OPTIMIZATION OF ENTEROCIN A PRODUCTION AND APPLICATION IN SPANISH STYLE DRY FERMENTED SAUSAGES

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BACKGROUND

Lactic acid bacteria (LAB) have been traditionally used in food processing because of their ability to improve the organoleptic and the wholesomeness characteristics of foodstuffs. The increasing consumers demands for natural food additives has focused on bacteriocins. Bacteriocins of LAB are considered natural biopreservatives because they are produced by GRAS organisms and moreover because they are biodegradable. Nisin is by now the only bacteriocin accepted in 45 countries as a biopreservative (de Vuyst and Vandamme, 1994). Pediocin AcH or PA-1, leucocin A, sakacin A/K and enterocin 1146 have been studied for their possible use in food preservation, the results of these studies seem very promising.

OBJECTIVES

The aims of this study were to demonstrate the antilisteria activity of enterocin A produced by *Enterococcus faecium* (Aymerich et al,1996) in sausages and to optimize the production of enterocin A for its application as a food additive. In this way, the effect of enterocin A in sausages, growth conditions and ingredients effect on enterocin A production were analized.

MATERIAL AND METHODS

Bacterial cultures and media.

Enterococcus faecium CTC492 (enterocin A producer) and *Lb.curvatus* CTC371 (standard starter culture (Bac^-) were incubated in MRS at 30ºC. *Listeria innocua* CTC1014 and *Listeria monocytogenes* CTC1011 were grown in TSBYE at 30ºC.

Sausage manufacture and sampling.

Four independent sausages treatments were conducted. Batch I: no lactobacilli added, batch II: standard starter strain (*Lb.curvatus* CTC371 Bac^-), batch III: *E.faecium* CTC492 (Bac^+) and batch IV: *E.faecium* CTC492 (Bac^+) plus 256 AU ml^{-1} of enterocin A. The starter cultures were added together with the additives to achieve 10^6 cfu g^{-1} in the sausage mixture. Meat was contaminated with *Listeria* at 10^3 cfu g^{-1}. Three sausages from each treatment were sampled at 0, 3, 7, 14 and 28 days after sausages preparation to determine lactic acid bacteria and listeria populations.

Bacteriocin assay

Bacteriocin quantification was performed by the agar spot test (Tagg et al, 1976). The total activity (TA) was obtained without centrifugation of the supernatant (TA) and the soluble activity (SA) after centrifugation, in order to remove the cells.

Determination of MICs and MKCs

The minimum inhibitory concentration (MIC) and the killing concentration (MKC) were determined as Nielsen et al (1990).

Influence of different parameters on enterocin A production.

Enterocin A production in modified MRS was studied: glucose (0% to 5%), 2% saccharose and 0.25% glucose plus 2% saccharose, sodium chloride (0% to 5%), black pepper, potassium nitrate, sodium nitrate, sodium nitrite, combinations of sodium chloride with the rest of the additives for sausage manufacture and tween (0 to 1%).

Initial pH was valorated ranging from 5.0 to 7.5 and final pH from 5.5 to 6.5, temperatures from 4° to 45ºC.

Desadsorption was assayed supplementing MRS with 0 to 1% of tween 80. The percentatge of adsorption was determined as ((TA-SA)/ TA) x 100.

Enterocin A production, OD_{600}, and pH were determined for every assay.

Statistical design to analyse optimization enterocin A parameters

Statistical analysis were carried out using the GLM procedure of the SAS software. Significant values in the different experiments were evaluated by the Bon Ferroni test (SAS, 1988). For analysis, bacteriocin activities were transformed to $\log_2$ (AU/ml)/100.

RESULTS AND DISCUSSION

Inhibition of *L.innocua* in dry fermented sausages

L.innocua was used instead of *L.monocytogenes* to minimize risks. The results of MIC and MKC indicated a greater sensitivity to the bacteriocin by *L.monocytogenes*. For *L.monocytogenes* CTC1011 a mean value of 19 AU ml^{-1} was estimated for MIC and 50 AU ml^{-1} for MKC. For *L.innocua* CTC1014, the MIC was estimated at 25 AU ml^{-1} and 100 AU ml^{-1} for MKC.

Listeria counts diminished 1.63 log cycles in batch I (non LAB inoculated), 1.58 log in the batch II (standard starter culture Bac^-), 1.67 log in the batch III (starter culture Bac^+) and 2.36 log in the batch IV (starter culture Bac^+ plus semipurified enterocin A (256 AU g^{-1})). Most important, listeria counts diminished down to 6 $NMPg^{-1}$ from the beginning until the end of the process in batch IV, thus representing 1.47 log reduction in listeria counts when comparing with batch I (non LAB inoculated) and 0.99 log reduction when comparing with batch II (standard starter culture Bac^- (figure 1).

