WHEY UTILIZATION FOR PRODUCTION OF BACTERIOCIN AND PROBIOTIC PREPARATIONS

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Abstract

Batch productions of nisin by Lactococcus lactis subsp. lactis CECT 539 and pediocin by Pediococcus acidilactici NRRL B-5627 were firstly investigated in MRS broth and in both deproteinized diluted (DDW) and concentrated (DCW) whey. Although both strains produced the higher amounts of biomass and bacteriocin titres on MRS broth, the productions on DDW were higher than on DCW. Therefore, the following experiment was focused on the optimization of the composition of DDW medium, by using factorial experiments and empirical modelling to study the effects of total sugar (lactose), nitrogen (glycine), phosphorous (KH_2PO_4) and buffer (potassium hydrogen phthalate–NaOH) concentrations on nisin and pediocin productions. The increase in buffer concentration led to a decrease in the production of both bacteriocins, and the supplements with lactose, glycine and KH_2PO_4 did not improve nisin and pedicoin productions.

Therefore, in an attempt for increasing bacteriocin production in DDW medium, both L. lactis CECT 539 and Ped. acidilactici NRRL B-5627 were grown in DDW media supplemented with lactose and four different nitrogen sources. Glycine and NH_4Cl were not good nitrogen sources, but the use of high concentrations of yeast extract and Casitone highly stimulated the production of both bacteriocins.

Since the use of these two complex nitrogen sources could be very expensive for bacteriocin production at a high scale, the production of bacteriocins and antibacterial factors by the two lactic acid bacteria was studied in DDW media by using re-alkalized fed-batch fermentations. These fermentations were mainly characterized with higher biomass and bacteriocin productions compared with the batch process in DDW medium and with the production of mixed acid metabolites (acetic acid, butane-2,3-diol and ethanol).

We also studied the fermentation kinetics of kefir grains CIDCA AGK1 on unbuffered and buffered DDW medium. In these studies, the highest levels of free biomass, lactic acid and acetic acid were obtained in the buffered medium. Subsequently, the effect of nitrogen and phosphorous supplementation on free biomass, lactic acid and acetic acid was studied in buffered DDW medium using factorial experiments and empirical modelling. Finally, the effect of agitation and aeration on the production of the three variables was studied by using the optimized DDW medium obtained in the latter experiment.

Keywords: *lactic acid bacteria, nisin, pediocin, kefir grains, probiotics, whey.*

INTRODUCTION

Cheese whey, which is a by-product of the dairy industry, contains usually high levels of lactose (about 50 g/L), low levels of nitrogen compounds and small amounts of vitamins and minerals (González, 1996; Guerra et al ., 2001). This effluent has been widely used for various bioproductions, such as organic acids, single-cell protein, enzymes, ethanol (González, 1996), bacteriocins (Guerra et al., 2001; Guerrae t.al., 2007; Fajardo, et.al., 2008) and probiotic preparations for animal feed (Guerra et.al 2007; Fajardo, et.al., 2008).

Nisin and pediocins are bacteriocins produced respectively by *Lactococcus lactis* and *Pediococcus* strains (organisms generally recognized as safe (GRAS)) (Guerra et al., 2001). Both bacteriocins exhibit a broad spectrum of antibacterial activity against Gram-positive spoilage and pathogenic bacteria present in foods such as *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Clostridium perfringens* (Bhunia et. al., 1988; Fajardo, et al., 2008). Both bacteriocins have proved to be effective food biopreservatives because they are innocuous, sensitive to digestive proteases and they do not produce changes in

the organoleptic properties of the foods. Since both bacteriocins 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 resistant Gram-positive bacteria (Kalchayanand,et al., 1992; Kalchayanand, Sikes et al., 1994; Fajardo, et al., 2008), there is a considerable interest in using them in current and potential applications in the veterinary and pharmaceutical areas.

Probiotics are living microorganisms that once ingested by humans and animals in sufficient numbers, can beneficially influence the health of the host (Salminen et al., 1998; Bogovič-Matijašić et al.j, 2004). The mode of action of probiotics is strain and host-dependent and may include the modulation of the host intestinal microbiota, modifications of the structure and function of the intestinal epithelium and stimulation of the immune response by activation and regulation of mucosa-associated and immune system responses (Gardiner et al., 2004;).

In recent years, some probiotic preparations, containing high concentrations of viable cells and antimicrobial substances, have been assayed as additives in animal feed as an alternative to the use of antibiotic growth promoters (Abe, et al 1995; Guerra et al., 2007; Fajardo, et al., 2008). This is basically due to the emergence of resistant bacteria and resistance genes as a consequence of the use of these latter compounds (Aarestrup, 2005). These antibiotic-resistant bacteria can spread among animal species and be transmitted to humans by food of animal origin (Abe et al., 1995; Tortuero,et al., 1995; Aarestrup, 2005).

Some lactic acid bacteria (LAB) strains (e.g. strains of the genus *Bifidobacterium*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and *Enterococcus*) have proven to be effective in prevention or treatment of some diseases in humans and animals (Gardiner et al., 1999; Bogovič-Matijašić et al., 2004; Ohashi et al., 2004; Anadón et al., 2006), due to their adhesion capability to gut intestinal epithelial cells and their ability to produce antibacterial products (mainly organic acids and bacteriocins) (Bogovič-Matijašić et al., 2004; Sablon, et al.,, 2000; Guerra,et al.,2007). However, there are discrepancies in the results obtained because more often such effects were not significant, except when the animals are challenged with selected pathogenic strains or in gnotobiotic animals (Bogovič-Matijašić et al., 2004) and in other cases administration of LAB have shown no effects (Gardiner et al., 1999).

