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Short Communication

Rapid quantifiable assessment of nutritional parameters influencing pediocin production by *Pediococcus acidilactici* NRRL B5627

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Abstract

A direct plate bioassay procedure was applied for rapid and quantifiable assessment of the influence of various nutritional parameters on pediocin production by *Pediococcus acidilactici* NRRL B5627. Solid-state cultivation of the microorganism was done on MRS-based media over 3-and 6-hours incubation periods. Nutritional parameters assessed included the carbon source (glucose, sucrose, fructose, galactose, glycerol), and various salts (NH_4PO_4, $CaCl_2$, KH_2PO_4, $MnSO_4 \cdot H_2O$). Glucose was found to be the optimal carbon source while glycerol exhibited the most suppressive effect. Using glucose as the carbon source, addition of various salts, in amounts used in liquid media commonly applied in the cultivation of the pediococci, was assessed with respect to bacteriocin production on a per cell basis. Experimental data obtained showed that several nutritional parameters repress pediocin production by *P. acidilactici*, while the direct plate assay proved to be a good pilot assay prior to conducting more intensive kinetic analysis in liquid cultivation.

Keywords: *Pediococcus acidilactici*; Pediocin; Bioassay; Nutritional parameters; Glucose

1. Introduction

The potential applications of bacteriocins from lactic acid bacteria in the food and health care sectors have attracted the strong interest of academia and the industry resulting in an impressive amount of published research on their production, purification, genetics and applications (Papagianni, 2003). So far, only nisin produced by *L. lactis*, is a commercial product and an approved food additive in most major food producing countries. Another bacteriocin that attracts research interest and will likely be the next to be used in the food industry is pediocin (Turcotte et al., 2004), which is an antilisterial bacteriocin (Guyonnet et al., 2000; Simon et al., 2002) produced by several *Pediococcus* strains. However, unlike nisin and *L. lactis,* studies on the physiology and genetics of pediocin producing *Pediococcus,* are still rather limited.

Several strains of *Pediococcus acidilactici* have been reported to produce bacteriocins (Gonzales and Kunka, 1987; Biswas et al., 1991; Kim et al., 1992; Elegado et al., 1997; Gurira and Buys, 2005). Some of these are designated as pediocin AcH by *P. acidilactici* H, E, F, and M (Bhunia et al., 1987; Ray et al., 1989; Kim et al., 1992) and pediocin PA-1 by *P. acidilactici* PAC 1.0 (Gonzales and Kunka, 1987). The production of pediocin AcH by *P. acidilactici* H has been studied in more detail and found to be greatly influenced by the media composition, incubation temperature and time, and initial and terminal pH of media (Ray, 1995). Studies in liquid media (Biswas et al., 1991; Ray, 1992, 1995) revealed that among the metabolizable carbohydrates by the strain H, pediocin AcH production was the highest on glucose, followed by sucrose, xylose, and galactose, even with terminal pH below 4.0, while no pediocin AcH was detected with arabinose, raffinose and trehalose.

The influence of some carbon sources, pH, and amino-acids, on the production of pediocin by *P. acidilactici* has

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been studied previously by Ray (1995), Vazquez et al. (2003, 2006). However, these studies did not differentiate between factors that influence pediocin production and the growth of the pediocin producer. Accurate quantification is an important prerequisite for assessment of factors influencing pediocin production by *P. acidilactici*. The original quantification method was a plate diffusion assay developed by Mocquot and Lefebvre (1956) for nisin, and later modified by Tramer and Fowler (1964). This method is used for routine measurements of bacteriocin activity (Cabo et al., 1999; Simon et al., 2002; Yin et al., 2003; Turcotte et al., 2004) and is presently adopted as the standard plate method for pediocin quantification.

In the present study, a direct and rapid plate bioassay procedure was applied that enabled the quantifiable assessment of growth parameters influencing pediocin production by *P. acilactici* NRRL B5627 growing directly on the solid medium containing the test factor. This assay was used to assess the influence of various carbohydrates and salts on pediocin production by *P. acilactici* on a per cell basis.

