ELSEVIER

Food Microbiology 22 (2005) 521–528

FOOD MICROBIOLOGY

www.elsevier.com/locate/fm

Bacteriocin production by *Lactobacillus pentosus* B96 can be expressed as a function of temperature and NaCl concentration

Amélia Delgado[a], Dulce Brito[b], Cidália Peres[b], Francisco Noé-Arroyo[c], Antonio Garrido-Fernández[c,*]

[a]*ITQB, Quinta do Marquês, apt 127 – 2781-901 Oeiras – Portugal*
[b]*EAN/INIAP, Quinta do Marquês, 2784-505 Oeiras – Portugal*
[c]*Instituto de la Grasa (CSIC), Avda. Padre García Tejero, 4. Seville 41012, Spain*

Received 4 August 2004; received in revised form 23 November 2004; accepted 23 November 2004

Abstract

Lactobacillus pentosus B96 is a bacteriocin-producing strain that was isolated from fermenting olive brines. The aim of the present work was the optimization of bacteriocin production, using response surface methodology (RS). A two-level screening Plackett–Burman design was used to select influencing factors. Then, a central composite design, with three repetitions in the centre, for pH, NaCl concentration, and temperature was carried out. Finally, an RS, which included the region of maximum accumulated bioactivity, was built as a function of NaCl concentration and temperature. Bioactivity accumulation was always observed during the exponential growth-phase, although no apparent correlation between maximum accumulated bioactivity and biomass formation was found. *L. pentosus* B96 is known to grow better at about 30 °C, neutral pH, and by the absence of NaCl; however, a suboptimal temperature (22 °C) and a moderate NaCl stress (0.65 mol l^{-1}) stimulated bacteriocin production. The research led to environmental conditions that maximized bacteriocin activity, which can be expressed as a polynomial function of temperature and NaCl concentration. The suboptimal growth conditions, which were found to produce the highest bacteriocin titres, resembled those prevailing during green table olive fermentation. This model can be used to improve "in situ" bacteriocin production thus contributing to the microbiological control of the process.

Keywords: *Lactobacillus pentosus*; Bacteriocin production; Response surface methodology

1. Introduction

The table olive is a Mediterranean fermented food based on a spontaneous fermentation, which is carried out in a mixed microbial population, mainly composed of *Enterobacteriaceae*, yeasts and lactic acid bacteria. Brine composition and physical conditions during these processes are major selection factors for the succession of microbial groups. Lactic acid fermentation is the last and most relevant step in the olive fermentation process and strains of genus *Lactobacillus* are required to obtain the correct flavor and texture profiles in the final product (Garrido-Fernández et al., 1997; Bobillo and Marshall, 1992; Montaño et al., 1993; Ciafardini et al., 1994; Sánchez et al., 2000). Thus, the growth in brines of suitably adapted strains of *Lactobacillus* is important from a technological point of view. Several authors consider bacteriocin production as a relevant factor in strain establishment, contributing to increase the quality and safety of fermented foods (Ruíz-Barba et al., 1994; Leroy and De Vuyst, 1999b). Some recent studies have helped clarify the effect of specific conditions from food environments on the production of bacteriocins (Leroy and De Vuyst, 1999a, b, 2003; Motta and Brandelli, 2003). Bacteriocin titres can dramatically change by

*Corresponding author. Tel.: +34 954 690850; fax: +34 954 691262.
E-mail address: garfer@cica.es (A. Garrido-Fernández).

0740-0020/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2004.11.015

altering environmental conditions and optimum production may require a certain combination of influencing factors (Leal-Sánchez et al., 2002). Regarding the complexity of food environments, a better knowledge of the interactions of these factors in bacteriocin production is needed. Although most of theses studies claim validation by a statistical test, usually the variance analysis, the combination of variables and their values and limits were arbitrarily chosen, based mainly on personal experience. However, the application of an adequate experimental design (optimization) has proven to be valuable in order to obtain maximum production. In synthesis, it consists of the performance of some previous prospective experiments aiming to reach the region of optimum production. Then, the surface response methodology can provide an empirical modeling of the activity, as a function of the diverse variables of interest (Myers and Montgomery, 2002). This technology is expanding its fields of application and is experiencing considerable development, due to the availability of extensive information and software packages to analyse data.

The goal of the present work was the optimization of bacteriocin production by *Lactobacillus pentosus* strain B96 in laboratory media, as a function of environmental conditions, using response surface technology.

2. Methods

2.1. Bacterial strain

The bacterial strain used was *L. pentosus* B96, which was isolated from fermenting green olives of "Azeiteira" cultivar, of Portuguese origin, although prepared according to the Spanish style. This strain is a presumable producer of plantaricin S (Jiménez-Díaz et al., 1993). Strain B96 possesses the same operon genes encoded for plantaricin S which were found in *L. plantarum* strain LPCO10, as demonstrated by PCR analysis according to the methodology described by Stephens et al. (1998).

2.2. Media

The bacteriocin producing strain B96 was propagated in MRS broth (Oxoid, England) or in modifications of this formulation, as described below. In those cases, the medium was prepared from single components (all of them obtained from Merck, Germany). When the initial pH was in a unit range from the set value it was adjusted before autoclaving, otherwise it was adjusted after it. The medium was finally filter-sterilized before inoculation.

2.3. Evaluation of the microbial population

The bacterial population was measured as the absorbance of cell suspensions at 600 nm, in a UV/VIS spectrophotometer (Jasco V-530, Japan). It was expressed as $g\,l^{-1}$ of dry biomass or as viable cell density ($cfu\,cm^{-3}$), based on the correlations between absorbance and cell dry weight, or between absorbance and viable cell density, respectively.

2.4. Bacteriocin assay

In the present work, bacteriocin titre was inferred from the area of the inhibition zone. This procedure relies on the linear dose–response relationship that reflects the proportionality between the natural logarithm of concentration/dilution and the inhibition zone on plates, as referred by several authors (Nuñez et al., 1996; Parente et al., 1995; Wolf and Gibbons, 1996). The sensitive organism was *Weissella paramesenteroides*, and a starting population of $10^7\,cfu\,cm^{-3}$ was used in the overlay. A total of 20 μl of each supernatant sample was applied on blank paper disks (Antimicrobial susceptibility test discs, Oxoid, England). Inhibition zone diameters were read using a 0.1 mm caliper (Dial 15, Tajima, Japan) in a colony counter with magnification and basal illumination (Gallenkamp, England). Accumulated bioactivity was expressed in mm^2 of the inhibition area, caused by the referred volume of the culture supernatant.

2.5. Culture conditions

Bacterial stocks were kept frozen at −20 °C in a solution of 1:4 v/v of glycerol to adequate broth and propagated twice before experimentation. Prior to experiments the inoculum was diluted to an OD (600 nm) equivalent to $10^7\,cfu\,cm^{-3}$ in the initial culture. Some combinations of variables from Table 1 resulted in medium modifications that were previously prepared and sterilized in tubes, accounting for inoculum volume ($0.1\,cm^{-3}$). After inoculation, each tube contained $5\,cm^3$. Incubation was static, according to selected temperature, in suitably accurate incubators or refrigerated water baths. Duplicate tubes were withdrawn every 2 h in order to detect the maximum bacteriocin activity and biomass concentration for the different treatments in each batch.

2.6. Experimental design

A preselection of the variables that might affect the production of bacteriocin was carried out using a two level screening Plackett–Burman design for 19 variables in 20 treatments. This design was replicated three times. Variables included compounds usually found in brines