For an effective application as additives in animal feed, a potential probiotic strain must be: i) non-pathogenic and non-toxic, ii) beneficial to the host in some way after consumption, iii) amenable to industrial scale cultivation, vii) able to produce high amounts of antimicrobial substances with antagonistic activity against noted pathogenic bacteria and/or viruses, iv) able to attach to the gut epithelial tissue, v) able to colonise and persist within the gastrointestinal tract, vi) able to survive passage through the gastrointestinal tract, and viii) stable during preparation and storage of the carrier feed (Berg, 1998; Fajardo, et al., 2008).

The use of cheaper culture media (*e.g*. wastes from food industry) and an efficient cultivation method could be an appropriate alternative to produce probiotic culture highly concentrated at a low production cost (Guerra and Pastrana, 2003; Guerra et al 2007; Fajardo et al., 2008b; Guerra et al., 2010). Considering that substantial amounts of whey are available free, or commercialized at very low cost by local dairy of Galicia, the use of this waste as culture medium could provide a profitable substrate for production of probiotic culture concentrates. This approach allows an effective reduction of the initial chemical oxygen demand (COD) of the whey (Guerra et al., 2001; Guerra and Pastrana, 2003) and the recycling of this waste in production of probiotic culture concentrates (Guerra and Pastrana, 2003; Guerra, Fajardo and Pastrana, 2007; Fajardo,et al.,2008b; Guerra et al., 2010). With regard to the cultivation method, a fed-batch technique based on periodical re-alkalizations of the culture medium has proved to be effective for enhancing biomass and antimicrobial products synthesis by some LAB in different culture media (Cabo, et al., 2001; Guerra and Pastrana, 2003; Guerra, Fajardo and Pastrana, 2007; Guerra et al., 2010; Fajardo et al

Guerra, 2008b). In fact, the concentrations of biomass and antibacterial products obtained in these cultures were higher than those obtained in the corresponding batch cultures on the same culture media.

Taking into account the large number of microorganisms (LAB, acetic bacteria and yeast) present in the kefir grains, as well as the variety of bioactive products than can be produced by them (Farnworth, 2005), the use of kefir grains as a starter culture in whey fermentation could be more advantageous than the use of a single LAB. This approach could allow the production of a more concentrated probiotic product containing a larger number and variety of microorganisms and products than those with a single LAB. In addition, in vitro and animal trials have shown kefir and its constituents to have anticarcinogenic, antimutagenic, antiviral and antifungal properties (Farnworth, 2005).

Although the stability of the microbiota present in the kefir grains is maintained when it is preserved and cultivated under appropriate conditions (Simova et al., 2002), several studies indicate that the balance and presence of species and strains depends on both the kefir origin and process conditions (Garrote et al., 1998). It is therefore crucial to know the possible deleterious effects that the operation mode can produce on the microbial populations and consequently, on the quality and stability of the fermented product, which are two essential requirements for the marketing of the product. However, only a few studies deal with the study of the effects of the process and culture conditions on the fermentation kinetics of whey by kefir grains (Schoevers and Britz, 2003; Koutinas et al., 2005; Tramšek and Goršek, 2008).

For these reasons, the main purpose of this work was to give an overview on the use of whey and different fermentation strategies for the production of antimicrobial compounds (mainly bacteriocins, lactic acid and acetic acid) and probiotic preparations with two LAB (*Lactococcus lactis* subsp. *lactis* CECT 539 and *Pediococcus acidilactici* NRRL B-5627) and with kefir grains CIDCA AGK1.

EXPERIMENTAL

All experimental details about strains, culture conditions, analytical determinations and statistical analysis were taken from our previous works (Guerra and Pastrana, 2001a; 2001b; Guerra et al., 2001; Guerra,et al., 2007; Fajardo et al., 2008; Fajardo et al., 2008a).

RESULTS AND DISCUSSION

1. Whey fermentations

1.1. Whey fermentation with lactic acid bacteria

The production of bacteriocins (nisin and pediocin) and probiotic preparations by *L. lactis* subsp. *lactis* CECT 539 and *Ped. acidilactici* NRRL B-5624 was assayed in culture media prepared with both concentrated (DCW) and diluted (DDW) whey. The composition (g/L) of the DCW medium was, total sugars, 48.11; proteins, 5.02; total nitrogen, 1.05 and total phosphorus, 0.43. The composition (g/L) of the DDW medium was, total sugars, 20.54; proteins, 2.04; total nitrogen, 0.45 and total phosphorus, 0.25.

Although the results (**Figures 1 and 2**) showed that the DDW medium without supplementation is capable of promoting the growth of the two lactic acid bacteria (LAB), the productions of nisin (23 BU/mL) and pediocin (58 BU/mL) obtained (Guerra et al., 2001) were respectively lower than those produced in MRS broth by the strains CECT 539 (50 BU/mL) and NRRL B-5624 (493 BU/mL) (Guerra and Pastrana, 2001a). This observation suggested that DDW medium lacks of some nutrient necessary for biomass and bacteriocin production. Therefore, we studied the effects of supplementation with glycine (as nitrogen (N) source), KH_2PO_4 (as phosphorous (P) source) and lactose (as carbon (C) source) on nisin and pediocin synthesis by strains CECT 539 and NRRL B-5624 in whey. Since the pH was found to be an important variable in bacteriocin production (Guerra and Pastrana, 2001a; Guerra et al., 2001), the effect of the concentration of the buffering (B) agent (Potassium hydrogen phthalate–NaOH) was

included in this study, which was carried out by using a 2^4-full factorial design with four replicates of the central treatment (Guerra et al., 2001). The experimental ranges selected for each variable were between 0-0.1 M (in case of B), 26-48 g/L (in case of C), 0.43-7.55 g/L (in case of N) and 0.27-1.56 g/L (in case of P).

