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Combination of food wastes for an efficient production of nisin in realkalized fed-batch cultures

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ABSTRACT

Nisin production by *Lactococcus lactis* CECT 539 was studied in four realkalized fed-batch cultures in diluted whey with feeding with lactose- and glucose-containing substrates. The first and third cultures were fed with mixtures of whey (W) and a 400 g/L concentrated glucose (CG), or with a concentrated mussel processing waste (CMPW) and CG, respectively.

The second and fourth cultures were respectively performed under the same conditions as in the first and third fermentations. However, these cultures were supplemented with mixtures of W plus a 2% (w/v) yeast extract (WYE2) and CG (second culture), or with CMPW plus a 2% (w/v) yeast extract (CMPWYE2) (fourth culture) after sample extractions at 132 and 168 h, respectively. From these times, each culture was fed with mixtures of WYE2 and CG, or CMPWYE2 and CG, respectively.

The final concentrations of nisin obtained in the third (223.98 BU/mL) and fourth (350.61 BU/mL) cultures, fed with glucose-containing substrates (CG and CMPW), were considerably higher than those obtained in the first (108.00 BU/mL) and second (158.53 BU/mL) cultures fed with the mixture of lactose- and glucose-containing substrates (W and CG).

1. Introduction

Nisin is a peptide produced by *Lactococcus lactis* strains, with a broad spectrum of antibacterial activity against important undesirable spoilage bacteria and food-borne pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium botulinum*. In addition, this bacteriocin is innocuous, stable to heat at low pH levels and sensitive to the gastric proteinases. For these characteristics, nisin is used as a safe preservative for different foods in many countries [1,2].

Optimization studies in batch cultures showed that nisin production was mainly affected by substrate inhibition at high levels of the carbon source [3–6], by nutrient limitation [1,5,7] or by the culture pH profiles during the fermentations [1,4,5,7]. To address these drawbacks, different fed-batch modalities have been used for increasing nisin production, including the variable or fixed volume fed-batch fermentations with or without pH control, and with constant or intermittent feeding [8–14].

In the variable volume fed-batch fermentation, the volume increases with the fermentation time as a consequence of the intermittent or continue feeding of the sterile fresh substrate [9–13]. Since this type of fermentation is started as a batch process, a low volume of fermentation medium must be used to reserve a fermentor volume for the addition of the feeding substrates and consequently, much of the fermentor volume is not utilized until the end of the process [14].

In the fixed volume fed-batch fermentation, the culture volume is maintained practically constant by feeding the limiting nutrient in undiluted form or as a very concentrated solution to avoid diluting the culture [14,15]. In a variant of this type of fed-batch fermentation, the culture volume can also be kept constant by periodically withdrawing a volume of fermented medium and adding the same volume of a sterile highly concentrated solution or medium containing the limiting nutrient [8]. This type of fermentation, known as cyclic or repeated fed-batch culture [8,14–16], has been successfully used for production of nisin [8,14], pediocin [15] and highly concentrated probiotic cultures [16] in whey.

However, the design of an efficient fed-batch process is a complex task that includes different steps. Firstly, the behavior and nutrient requirements of the nisin-producing bacterium should be

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studied in batch cultures to select the best fermentation medium [4,5,14]. Secondly, it is necessary to select the best feeding strategies to keep the carbon source concentration at non inhibitory levels for nisin production [8,11,13–15]. The latter step includes the selection of the most appropriate fed-batch process (fixed or variable volume), feeding substrate and feeding rate [8,14]. Thirdly, it is very important to develop the appropriate mass balance equations to analyze and control the evolution of the main culture variables during the fermentation process [8,14–17].

Previous optimization studies in batch cultures showed that the unsupplemented diluted whey (DW, 22.62 g of lactose/L) was the best fermentation medium for nisin production by *L. lactis* CECT 539, in comparison to the same medium supplemented with lactose, glycine and KH_2PO_4 [4] or glucose [14]. In addition, the higher nisin titres in DW medium were obtained in the batch cultures at uncontrolled pH [4]. On the other hand, the nisin concentration obtained in a realkalized fed-batch culture in whey fed with a 400 g/L concentrated glucose (60.35 BU/mL) was 1.2 times higher than that obtained in a similar culture fed with a 400 g/L concentrated lactose (50.57 BU/mL) [14].

Therefore, the aim of this work was to study nisin production by *L. lactis* CECT 539 in realkalized fed-batch cultures by using a combination of feeding substrates containing carbon, nitrogen and phosphorus sources similar or different to those of the fermentation medium (DW). Thus, the different mixtures of substrates used to feed the growing cultures were: (i) whey (W medium, 51.35 g of lactose/L) and a 400 g/L concentrated glucose (CG) in the first culture, (ii) W medium and CG (during the first 132 h of incubation), and W supplemented with a 2% (w/v) yeast extract and CG (from the 132 h to the end of the incubation) in the second culture, (iii) a concentrated mussel processing waste (CMPW medium, 101.72 g of glucose/L) and CG in the third culture and, (iv) CMPW and CG (during the first 168 h of incubation), and CMPW supplemented with a 2% (w/v) yeast extract (from the 168 h to the end of the culture) in the fourth fermentation. With this approach, the most appropriate feeding strategy was selected for an efficient production of nisin in realkalized fed-batch cultures in DW medium.

