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Short communication

High-performance liquid chromatography determination of phenyllactic acid in MRS broth

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Abstract

Phenyllactic acid (PLA) is an organic acid produced by some strains of lactic acid bacteria (LAB) and concentrations higher than 7.5 mg/ml inhibit growth of moulds and yeasts. Since PLA can be used to select LAB, a rapid, simple and cheap method for its determination is desirable. Typical methods for its analysis in broth are time-consuming, analytically complicated, and have poor recoveries. Herein we propose a simple and rapid method that does not require extraction, but only microfiltration of broth before injection in HPLC. The improved chromatographic conditions allow separation and quantification of PLA with a recovery of 98.7%. The method is highly reproducible with an intraday repeatability of the total peak area of 2.00%, while an interday repeatability of 2.69%.

Keywords: Phenyllactic acid determination; HPLC; Lactic acid bacteria (LAB)

1. Introduction

Production of fermentative compounds with antagonist action against moulds, yeasts or other microorganisms, is object of investigation for technologies of production of fermented foods. The capacity of lactic acid bacteria (LAB), to produce bacteriocin, compounds with inhibitory action against other bacteria or strains of the same species, has been known from long time [1–8]. *Lactobacillus plantarum* is a LAB of enormous technological relevance and is generally used as starter culture for production of several fermented foods [9–13]. Lavermicocca et al. [14] characterized a *L. plantarum* strain that is able to produce both phenyllactic and 4-hydroxyphenyllactic acid, which are two organic acids able to inhibit the growth of moulds and yeasts. Phenyllactic and hydroxyphenyllactic acids have been also found as metabolites involved in formation of cheese flavor produced by LAB strains through degradation of phenylalanine and tyrosine, respectively [15–17]. However, PLA is the main responsable of inhibition activity and for this reason it is an effective marker of antifungal action of LAB. Production of this organic acid from LAB starter, or non-starter LAB in fermented foods, acquire significant technological importance due to their characteristic of antimicrobial action and impact on the formation of flavor-forming compounds. Therefore, a quantitative, simple, rapid and cheap determination of this organic acid from broth is required when its production could be used as a selection criteria for starter LAB.

Determination of PLA of previous works concerned especially analysis of rumen fluid [18,19]. In these works, PLA was determined as related product of metabolism of phenylalanine by rumen microorganism. An HPLC system with UV detection (215–220 nm) was employed for this determination and isocratic runs with methanol–50 mM sodium acetate buffer pH 6.5 (8:92, v/v) were performed after sample preparation. Most recent works looked at PLA as antifungal compounds produced by LAB. In these works, PLA determination requires liquid–liquid extraction with 4 × 30 ml of ethyl acetate after centrifugation and modification of pH to 2.0 with 10 M formic acid [20], or purification by solid-phase extraction (SPE) [21] after centrifugation and filtration of bacterial supernatant. In both cases, quantification was carried out by HPLC/UV (210 nm) with water–acetonitrile gradient and run total time of 15 min for both methods. Herein we propose a simple and reliable method that does not require extraction or purification of bac-

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terial supernatants for determination of PLA uisng HPLC with UV detection.

2. Experimental

2.1. Reagents and materials

The PLA-producing strain VLT01 of *L. plantarum,* from the DIPROVAL collection (Bologna University), was grown on MRS liquid broth (Oxoid, Basingstoke, UK). HPLC-grade water, acetonitrile, and sulfuric acid (96%) were provided by Carlo Erba reagents (Milan, Italy). DL-3-Phenyllactic acid (purity 97%) was purchased from Fluka (Buchs, Switzerland) and the SPE column (C18 EC) were obtained from Isolute, International Sorbent Technologies Ltd. (Hengoed, UK). Filters (0.45 μm GMF w/GMF) were provided by Whatman (Middlesex, UK), while 0.22 μm type GS filters were purchased from Millipore (Billerica, MA, USA).

2.2. Bacterial supernatant preparation

Cells of the strain VLT01 of *L. plantarum* harvested during the exponential growth phase was inoculated in 500 ml (5×10^5 ufc/ml) of MRS broth and placed in a thermostat at 30 °C under anaerobic conditions for 24 h. At the end of incubation, samples were centrifuged at $4400 \times g$ for 10 min and subsequently filtered through a 0.22 μm filter. The solution was then divided in two aliquots to test two different sample preparations methods.

2.3. Sample preparation

2.3.1. Method 1

A C18 EC column was used for SPE purification. Column was activated with 20 ml of acetonitrile and equilibrated with 20 ml of HPLC grade water before sample loading. A washing step with 5% aqueous acetonitrile was followed by performing elution with 95% aqueous acetonitrile [18]. Chromatographic determination was carried out on washing extract (W) and elution extract (E) to evaluate possible repartition of phenyllactic acid in different fractions.

2.3.2. Method 2

Samples of bacterial supernatant were centrifuged and microfiltered using the same condition indicated in Section 2.2 and directly injected in the HPLC system.

2.4. Chromatographic conditions

Analysis was carried out with a Waters (Milan, Italy) HPLC, equipped with a Waters 1525 binary pump, dual wavelength absorbance detector Waters 2487 set at 210 nm, a Symmetry column C18 RP (150 mm × 4.6 mm, Waters, particle size 5 μm) at room temperature. Breeze 3.30 SPA software (Waters) was used for data acquisition and processing on a personal computer. The gradient used for elution is shown in Table 1. Phenyllactic acid was identified by comparison with retention time and co-elution of authentic standard solution.

Table 1
HPLC elution profile program

Time (min)	Acetonitrile (%)	Water (%)	Flow (ml/min)
	25	75	1
3	25	75	1
4	50	50	1
6	50	50	1
8	100	0	1.3
12	100	0	1.3

2.5. Evaluation of recovery

Two ml of a standard solution of phenyllactic acid in MRS broth (4 mg/ml) in 200 ml with bacterial supernatant of VLT01 strain was prepared. Evaluation of recovery of phenyllactic acid was carried out through comparison of the spiked sample with the supernatant from the two different sample preparation methods.

2.6. Calibration and production curve

The quantification procedure was performed over the range of 5–500 μg/ml for above-mentioned sample using the peak area versus analyte concentration to make the calibration curves. The linear range was assessed using seven different concentrations that were injected three times.

The curve of production was created monitoring PLA quantification during the exponential phase of microbial growth under the same conditions of the bacterial supernatant preparation indicated in Section 2.2 without anaerobiosis. Samples were taken 8 h after inoculation and every hour analyzed in duplicate during the successive 10 h.

2.7. Statistical analysis

The results reported are the average of three repetitions for sample analyzed with method 1, while microfiltered samples prepared using method 2 were injected 10 times on the same day (intraday precision, $n = 10$) and on two consecutive days (interday precision, $n = 20$). The relative standard deviations of the peaks area and migration times were determined.

3. Results and discussion

Some LAB produce proteic antifungal compounds [22,23], although these are formed during late inhibition due to cellular death with subsequent release of cellular material into the matrix through autolysis. In contrast, PLA is produced during the first hours of development reaching concentrations of 14 ppm within 8 h of inoculation. Determination of PLA can represent an important selection criteria for LAB since they are utilized as a starter culture in fermented foods. This organic acid is an effective marker of the ability of LAB to produce compounds with