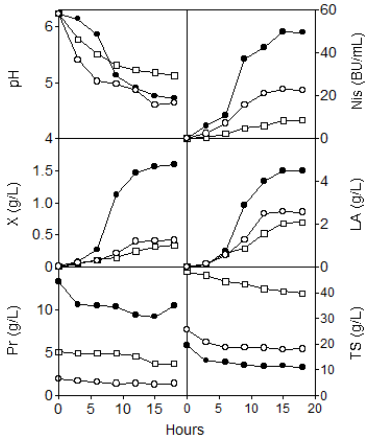

Figure 1. Batch fermentations of *L. lactis* CECT 539 on MRS broth (black circle), DDW medium (white circle) and DCW medium (white square). Nis, nisin; X, biomass; LA, lactic acid; Pr, protein; TS, total sugars.

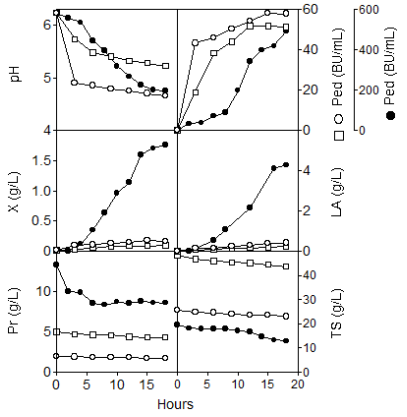

Figure 2. Batch fermentations of *Ped. acidilactici* NRRL B-5627 on MRS broth (black circle), DDW medium (white circle) and DCW medium (white square). Ped, pediocin; X, biomass; LA, lactic acid; Pr, protein; TS, total sugars.

The results obtained showed that the supplementation with lactose, glycine and KH_2PO_4 and the use of a buffering agent did not improve the productions of nisin and pediocin by *L. lactis* CECT 539 and *Ped. acidilactici* NRRL B-5624 in DDW medium (**Figure 3**).

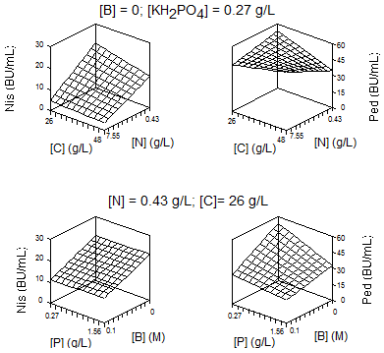

Figure 3. Response surfaces showing the effect of the concentrations of lactose (C), glycine (N), KH_2PO_4 (P) and the buffering agent (B) on nisin (Nis) and pediocin (Ped) production by *L. lactis* CECT 539 and *Ped. acidilactici* NRRL B-5627 in DDW medium.

For this reason, in a following step, our group investigated the effect of different nitrogen sources (glycine, NH_4Cl, yeast extract and Casitone) on nisin and pediocin production in batch cultures in DDW medium (Guerra and Pastrana, 2001b). In this study, both the amino acid and the inorganic salt were not good nitrogen sources for bacteriocin production. However, enhanced concentrations of nisin (59 and 74 BU/mL) and pediocin (185 and 195 BU/mL) were obtained when the whey was supplemented with casitone or yeast extract, respectively (**Figure 4**). However, this approach could be uneconomic for a high-scale bacteriocin production due to the high cost of the complex nitrogen sources in comparison with the low cost of whey.

The results obtained in the above batch cultures (Guerra et al., 2001; Guerra and Pastrana, 2001b) showed that low carbon source concentrations and high pH drops are needed for stimulating bacteriocin production. These observations were taken into account to develop the re-alkalized fed-batch fermentations, in which the DDW medium was used as a fermentation medium instead of the DCW medium. In addition, the fed-batch fermentations were carried out without pH control, allowing the pH to freely drop from its initial value until the 12 h of fermentation, justly when the nutrients (sugars, nitrogen,

phosphorous) utilization, cell growth and bacteriocin production stopped (Guerra et al., 2001).

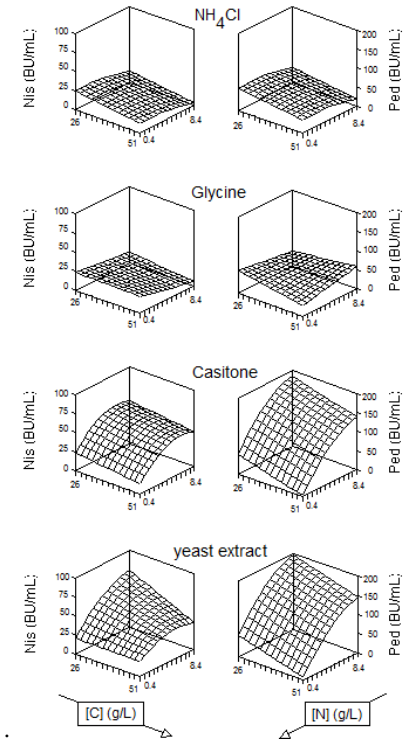

Figure 4. Response surfaces showing the effect of the concentrations of lactose (C) and four nitrogen (N) sources on nisin (Nis) and pediocin (Ped) production by *L. lactis* CECT 539 and *Ped. acidilactici* NRRL B-5627 in DDW medium.

