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Fed-batch pediocin production on whey using different feeding media

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Abstract

Pediocin production by *Pediococcus acidilactici* NRRL B-5627 was followed in both batch and re-alkalized fed-batch fermentations on diluted whey (DW) supplemented with 2% (w/v) yeast extract (DWYE2 medium). In the first fed-batch culture, the fermentor was fed with a mixture of a 400 g/L concentrated lactose and concentrated whey (CW) supplemented with 2% (w/v) yeast extract. The maximum pediocin concentration obtained was only 1.2-fold higher than in batch fermentation. Since supplementing the DW with increasing amounts of glucose inhibited pediocin production, a second re-alkalized fed-batch culture was carried by using a 400 g/L concentrated glucose as feeding substrate. The pediocin concentration obtained was 2.2-fold higher than that in the first fed-batch culture. The third fed-batch fermentation was carried out by feeding the growing culture with a mixture of a 400 g/L concentrated glucose and CW medium supplemented with 4% (w/v) yeast extract. The concentration of biomass and the volumetric pediocin productivity obtained in this last culture were higher than those obtained in both the batch and the two fed-batch cultures ($P < 0.05$). Therefore, the use of feeding substrates containing glucose instead of lactose could be an appropriate alternative for increasing fed-batch production of pediocin in DWYE2 medium.

Keywords: *Pediococcus acidilactici*; Biomass; Pediocin; Whey; Re-alkalizations; Fed-batch fermentation

1. Introduction

Bacteriocins are biologically active proteins with antibacterial activity against Gram-positive bacterial species related to the producer strain. Some have a very narrow spectrum of activity but others have a relatively broad spectrum of antibacterial activity [1]. In recent years there has been an increased interest in using broad-spectrum bacteriocins as natural food preservatives and antimicrobial agents in veterinary and pharmaceutical areas [2]. Pediocins family, that are produced by *Pediococcus* strains, organisms generally recognized as safe (GRAS), have a wide inhibitory spectrum of activity against Gram-positive bacteria, including both spoilage and pathogenic organisms, such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Clostridium perfringens* [3]. This bacteriocin in combination with other stress inducing processes (such as heating, freezing, acid treatment, chelating agents, high hydrostatic pressure and electroporation) can also be effective against Gram-negative or pediocin-resistant Gram-positive bacteria [4]. Because of these characteristics, pediocin can potentially be used as a non-toxic food preservative to improve the quality, naturalness and safety of food products [5–7].

To produce the large amounts of bacteriocins needed for studies of their effectiveness in controlling the growth of undesirable bacteria to preserve functional foods and health products, the use of cheaper culture media [1] and fermentations techniques enhancing the growth and bacteriocin concentrations are needed [7].

Whey, which is a by-product of the dairy industry, has been widely used for various productions including organic acids, single-cell protein, enzymes, ethanol [8] and bacteriocins [1,9–14]. With regard to the fermentation technique, it is well known that fed-batch processes provide higher amounts of bacteriocin than batch cultures [7,15,16], especially when the cultures were also stressed by periodical re-alkalizations [7,13,17,18]. This fermentation technique has been used to reduce or prevent substrate inhibition [19], carbon catabolite repression [20] or nutrient depletion [21], phenomena which produce harmful effects on both cell growth and bacteriocin production [1].

In the present work, the batch production of pediocin by *Pediococcus acidilactici* NRRL B-5627 was studied on diluted whey supplemented with 2% (w/v) yeast extract (DWYE2

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medium). Secondly, the production of pediocin was followed in a re-alkalized fed-batch culture, using a mixture of a 400 g/L concentrated lactose and concentrated whey supplemented with 2% (w/v) yeast extract (CWYE2 medium) as feeding substrates. Subsequently, we evaluated the effect that the addition of other carbon source (glucose) produces on pediocin production. Thus, the effects of initial concentrations of glucose and yeast extract on the batch pediocin production were determined in order to optimize the composition of the fermentation (DW) medium. From the results obtained, other two re-alkalized fed-batch cultures were carried out in DWYE2 medium. In the first, the feeding substrate used was a 400 g/L concentrated glucose. The last re-alkalized fed-batch culture was fed with a mixture of concentrated lactose (400 g/L) and concentrated whey supplemented with 4% (w/v) yeast extract (CWYE4 medium).

2. Materials and methods

2.1. Bacterial cultures

Pediocin-producing strain *Ped. acidilactici* NRRL B-5627 was obtained from the Northern Regional Research Laboratory (NRRL, Peoria, Illinois, USA). Indicator strain *Carnobacterium piscicola* CECT 4020 was obtained from the Spanish Type Culture Collection (CECT). Stock cultures were maintained at 4 °C on agar slants (MRS). Working cultures were grown in MRS broth at 30 °C and 200 rpm.

2.2. Culture media and fermentation conditions

Whey obtained from a local dairy plant, was used in two forms: as concentrated whey (CW: the liquid remaining after the first cheese pressing) and as diluted whey (DW: CW mixed with wash waters). Before being used as culture media, both wastes were deproteinized as described previously [1]. The resulting diluted whey medium contained (g/L): lactose, 20.06; total nitrogen, 0.45; total phosphorous, 0.25 and soluble proteins, 2.04. The resulting concentrated whey medium contained (g/L): lactose, 48.51; total nitrogen, 1.05; total phosphorous, 0.43 and soluble proteins, 5.02. Both media were supplemented with yeast extract, adjusted at pH 7.0, sterilised at 121 °C for 15 min and used as culture media.