2. Materials and methods

2.1. Bacterial cultures

L. lactis subsp. *lactis* CECT 539, the nisin-producing bacterium, and *Carnobacterium piscicola* CECT 4020, the nisin-sensitive indicator strain, were acquired from the Spanish Type Culture Collection (CECT, Valencia, Spain). Both bacteria were grown on MRS (De Man, Rogosa and Sharpe, Merck, Germany) broth and maintained as frozen stock held at −40 °C in Nutrient broth containing 15% (v/v) glycerol. Before their use in fermentation experiments, working cultures of both strains were grown on MRS as agar slants. The latter cultures were maintained at 4 °C and propagated twice in liquid cultures (30 °C, 12 h) in the same medium [1].

2.2. Culture media and fermentation conditions

The culture media used in this work were prepared with diluted (DW) and undiluted whey (W) and with a concentrated mussel processing waste (CMPW). The DW and W media, which were obtained from a local dairy plant, were prepared as follows: after adjusting the pH to 4.5 with 5 N HCl, both media were heated at 121 °C for 15 min to denature the proteins, and then centrifuged at 12,000 × *g* for 15 min [4]. The supernatants were used as culture media.

The mussel processing wastes (MPW, glycogen as main component: 5–10 g/L), obtained from a local mussel processing plant, were firstly concentrated by ultrafiltration at 100 kDa to produce a medium with an average glycogen level of 100 g/L (CMPW medium) [18,19]. The CMPW medium was acidified to pH 4.5 with 5 N HCl and centrifuged at 12,000 × *g* for 15 min to eliminate the precipitate. Subsequently, the glycogen contained in the supernatant was saccharified to glucose by enzymatic hydrolysis at 40 °C for 1 h, with a San Super 240 L commercial preparation of α-amylase (Novo Nordisk, Denmark). The commercial preparation of enzyme:culture medium ratio was 1:1000 (v/v) [20].

After the treatment, the DW medium contained (in g/L): total sugars, 22.62; total nitrogen, 0.44; total phosphorus, 0.26 and soluble proteins, 2.08. The W medium contained (in g/L): total sugars, 51.35; total nitrogen, 0.99; total phosphorus, 0.47 and soluble proteins, 4.57. The CMPW medium contained (in g/L): total sugars, 101.72; total nitrogen, 0.54; total phosphorus, 0.06 and soluble proteins, 3.47. The mean composition (in g/L) of the W medium supplemented with a 2% (w/v) yeast extract (Panreac AppliChem, Barcelona, Spain), named as WYE2 medium, was: total sugars, 54.85; total nitrogen, 3.69; total phosphorus, 1.15 and soluble proteins, 16.31. The mean composition (in g/L) of the CMPW medium supplemented with a 2% (w/v) yeast extract (Panreac AppliChem, Barcelona, Spain), named as CMPWYE2 medium, was: total sugars, 105.22; total nitrogen, 3.24; total phosphorus, 0.73 and soluble proteins, 15.22.

The five culture media were adjusted to initial pH 7.0 and sterilized at 121 °C for 15 min [1,4].

All the realkalized fed-batch cultures were carried out in duplicate in two 13-L fermentors placed in a twin configuration, both equipped with aerators, baffles, two 6-bladed Rushton-type impellers and sensors for pH and temperature.

The four fermentations were initiated as batch processes, with a working volume of 10 L of DW medium (pH 7.0). The temperature, aeration flow rate and the agitation speed were maintained during all the incubation periods at 30 °C, 0.5 L/h and 200 rpm, respectively. The fermentation medium was inoculated with a 2% (v/v) of a 12-h culture of *L. lactis* CECT 539 in DW medium, adjusted previously to give an initial biomass concentration in the fermentation medium of 0.03 g/L [8,14]. The batch fermentations were converted into repeated re-alkalized fed-batch mode each 12 h, by withdrawing a volume of fermented culture medium (V_{FM}) from the fermentor, when the culture reached the optimum pH value for nisin production in DW medium [4]. The samples taken were used to detemine the culture pH and the concentrations of biomass (X), total sugars (TS), nitrogen (TN), phosphorus (TP), proteins (Pr), glucose (G), lactose (L), lactic acid (LA), acetic acid (AA) and butane-2,3-diol (B), as well as the nisin activity (Nis).

After determining the total sugars concentration in the sample withdrawn, the DW medium was realkalized up to the initial pH of 7.0 with 5 N NaOH. Subsequently, the necessary volumes of feeding substrates (V_{FS}) to bring the culture up to the initial total sugars concentration (22.62 g/L) were calculated by taking into account the volumes of 5 N NaOH (V_{NaOH}) added to the fermentor: $V_{FS} = V_{FM} - V_{NaOH}$. With this approach the culture volume was kept constant and the TS levels were not higher than that of the DW medium to avoid the manifestation of the substrate inhibition phenomenon observed in the batch cultures [4]. The volumes of feeding media and NaOH were added to the fermentor using a peristaltic pump (LKB, Pharmacia, Uppsala, Sweden).

In the first realkalized fed-batch culture (WCG-I), the feeding substrate consisted in a mixture of two substrates composed of a 400 g/L concentrated glucose (CG) and W medium. The latter medium was used as a feeding substrate to supplement the fermentor with carbon (lactose), nitrogen and phosphorus sources similar to those of the fermentation medium (DW) in each feeding cycle.

In the following realkalized fed-batch culture (WCG-II), the fermentation and feeding media, as well as the fermentation conditions were identical to those used in fermentation WCG-I during