Re-alkalized fed-batch fermentations (Guerra and Pastrana 2003; Guerra et al, 2005) were carried out in a 6 L bench top fermentor (New Brunswick Scientific, New Jersey) with a 4 L working volume of medium at controlled temperature (30ºC), agitation (200 rpm) and aeration flow rate (0.5 L/h). Fermentations were initiated as a batch fermentation without pH-control during the first 12 h of culture. 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. The necessary volumes of feeding substrates to restore the initial total sugars concentration (~ 20 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 taken into account. These sampling, feeding and re-alkalization strategies were repeated every 12 h until the strains were unable to bring about the decrease of pH.

In the re-alkalized fed-batch culture of *L. lactis* in DDW medium (fermentation 1), the fermentor was fed with a mixture of a 400 g/L concentrated lactose and DCW medium (Guerra and Pastrana 2003). In case of *Ped. acidilactici* (fermentation 2) (Guerra et al., 2005), the DDW medium was supplemented with a 2% (w/v) yeast extract (DDWYE2 medium) due to the negligible growth observed during the batch culture in DDW medium (Guerra et al., 2001), in contrast with the exuberant growth observed in the same culture medium supplemented with varying concentrations of yeast extract (Guerra and Pastrana, 2001b). In this re-alkalized fed-batch fermentation, the fermentor was fed with a mixture of a 400 g/L concentrated glucose and DCW medium supplemented with 2% (w/v) yeast extract (DCWYE2 medium). The inoculum in the re-alkalized fed-batch cultures consisted in 2% (v/v) of a 12-h culture in DDW medium (in case of *L. lactis*) or DDWYE2 medium (in case of *Ped. acidilactici*).

In the *L. lactis* re-alkalized fed-batch culture (**Figure 5**), the accumulated concentrations of biomass (3.5 g/L) and nisin (125 BU/mL) (Guerra and Pastrana, 2003), were respectively 2.2 and 2.5 times higher than those obtained on MRS broth (1.6 g/L and 50 BU/mL) (Guerra and Pastrana, 2001a). In this culture, the nitrogen source seemed to be consumed in two steps and consequently, biomass concentration displayed two exponential growth phases and two nonexponential growth phases. In addition, biomass concentration stopped when the cultures reached a low nitrogen concentration, even though the carbon source levels were still sufficiently available. These observations suggested that the nitrogen source was the

growth limiting substrate for *L. lactis* CECT 539 in this re-alkalized fed-batch fermentation.

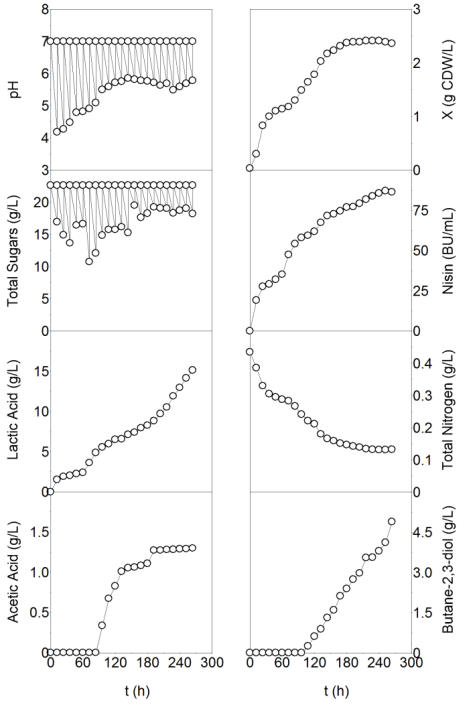

Figure 5. Re-alkalized fed-batch culture of *Lactococcus lactis* subsp. *lactis* CECT 539 in DDW medium with feeding with DCW medium and a 400 g/L concentrated lactose. X: biomass.

Taking into account the organic acids production, there were an initial homolactic phase (first 84-h of incubation) in which, only lactic acid was produced) followed by a second mixed acid fermentation phase (84 h to the end of the cultivation), in which lactic acid, acetic acid and butane-2,3-diol accumulated in the medium. The active period increased from 12 h in batch cultures to 264 h in the re-alkalized fed-batch culture.

In the re-alkalized fed-batch culture of *Ped. acidilactici* NRRL B-5627 (**Figure 6**), the concentrations of biomass (6.57 g/L) and bacteriocin (518 BU/mL) (Guerra et al., 2005) produced were respectively, 3.7 and 1.05 times higher than those levels obtained on MRS broth (Guerra and Pastrana, 2001a). However, in this case, a mixed acid fermentation phase was observed from the start of the fermentation, and increased concentrations of lactic acid, acetic acid and ethanol accumulated in the culture medium. The active period increased from 15 h in batch cultures (Guerra et al., 2001) to 240 h in the re-alkalized fed-batch culture (Guerra et al., 2005).