2. Methods

2.1. Microorganism

Pediococcus acidilactici NRRL B5627 (Vazquez et al., 2006) was the microorganism used throughout this work. It was grown for 20 h in MRS broth (Sharlau, Spain), in 250 ml Erlenmeyer flasks containing 100 ml medium, at 30 °C, under static conditions. Flask cultures were either used as precultures for bioreactor cultures or were samples for the plate biossay. In the second case, 1 ml of the culture was pelleted, washed twice and re-suspended in 900 μl saline. A 1:10 dilution of the culture was used for the 3 h assay and a 1:100 dilution was used for the 6 h assay.

2.2. Plate bioassay

Sterile filter discs (0.45 μm pore size filter membranes, Whatman, UK) were placed onto MRS agar-based media (Sharlau, Spain) plates containing 1% Tween-20 and the test factor (which substituted glucose in MRS medium). A 5 μl aliquot of the diluted culture was spotted onto the disc membranes and the plates were incubated at 30 °C for 3 and 6 h, after which the membranes were removed and transferred to sterile microcentrifuge tubes containing 150 μl saline and centrifuged at medium speed (5000*g*) for 30 s. These parameters were experimentally determined to be sufficient to release the cells off the membrane and also break the pediococcal chains formed during the incubation period. The plates were refrigerated at 4 °C for 24 h and then overlaid with TM17G top agar (M17 agar, Sharlau, Spain, containing 1% Tween-20) seeded with *M. luteus* CECT 241, followed by incubation at 37 °C for 24 h. The zones of inhibition were measured and compared to known pediocin standards treated similarly and to nisin standards (Sigma–Aldrich, UK). To determine the time required by the organism to form and inhibition zone, *P. acidilactici* was inoculated onto membranes placed onto plates and incubated at 30 °C for various time intervals. The membranes were then taken off to determine the CFU (colony forming units), while the plates were overlaid with the indicator microorganism. AU (arbitrary units) of pediocin/10^6 CFU were determined and the experiments were carried out in triplicates. Test factors were the following: Glucose, sucrose, glucose + sucrose, galactose, fructose, glycerol (10 g/l each), and glucose (10 g/l) + NH_4PO_4 (1 g/l), glucose (10 g/l) + KH_2PO_4 (1.13 g/l), glucose (10 g/l) + $CaCl_2$ (14.7 g/l), glucose (10 g/l) + $MnSO_4 \cdot H_2O$ (10 g/l). Salts concentrations were according to suggested compositions of standard substrates used for *Pediococcus* cultivation by Atlas (2004). To prevent adsorption of pediocin onto glass and polypropylene surfaces, HCl (0.02 M) containing 0.1% Tween-80 was used for preparation of the stock solutions and dilutions of both standards and filtrates.

2.3. Submerged fermentations

Following growth in flasks as described in Section 2.1, at OD_{600} 1.4, the culture was transferred (2% v/v) in a stirred tank bioreactor (BIOFLO 110, New Brunswick Scientific) with a working volume of 2 l. The reactor was operated at 150 rpm, at 30 °C and without pH control. Experiments were carried out with standard, glucose containing, MRS and MRS in which glucose was substituted with sucrose (same concentration), under semi-aerobic conditions, with the DOT (dissolved oxygen tension) maintained at 60% of saturation (by sparging the reactor with a mixture of N_2 and atmospheric air, the DOT was kept constant by feedback regulation of the ratio). Samples were taken every two hours for biomass, lactate, residual sugar and bacteriocin activity determination. All runs were carried out in triplicate (mean values presented) and repeated if experimental variation exceeded 10%.

2.4. Determination of biomass, lactate, glucose, sucrose, and bacteriocin activity

Biomass concentration was measured spectrophotometrically by measuring the optical density at 600 nm and correlating it with cell dry weight (CDW) measurements. One unit of OD at 600 nm was shown to be equivalent to 0.25 g of cells per liter. CDW was determined by filtering 10 ml of broth through nitrocellulose filters (pore size, 0.45 μm). The filters were tared following drying in a microwave oven at 150 W for 15 min. The collected biomass was washed twice with 10 ml distilled water before being dried and tared.

Lactate was determined with the EnzyPlus D/L Lactic Acid kit by Diffchamb AB (Diffchamb, Sweden). Glucose was determined using the glucose oxidase/peroxidase method of Kunst et al. (1986). Sucrose was determined