This is the first report in meat products of an *in situ* assay showing an effective inhibition of *Listeria* by a bacteriocin, enterocin A, produced by a species of *Ent.faecium*. Enterocin A may be considered as a good antilisterial substance to be applied in fermented sausages as a food additive.

Effect of different parameters on enterocin A production

The addition of single additives currently used in the Spanish style sausages, significantly inhibited bacteriocin production, with the exception of nitrate. Sodium chloride had a negative effect in growth and bacteriocin production. The addition of 0.5% glucose and nitrate increased the tolerance of *Ent. faecium* CTC492 to salt and positively affected the bacteriocin production. The combination of

salt and pepper strongly inhibited the bacteriocin production (300 AU ml^{-1}), although growth was not affected when comparing with standard MRS (4815 AU ml^{-1}). This negative effect of salt and pepper together with the results obtained in the *in situ* experiments in fermented sausages have suggested new assays for the optimization of the enterocin A in *Ent.faecium* in order to be able to be applied as a good antilisterial food additive.

Enterocin A production was enhanced at initial pH ranging from 6 to 7.5, at pH 5.0 was strongly inhibited (figure 2). The optimum temperature for production in MRS was 30-35ºC (figure 4).

A limitation of glucose in the growth media favoured enterocin A production, the maximum activity was achieved at 0.5% glucose (7800 AU ml^{-1} in the supernatant). The addition of 2% saccharose plus 0.25% glucose was the best sugar combination to increase growth and bacteriocin production when comparing with the standard MRS (figure 3). An activity of 11200 AU ml^{-1} in the supernatant, a total activity of 24137 AU ml^{-1} and a final optical density of 3.8 (ls means) was achieved in these conditions.

By increasing the concentration of tween up to 0.75%-1%, a significantly higher bacteriocin production in the supernatant (13000-11000 AU ml^{-1}), together with a decrease of the adsorption to the cell wall from 59% to 4-27% was observed. When combining 1% tween with the best carbohydrate combination an increase in the yield of total supernatant bacteriocin production was significantly observed (23000 AU ml^{-1}), while the percentage of absorption was stable (28705 AU ml^{-1} for total activity). Moreover, combining 1% tween, 2% saccharose plus 0.25% glucose and controlling the final pH to pH 5.5 (mMRS), a synergistic effect of the three parameters was observed and the highest bacteriocin production and growth of the producer cells were obtained: a maximum production of 66000 AU ml^{-1} of enterocin A in the supernatant and 96000 AU ml^{-1} in the whole culture with a favourable adsorption of 21%. Thus representing a total significant increase of 13.7-fold when comparing to the standard MRS (figure 3).

CONCLUSIONS

- Enterocin A is an effective antilisterial substance in fermented sausages.
- Enterocin A production in *Ent.faecium* CTC492 is highly inhibited by salt and pepper, essencial sausages ingredients.
- *In vitro* production of enterocin A by *Ent.faecium* CTC492 is pH, temperature, salt and pepper, carbohydrate source and tween dependent.
- A modified MRS supplemented with saccharose and glucose, 1% of tween and a controlled final pH of 5.5 allows an increase in enterocin A production by 13.7-fold when compared with the standard MRS.

ACKNOWLEDGEMENTS

This project was supported by the Comision Interministerial de Ciencia y Tecnologia (CICYT). Project nº: .ALI 94-0207.

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Figure 1: Antilisterial effect of enterocin A in fermented sausages

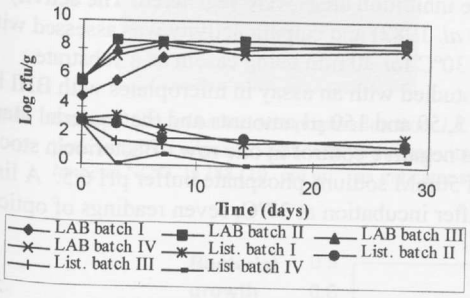

Figure 2: Effect of pH on enterocin A production

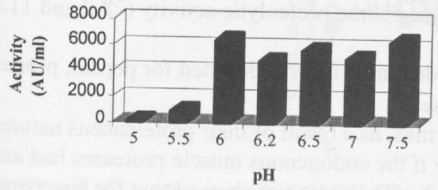

Figure 3: Improving enterocin A production at 30ºC

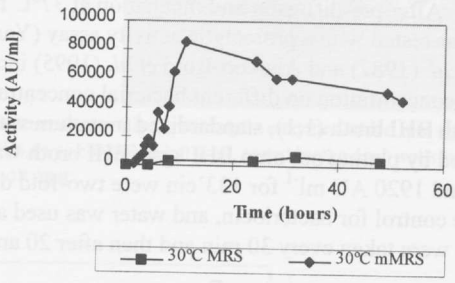

Figure 4: Effect of temperature on enterocin A production