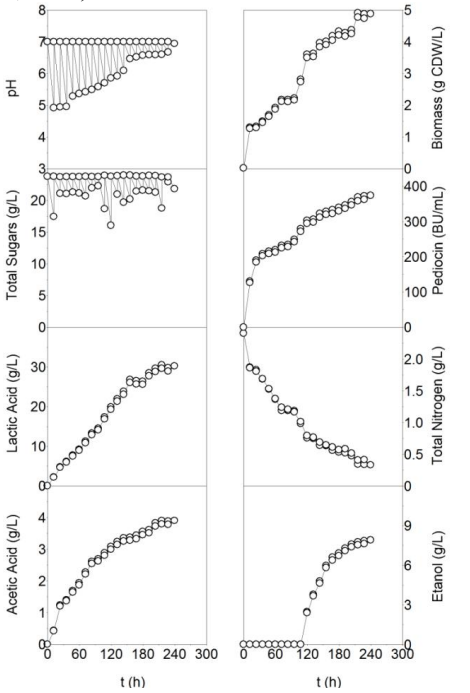

Figure 6. Re-alkalized fed-batch culture of *Pediococcus acidilactici* NRRL B-5627 in DDWYE2 medium with feeding with DCWYE2 medium and a 400 g/L concentrated glucose.

Taking into account the high concentrations of biomass and antimicrobial metabolites produced in the re-alkalized fed-batch cultures in whey, the following experiment was focussed on the evaluation of highly concentrated and viable probiotic culture (probiotic preparations) of four LAB (*Ped. acidilactici* NRRL B-5627, *L. lactis* CECT 539, *Lactobacillus casei* CECT 4043) as additives in animal feed. The results obtained showed that the use of the three potentially probiotic strains as additives in animal feed, stimulated the growth of post-weaning piglets and reduced coliform counts in the faeces of the animals. This indicated that these LAB could be suitable strains for widespread use in the pig industry (Guerra, Fajardo, et al., 2007).

1.2. Whey fermentation with kefir grains

In the last years, our research group has been focused on the study of the kinetic of the batch fermentation of whey by kefir grains CIDCA AGK1 (Fajardo et al., 2010). The cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL of deproteinized whey medium on a rotary shaker with shaking at 200 rpm at 30 ºC for 80 h. A culture on skimmed milk (–Hacendado", Spain) obtained from a local supermarket, was carried out in the same conditions as those used in the culture on whey, to obtain data for comparisons.

The initial composition of the skimmed milk (in g/L) used was: lactose, 60.0; proteins, 35.0; phosphorous, 0.12 and initial pH, 6.8. Whey was obtained from a local dairy plant (–Queserías Prado", Lugo, Spain) with the following mean composition (in g/L): lactose, 34.7; reducing sugars, 24.8; proteins, 11.5 and initial pH, 4.86. Before being used as culture medium, whey was deproteinized as described previously (Guerra et al., 2001) and a final protein concentration of 5.0 g/L was obtained. The deproteinized whey was adjusted to pH 6.5 and sterilized at 121 ºC for 15 min.

The pre-inoculum in both cultures was prepared by transferring 0.5 g of kefir grain into 50 mL of skimmed milk (–Hacendado") and incubated for 24 h at 30 ºC with agitation at 200 rpm. An aliquot (1 mL) of this preculture and 0.5 g of the kefir grains were used to inoculate the deproteinized whey medium or skimmed milk (Fajardo et al., 2010). Samples were withdrawn at intervals during the incubation to perform the analytical determinations.

The results obtained in this assay are shown in **Figure 7**. Although the initial pH of whey was initially adjusted to 6.5 with 2N NaOH, it decreased to a value of 4.86 after medium sterilization, and then the culture was started at this pH level. Then, the culture pH dropped to 4.0 during the first 4 h of incubation and remained constant afterwards.

In this culture, the dry weight of the grain (GDW) increased only slightly during the incubation. Free biomass production (as cell dry weight, CDW) reached its highest level (2.0 g/L) after 24 h of fermentation, remained constant until the 60 h, and then began to fall, when relatively high concentrations of lactose (17.0 g/L) remained in the medium. Thus, this growth cessation could be due to the exhaustion of nutrients (the sources of nitrogen and phosphorus) or any essential micronutrient for biomass production, or due to the low pH levels (below 4.0) achieved in the culture medium.

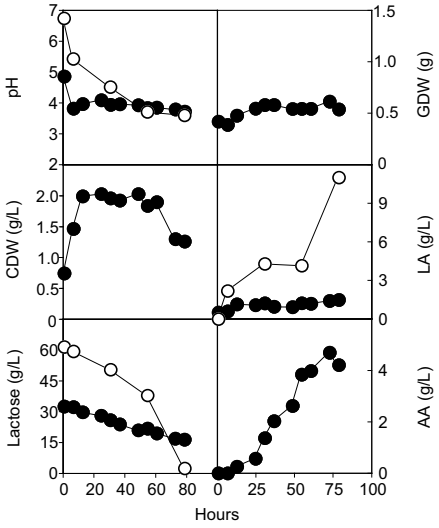

Figure 7. Kinetics of the batch fermentation of kefir grains CIDCA AGK1 on unbuffered whey (closed symbols) and skimmed milk (open symbols). CDW: cell dry weight of free biomass, AA: acetic acid, LA: lactic acid, GDW: grains dry weight.

Production of acetic acid (4.3 g/L) was only detected in whey, probably due to the metabolic activity of acetic bacteria or the development of an early heterolactic fermentation as a result of nutrient limitation due to low nitrogen content in the deproteinized whey medium. This behaviour has been observed before in other cultures with *L. lactis* CECT 539, *Ped. acidilactici* NRRL B-5627 and *Lactobacillus casei* subsp. *casei* CECT 4043 on whey (Guerra and Pastrana, 2003; Guerra et al., 2005; Fajardo, et al., 2008).