Batch cultures of *Ped. acidilactici* were performed in 250 mL Erlenmeyer flasks containing 50 mL of DW medium supplemented with 2% (w/v) yeast extract (DWYE2 medium), on a rotary shaker (Innova 4330, New Brunswick Scientific Co., Inc., New Jersey) at 30 °C and 200 rpm, during 52 h. Samples were withdrawn at regular intervals to perform analytical determinations.

Fed-batch fermentations were carried out in a 6 L bench top fermentor (New Brunswick Scientific, New Jersey) with a 4 L working volume of medium at a controlled temperature of 30 °C and at a controlled constant agitation of 200 rpm. A constant aeration flow rate of 0.5 L/h was used during the fermentations.

Fed-batch fermentations were carried out as a batch fermentation without pH-control during the first 12 h of culture, when the lower steady pH was reached. Then, a sample of 100 mL was taken from the fermentation medium to perform analytical determinations. After determining the total sugars concentration in the sample withdrawn, the medium was re-alkalized up to a set pH of 7.0 with 5 M NaOH. Then, the necessary volumes of feeding substrates to restore the initial total sugars concentration (~23 g/L) in the fermentation medium were calculated by applying mass balance equations for the total sugars around the fermentor. In these equations, the volumes of NaOH added to the fermentor in each re-alkalization cycle were also taken into account, as indicated below.

In the first re-alkalized fed-batch culture (fed-batch fermentation 1), the fermentor was fed with a mixture of a 400 g/L concentrated lactose and CW medium supplemented with 2% (w/v) yeast extract (CWYE2 medium). In the second fed-batch culture (fed-batch fermentation 2), a 400 g/L concentrated glucose was used to feed the growing culture. In the third fed-batch culture (fed-batch fermentation 3), the feeding substrate consisted in a mixture of a 400 g/L concentrated glucose and CW medium supplemented with 4% (w/v) yeast extract (CWYE4 medium).

These sampling, feeding and re-alkalization strategies were repeated every 12 h until the producer strain was unable to bring about the decrease of pH. The inoculum in both batch and fed-batch cultures consisted in 2% (v/v) of a 12-h culture in DWYE2 medium.

2.3. Analytical determinations

The concentrations of biomass (X) total phosphorous (TP), nitrogen (TN), protein (Pr) and sugars (TS) were determined by methods described in a previous work [13]. Lactose (L), glucose (G), lactic acid (LA), acetic acid (AA) and ethanol (Et) concentrations were measured by HPLC (Gilson, Inc., USA) using an ION-300 Organic Acids column (300 mm × 7.8 mm i.d.) with a pre-column IONGUARD^{TM} (Tecknokroma S. Coop. C. Ltda, Barcelona, Spain) equipped with a RI detector. The eluent consisted of 3 mM H_2SO_4 with a flow rate of 0.4 mL/min at (60–65) °C.

2.4. Bacteriocin assay

Culture samples were adjusted to pH 3.5 and then heated for 3 min to kill the cells. Subsequently they were centrifuged at 27,200 × *g* for 15 min at 4 °C to obtain the cell-free supernatants containing the total antibacterial activity. Pediocin activity was determined using 3 mL of convenient dilutions of the cell-free supernatants in sterile and distilled water and 3 mL of an overnight culture of the sensitive strain (*C. piscicola*) previously adjusted to an optical density of 0.2 at 700 nm. Inoculated tubes were incubated at 30 °C with an agitation speed of 200 rpm for 6 h, after which, the optical density of each tube was recorded. Dose–response curves were constructed from these data. One activity unit (BU) per millilitre was expressed as the inverse of the dilution that produced a 50% growth inhibition (inhibitory dose 50), which was obtained from the dose-response curves [13,22].

2.5. Effects of the carbon (glucose) and nitrogen (yeast extract) sources on the batch production of pediocin

A multifactorial composite rotatable design [23] based on five levels and two variables was used to study the combined effect of the glucose and yeast extract concentrations on the bacteriocin and biomass productions by *Ped. acidilactici*. The designs consisted of 13 experiments with four (2^2) factorial points, four axial points to form a central composite design with $\alpha = 1.267$ and five centre points for replication. Response surfaces were depicted from the empirical equations derived of design. The range and coding of the two variables are shown in Table 1.

2.6. Mass balance equations in the re-alkalized fed-batch fermentations

In this work, the volume of the fermentation medium (V) in the fed-batch fermentations was maintained constant ($(dV/dt) = 0$) by matching the volumes

Table 1
Experimental domain and codification of the variables used in the experimental design analysis for the combined influence of glucose (G) and yeast extract (YE) concentrations on pediocin production in DW medium

Codified values	Natural values	
	G (g/L)	YE (g/L)
−1.267	0.00	0.00
−1	1.05	2.10
0	5.00	10.00
+1	8.95	17.89
+1.267	10.00	20.00
Increments		
1	3.95	7.89