al., 2008), as a starter in cheese production (Dimitrellou, Kandylis, Kourkoutas, Koutinas, & Kanellaki, 2015; Kourkoutas et al., 2006; Koutinas et al., 2010), and recently for fermentation of various vegetable and fruit juices (Corona et al., 2016; Randazzo et al., 2016). However, to the best of the authors' knowledge, it has not be tested for alcoholic and/or ML fermentations of cider.

Hence, the aim of the present study was to assess application of free or immobilized kefir culture on apple pieces and DCM in cider fermentations. Data documenting the ability of the kefir culture to conduct simultaneous alcoholic and ML fermentations successfully, avoiding biological competition among species, and quality improvement are presented.

2. Materials and methods

2.1. Kefir culture

Kefir starter culture was grown in synthetic medium consisting of 4% w/v glucose (Fluka, Switzerland), 0.4% w/v yeast extract (Fluka), 0.1% w/v $(NH_4)_2SO_4$ (Merck, Germany), 0.1% w/v KH_2PO_4 (Fluka), and 0.5% w/v $MgSO_4$ $7H_2O$ (Merck) at 30 °C for 24 h. The synthetic medium was sterilized at 121 °C for 15 min prior to use.

2.2. Apple juice

The concentrated apple juice used (100% apple juice Mr. Grand) was supplied by a local store. It had a pH value 3.1 and contained 112.8 g/L sugars, 0.1 g/L citric acid, 5.1 g/L malic acid, 0.3 g/L propionic acid, and 0.1 g/L glycerol.

2.3. Cell immobilization and determination of immobilized cell counts

Kefir culture was immobilized on apple pieces and DCM as previously described (Agouridis et al., 2008; Kourkoutas et al., 2001).

For the determination of the immobilized cell counts, 5 g of immobilized cells were blended with 45 mL of sterile ¼ Ringer's solution, serially diluted and subsequently plated. Then, lactobacilli, lactococci, and yeasts/molds counts were determined as described by Dimitrellou et al. (2008).

All analyses were performed in triplicate and the number of immobilized cells was determined as colony forming units (log cfu/g).

2.4. Fermentations

A series of repeated batch fermentations of commercial apple juice (250 mL) were carried out in batch bioreactors (0.5 and 1 L) using either free cells (10 g/L) or immobilized cells (480 g/L DCM and 1420 g/L apple pieces). Two separate experiments were carried out as follows: experiment A: a series of 13 repeated batch fermentations were performed at 30, 20, and 5 °C; experiment B: a series of 11 repeated batch fermentations were performed at 30, 37, and 45 °C.

All fermentations were carried out until all sugar content was utilized or when no fermentation activity was observed (stuck fermentations). Both free and immobilized cells were washed twice with apple juice and reused in the next batch fermentation. At the end of each batch fermentation, samples were collected and analyzed for residual sugars, ethanol, glycerol, organic acids, and volatile by-products.

2.5. Molecular analyses

2.5.1. PCR-DGGE analysis on biocatalysts

Samples of free (5 mL) or immobilized (5 g) cells were collected after fermentations at various temperatures and homogenized with 45 mL sterilized buffered peptone water (Lab M). Debris was allowed to deposit for 1 min and 1 mL of supernatant was used for DNA extraction using a DNeasy Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol. PCR-DGGE analysis was performed, as previously described (Sidira, Galanis, Nikolaou, Kanellaki, & Kourkoutas, 2014; Sidira, Karapetsas, Galanis, Kanellaki, & Kourkoutas, 2014).

Bacterial DNA was amplified with primers V3f (5′ CCT ACG GGA GGC AGC AG 3′) and V3r (5′ ATT ACC GCG GCT GCT GG 3′) (Kesmen & Kacmaz, 2011; Pepe, Blaiotta, Moschetti, Greco, & Villani, 2003), while for eukaryotic DNA amplification primers NL1 (5′ GCC ATA TCA ATA AGC GGA GGA AAA G3′) and LS2 (5′ ATT CCC AAA CAA CTC GAC TC 3′) (Cocolin, Bisson, & Mills, 2000) were used. The PCR products were subjected to DGGE analysis using an INGENYphorU DGGE system (Ingeny, The Netherlands) (Sidira, Galanis et al., 2014; Sidira, Karapetsas et al., 2014). Followed the electrophoresis, the gels were scanned with a fluorescent imager (Molecular Imager FX, BioRad) and the bands of interest were excised.

2.5.2. Sequencing of DGGE fragments and data analysis

Sequencing of DGGE fragments and data analysis was carried out as previously described (Sidira, Galanis et al., 2014; Sidira et al., 2014).

2.6. Chemical analyses

2.6.1. pH, total and volatile acidity

pH was determined using a pH-330i pH meter (WTW GmbH, Germany). Total acidity was estimated by titration with 4 g/L NaOH solution and volatile acidity by titration with 4 g/L NaOH after steam distillation using an Electronic Distiller (DUALSTILL Exacta + Optech Labcenter S.p.a., Italy).

2.6.2. Determination of residual sugars, ethanol, glycerol, and organic acids concentration

Residual sugars (fructose & glucose), ethanol, glycerol, and organic acids (malic, lactic, acetic, citric, and propionic acids) concentration was determined by HPLC, using a Shimadzu chromatography system (Shimadzu Corp., Germany) equipped with a Nucleogel ION 300 OA column (Macherey-Nagel, Germany), a DGU-20A5R degassing unit, a LC-20AD pump, a CTO-20AC oven at 85 °C, and an RID-10A refractive index detector. A solution of 0.049 g/L