Determination of biomass in the milk culture was not possible due to the high amount of material precipitated after performing the centrifugation of the medium. As expected, lactic acid concentration in milk (11.0 g/L) was 7 times higher than in whey (1.5 g/L). This was probably due to the fact that milk

contains a higher concentration of nutrients (sources of carbon, nitrogen, phosphorus, vitamins, etc.) than the whey medium. Consumption of lactose in milk (59.0 g/L) was also higher than that in whey (16.1 g/L) during the whole fermentation period.

In general, the differences observed between the productions obtained in milk and whey could be related to the different initial media composition, initial pH levels (6.8 in milk, and 4.86 in whey) and pH evolution. According to Poolman and Konings (1988), the amino acid or peptide transport, which is one of the growth-rate-determining steps, depends on the culture pH. For *L. lactis* and *L. cremoris* strains, the optimum pH value for amino acid transport varied between 6.0 and 6.5, decreasing rapidly at higher and lower pH values (Poolman and Konings, 1988). In addition, a low nutrient consumption and consequently, a low biomass production by *Lact. casei* CECT 4043 (Fajardoet al, 2008) and *Ent. faecium* CECT 410 (Guerra et al., 2010) was observed in batch cultures in whey when the media reached a pH value below 5.0. In both cases, the observed reduction in the growth was related not only to a limitation in nutrient transport, but also to the exhaustion of some essential micronutrients (vitamins or minerals) or amino acids in the medium (Hofvendahl, et al, 1999; Fajardo et al 2008; Guerra et al., 2010).

In order to verify if the low initial pH and the low pH levels reached in the medium during fermentation affected the fermentation kinetic of kefir grains in whey, a new culture was performed with a whey medium buffered to an initial pH of 6.5 with 0.10 M potassium hydrogen phthalate-NaOH. The fermentation conditions were the same as in the previous culture.

The results obtained (**Figure 8**) showed that the pH dropped from its initial level of 6.5 to a value of 5.4 during the first 12 h of fermentation and remained almost constant afterwards. This fact did not affect the growth of the grains, because the final GDW obtained was similar to that of the previous culture. However, the productions of biomass (4.4 g/L) and lactic acid (6.2 g/L), as well as the lactose consumption (25.4 g/L) were higher than those obtained in the unbuffered whey medium, showing the positive effect of pH control on these variables.

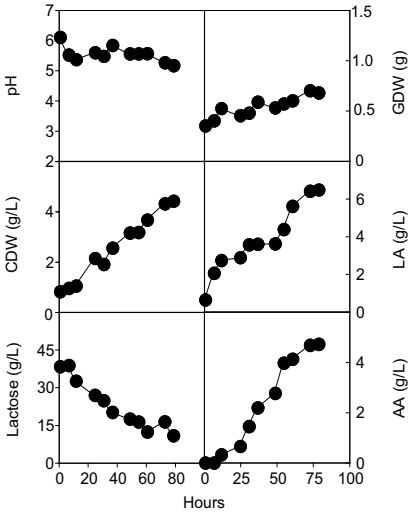

Figure 8. Kinetics of the batch fermentation of kefir grains CIDCA AGK1 on whey buffered at initial pH of 6.5 with 0.10 M potassium hydrogen phthalate–NaOH. Notations are as in Figure 7.

Surprisingly, acetic acid production reached the same final level as in the previous fermentation (Figures 7 and 8). Since the pH values never dropped below 5.0, it could be considered that a limitation of nutrients uptake and consequently the shift from homolactic to heterolactic fermentation did not occur in the culture in buffered whey. Thus, it is reasonable to suppose that the accumulation of acetic acid in this culture was probably due to the increase of the metabolic activity of the acetic bacteria present in the kefir grains.

After clarifying the positive effect that the initial pH and its evolution produced on the production of biomass and lactic acid, the following experiment was focussed on the optimization of the composition of buffered whey medium.

Since the carbon source is not completely consumed during the fermentations (see **Figures 7** and **8**), it could be considered that the initial lactose in the whey medium (~ 35 g/L) is suitable for the growth of the microbial population present in the kefir grains. For this reason, in the following experiment, the whey was supplemented with

phosphorus (P) and nitrogen (N) sources, whose concentrations in this medium are lower than those of the complex culture media such as milk or MRS medium (Guerra and Pastrana, 2001a). With this experiment, the production of free biomass, lactic acid and acetic acid after N and P supplementation could be studied in buffered whey without nutrient limitation.

Thus, a central composite design (Akhnazarova and Kafarov, 1982; Box et al, 1989) based on two levels and two variables was used to study the effect of the initial concentrations of total nitrogen and phosphorous on free biomass production and product (lactic and acetic acid) synthesis on buffered whey. The design 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 (**Table 1**)

.Table 1. Experimental domain and codification of the variables in the study on the effect of the nitrogen (N) and phosphorous (P) sources on production of free biomass and product formation by kefir grains CIDCA AGK1 on buffered whey.

Codified values	Natural values	
	N (g/L)	P (g/L)
-1.267	0.00	0.00
-1	2.32	0.21
0	11.01	1.00
1	19.70	1.79
1.267	22.00	2.00

Those concentrations corresponding to the buffered whey medium (initial pH = 6.5) without supplements were taken as the inferior levels ($\alpha = -1.267$) for the N and P variables. To reach the superior limit ($\alpha = 1.267$), the media were supplemented with the sources of phosphorus (K_2HPO_4) and nitrogen (a mixture of bacteriological peptone, meat extract and yeast extract) to obtain the same concentrations as in the MRS medium. This approach would provide, *a priori*, an increase in the production of free biomass and antimicrobial substances (Guerra and Pastrana. 2001b; 2002).

Results were analyzed by Experimental Design Module of the Statistica software package (Statistica 5.1 for Windows computer program manual; StatSoft Inc. Tulsa, OK, USA, 1996). The Student's *t*-test was employed to check the statistical significance of the regression coefficients. The Fisher's *F*-test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the models.

Table 2 shows the experimental matrix, as well as the corresponding results for the productions of free biomass (CDW), lactic acid (LA) and acetic acid (AA) and the final pH reached in the cultures. As can be observed, the final pH values obtained in the cultures were from 4.41 to 4.64. Therefore, the effect of this variable on the metabolic activity of the microbiota present in the kefir grains appeared to be not significant.

The corresponding analysis of variance (ANOVA) of each empirical model obtained along with the values of the determination coefficient (R^2) and the adjusted determination coefficient are presented in **Table 3**. In each case, the equations obtained for CDW, LA and AA productions were highly significant in Fisher's *F*-tests ($\alpha < 0.05$) applied to the quotients total error/experimental error and lack of fitting/experimental error. In addition, the higher values of R^2 obtained (0.847 or higher) indicated that the fitted models could explain at least 85 % of the total variation in the responses. These facts indicate that the quadratic models were appropriate to fit and describe satisfactorily the experimental data.

After eliminating the non-significant coefficients, the corresponding empirical equations obtained for CDW, LA and AA productions as a function of the concentrations of N and P, in coded units, were:

$$\text{CDW (g/L)} = 5.70 + 0.56 \cdot N + 0.56 \cdot P - 0.61 \cdot P^2$$

$$\text{LA (g/L)} = 8.84 + 0.75 \cdot N + 1.02 \cdot P - 0.99 \cdot P^2$$

$$\text{AA (g/L)} = 22.06 + 1.76 \cdot N + 2.77 \cdot P - 6.91 \cdot N^2 \quad (3)$$

Table 2. Experimental concentrations of free biomass (CDW), lactic acid (LA), acetic acid (AA) and final pH values reached in the buffered whey media supplemented with different concentrations of N and P according to the experimental matrix defined in Table 1.

Codified values		Natural values		Response variables			
N	P	N (g/L)	P (g/L)	LA (g/L)	AA (g/L)	CDW (g/L)	Final pH
1	1	19.70	1.79	10.02	20.35	6.27	4.45
1	-1	19.70	0.21	8.20	15.96	5.16	4.41
-1	1	2.32	1.79	7.99	12.73	5.15	4.56
-1	-1	2.32	0.21	6.36	9.57	4.09	4.53
1.267	0	22.00	1.00	8.99	9.47	6.46	4.64
-1.267	0	0.00	1.00	7.79	10.50	5.01	4.64
0	1.267	11.01	2.00	8.40	23.92	5.40	4.55
0	-1.267	11.01	0.00	5.29	14.09	3.97	4.54
0	0	11.01	1.00	9.06	22.02	5.72	4.51
0	0	11.01	1.00	9.20	23.02	5.91	4.45
0	0	11.01	1.00	8.54	20.02	5.49	4.55
0	0	11.01	1.00	9.20	24.02	5.91	4.48
0	0	11.01	1.00	8.54	22.02	5.49	4.48

Table 3. Significance analysis of the proposed models for the free biomass (CDW), lactic acid (LA) and acetic acid (AA) production. SS: Sum of Squares; df: degrees of freedom; QM: Quadratic Means; E: total error; Ee: Experimental error; LF: Lack of Fitting.

	CDW (g/L)			LA (g/L)			AA (g/L)		
QME/QMEe	0.50			2.15			2.96		
F ($\alpha = 0.05$)	$F^9_4 = 6.00$			$F^9_4 = 6.00$			$F^9_4 = 6.00$		
QMLF/QMEe	0.10			3.07			4.52		
F ($\alpha = 0.05$)	$F^5_4 = 6.26$			$F^5_4 = 6.26$			$F^5_4 = 6.26$		
R^2	0.969			0.882			0.847		
Adjusted R^2	0.959			0.842			0.800		
	SS	df	QM	SS	df	QM	SS	df	QM
Model	6.34	3	2.11	16.67	3	5.56	323.88	3	107.96
Error	0.20	9	0.02	2.24	9	0.25	58.55	9	6.51
Exp. error	0.18	4	0.04	0.46	4	0.12	8.80	4	2.20
Lack of fitting	0.02	5	0.00	1.78	5	0.36	49.75	5	9.95
Total	6.55	12	0.55	18.91	12	1.58	382.44	19	0.028

Table 4. Experimental conditions to study the effect of aeration, agitation and inoculum size in the kinetic behaviour of whey fermentation by kefir grains CIDCA AGK1.

Series	Erlenmeyer flask volume (mL)	Medium volume (mL)	Inoculum	Agitation speed (rpm)
1	250	50	0.5 g of kefir grain 1.0 mL of fermented milk	200
2	250	100	1.0 g of kefir grain 2.0 mL of fermented milk	200 (0 - 24 h), 0 (24 - 48 h), 200 (48 - 72 h), 0 (72 - 96 h)
3	150	90	0.9 g of kefir grain 1.8 mL of fermented milk	0

The significant coefficients for the quadratic term P^2 in models (1) and (2), and the quadratic term N^2 in model (3), imply the existence of optimum values for P and N inside the experimental domain for CDW, LA and AA. For CDW and LA productions, the coefficient for the quadratic term N^2 was found to be non-significant and the linear term N had a positive coefficient. For AA production, a non-significant value was obtained for the quadratic term P^2 and the linear term P had a positive coefficient.

Therefore, the optimum value of N for CDW and LA productions was located in the maximum level (N = 22.0 g/L) assayed for this independent variable. In case of AA production, the optimum value of P was also located at its maximum value (P = 2.0 g/L).
The response surfaces obtained according to the three above models showed that the productions of CDW, LA and AA were highly influenced by both the phosphorus and nitrogen source concentrations (**Figure 9**).

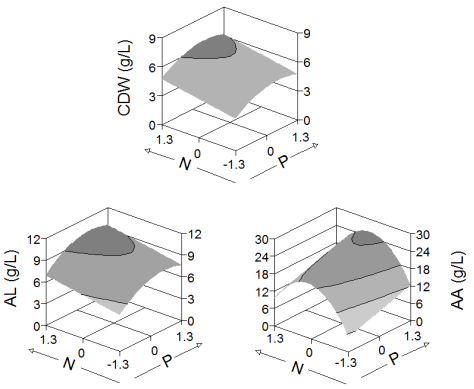

Figure 9. Response surfaces showing the effect of the nitrogen and phosphorous sources on the batch production of free biomass (CDW), lactic acid (LA) and acetic acid (AA) by kefir grains CIDCA AGK11, during 96 hours, on whey buffered at initial pH of 6.5 with 0.10 M potassium hydrogen phthalate–NaOH.

Thus, the optimum concentrations of CDW (5.7 g/L), LA (10.0 g/L) and AA (25.7 g/L) were obtained for N = 22.00 g/L and P = 1.36 g/L, for N = 22.00 g/L and P = 1.41 g/L and for N = 12.12 g/L and P = 2.00 g/L, respectively. These optimum concentrations of CDW, LA and AA were higher than those (4.5, 5.5 and 5.2 g/L, respectively) obtained in the previous experiment. This indicates that there was not nutrient limitation in the cultures on buffered whey supplemented with the complex nitrogen sources and K_2HPO_4, and consequently, the metabolic activity of both the acetic and lactic acid bacteria was favoured.
Taking into account that in the fermentation processes, other variables such as the agitation, aeration and inoculum size have a marked influence on the evolution of the fermentation process (Cabo et al., 2001; Pérez and Guerra, 2009), the following experiment was focused on the study of the effect of these independent variables on CDW and product formation (as response variables) by kefir grains in supplemented buffered whey.
The results obtained in the previous experiment showed that the optimum values for the response variables were obtained for different concentrations of the nitrogen and phosphorus sources. Therefore, the following experiment was carried out by supplementing the buffered whey with the mean concentrations of N and P (18.71 and 1.59 g/L, respectively) that provided the optimum CDW, LA and AA concentrations.
The culture conditions defined for this experiment are shown in **Table 4**. In this study, different Erlenmeyer flask volume/medium volume relationships were used to ensure different levels of aeration in the culture media.
The results obtained (**Figure 10**) showed that the evolution of the culture pH was not affected by changes in agitation, inoculum size and aeration. In addition, the different agitation and aeration strategies had a poor influence on the production of lactic acid, but there was a slight stimulation of production of ethanol and acetic acid in the static conditions (third series).

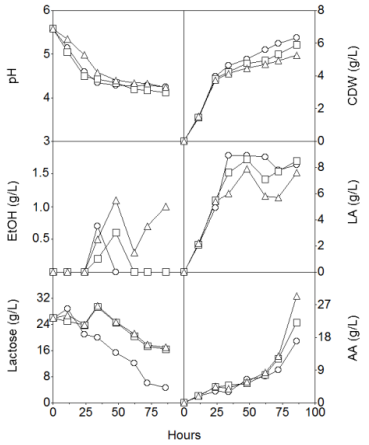

Figure 10. Kinetics of the batch fermentations of kefir grains CIDCA AGK1 on buffered whey supplemented with N and P sources (for more details see the text). The symbols correspond to the series 1 (○), series 2 (□) and series 3 (Δ). EtOH: ethanol. Notations are as in Figure 7.

On the other hand, the continue agitation favoured the consumption of the carbon source, since a concentration of lactose below 4.0 g/L was obtained in the culture carried out at a constant agitation speed of 200 rpm. Since in this culture, the production of lactic acid and acetic were respectively similar and lower, than those of the other two series, it is reasonable to suppose that the higher amounts of lactose consumed in the first culture was mainly used for biomass production. In fact, the final concentration of CDW obtained in the first series (6.32 g/L) was higher than those obtained in the second (5.88 g/L) and third (5.27 g/L) series (**Table 1** and **Figure 10**).

Ethanol production was slightly higher in the static culture (series 3) than in the other two fermentations. However, in the series 1 and 2, ethanol concentration dropped to zero before the end of the cultivations (**Figure 10**). The disappearance of ethanol in these cultures could be due to its assimilation as carbon source by the acetic bacteria to produce acetic acid (Nanda et al., 2001; Sueki, et al, 2005).

These promising results indicated that whey could be used a profitable substrate for production of highly concentrated probiotic cultures to be used as additives in animal feed.

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