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Media Optimization of Bacteriocin ST22Ch Production by *Lactobacillus Sakei* ST22Ch Isolated from Salpicão, a Traditional Meat-Product from Portugal

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Bacteriocins are known for anti-microbial properties against various pathogens. In the present study, bacteriocin ST22Ch produced by *Lactobacillus sakei* isolated from *salpicão*, a traditional pork product from the northwest of Portugal, inhibited the growth of *Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae, Lactobacillus rhamnosus, Listeria ivanovii* subsp. *ivanovii, Listeira monocytogenes*, *Pseudomonas* spp., *Staphylococcus aureus, Streptococcus caprinus* and *Streptococcus* spp. In particular, low levels of bacteriocin ST22Ch activity (approximately 200 AU/mL) was detected after 3 h of growth in MRS broth. Maximal production (1600 AU/mL) of bacteriocin ST22Ch was recorded after 19 h in MRS broth, stay constant only for 5 h and decrease to 800 AU/mL and only when incubated at 30 °C. During 35 h of growth, the optical density increased from 0.15 to 5.73. Highest bacteriocin ST22Ch production (3200 AU/mL) was records in presence of yeast extract as single organic nitrogen source (20.0 g/L). Presence of tryptone (20.0 g/L) or combination of tryptone and meat extract (12.5 g/L and 7.5 g/L) or yeast extract and meat extract (10.0 g/L and 10.0 g/L, respectively) yielded 1600 AU/mL bacteriocin production. Presence of glycerol at 1.0 g/L to 5.0 g/L not effect the bacteriocin ST22Ch production. Tween 80 added to the MRS medium (5.0 g/L) increase bacteriocin ST22Ch production up to 6400 AU/mL. Exclusion of manganese sulphate results in reduction of activity in the cell-free supernatant. Exclusion of magnesium sulphate from the media formula increases the bacteriocin production to 3200 AU/mL.

1. Introduction

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides (De Vuyst and Vandamme, 1994). Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms (De Vuyst and Vandamme, 1994). They are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglican syntesis (De Vuyst and Vandamme,1994).

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Recently, according to a complete genome analysis, it was proposed that *L. sakei* can be used to control pathogens in meat because its metabolism is particularly well adapted to a meat medium (Chaillou et al., 2005).
Recent approaches in the preservation of meat products are increasingly directed toward biocontrol using bacteriocinogenic *Lactobacillus* species as protective microflora to inhibit growth of *L. monocytogenes* and other undesired microorganisms.
Bacteriocin production does not always correlate with the increase in cell mass or growth rate of the producer strain (Bogovic-Matijasic and Rogelj, 1998). Higher bacteriocin levels are often recorded in the absence of growth stimulating nutrients, or at temperatures and pH conditions lower than required for optimal growth (Todorov et al., 2000). Optimal bacteriocin production is often recorded in medium with limiting concentrations of sugars, nitrogen sources, vitamins and potassium-phosphate, or when the medium pH is regulated (Vignolo et al., 1995).
The objective of this study was to characterize bacteriocin ST22Ch produced by *Lactobacillus sakei* ST22Ch isolated from *Salpicão*, with the aim of using the strain as a co-starter culture in meat fermentations.

2. Materials and methods

2.1 Isolation of lactic acid bacteria and screening for bacteriocin activity

Samples of 50 g each Salpicão obtained from the Local market (Viana do Castelo, Portugal), were macerated in a stomacher for 10 min at 20 °C. Serial dilutions of the sample were made with sterile saline (0.85 %, w/v NaCl), plated onto MRS agar (Merck) and incubated at 30 °C for 24 h. Screening for bacteriocin-producing isolates was carried out according to the triple-agar-layer method described by Todorov and Dicks (2005). Antimicrobial activity was confirmed by using the agar-spot-test method (Todorov and Dicks, 2005). E. faecium was used as a sensitive test strain.

2.2 Identification of strain ST22Ch

The morphology of strain ST22Ch was determined by using an AFM. Identification was by physiological and biochemical tests. Carbohydrate fermentation reactions were recorded by using API50CHL. Identification to species level was by PCR with primers specific for *L. sakei*. Confirmation of identification was obtained by amplifying the genomic DNA with primers F8 and R1512. The amplified fragments were cleaned using SigmaSpin™ Post-Reaction Clean-Up Columns (Sigma, St Louis, MO, USA), sequenced, and compared to sequences in GenBank using BLAST, Basic Local Alignment Search Tool.

2.3 Isolation and determination of molecular size of bacteriocin ST22Ch

Strain ST22Ch was cultured in MRS broth (Merck) for 24 h at 30 °C. The cells were harvested (8000 x *g*, 10 min, 4 °C), the cell-free supernatant was adjusted to pH 5.0 with 1 M NaOH, heat-treated (80 °C for 10 min) and the bacteriocin ST22Ch precipitated with 60 % saturated ammonium sulphate. The precipitate was resuspended in 20 mL of 25 mM ammonium-acetate (pH 6.5) and the amount of antimicrobial activity determined by testing against *E. faecium*, as described above. The molecular size of bacteriocin ST22Ch was determined by tricine-SDS-PAGE (Todorov and Dicks, 2005).

2.4 2.4 Effect of medium components of bacteriocin ST22Ch

Strain ST22Ch (100 µL of a 24 h-old culture) were inoculated into 10 mL MRS broth, modified as indicated in Table 2.

3. Results and discussion

3.1 Spectrum of antimicrobial activity

Bacteriocin produced by *L. sakei* ST22Ch, isolated from Salpicão inhibited the growth of *Enterococcus faecium, Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *Listeira monocytogenes* NCTC 11944, NCTC 7973 and Scott A. This strain was screened against a panel of sensitive strains (Table 1). In addition cell-free supernatant from strain ST22Ch, adjusted to pH 6.0, inhibited the growth of *Escherichia coli, Pseudomonas* spp., *Staphylococcus aureus, Streptococcus* sp. and *Lactobacillus*

rhamnosus. This narrow-spectrum of activity is unique for a bacteriocin produced by *L. sakei*. Most of the bacteriocins described for *L. sakei* are active against a much broader range of genera and species (De Vuyst and Vandamme, 1994).

Table 1: Spectrum of antimicrobial activity of bacteriocin ST22Ch

Test microorganisms	Bacteriocin ST22Ch
Enterobacter cloaca	0 / 2
Enterococcus faecalis	0 / 6
Enterococcus faecium	1 / 3
Escherichia coli	1 / 3
Lactobacillus acidophilus	0 / 1
Lactobacillus casei	0 / 2
Lactobacillus curvatus	0 / 4
Lactobacillus delbruekii	0 / 1
Lactobacillus fermentum	0 / 2
Lactobacillus jonhsonii	0 / 1
Lactobacillus paracasei subsp. *paracasei*	0 / 1
Lactobacillus paraplantarum	0 / 1
Lactobacillus pentosus	0 / 3
Lactobacillus plantarum	0 / 8
Lactobacillus rhamnosus	1 / 1
Lactobacillus salivarius	0 / 1
Lactococcus lactis subsp. *lactis*	0 / 1
Listeria innocua	2 / 3
Listeria ivanovii subsp. *ivanovii*	1 / 1
Listeria monocytogenes	4 / 4
Pediococcus acidilactici	0 / 1
Pediococcus pentosaceus	0 / 1
Pseudomonas spp.	1 / 3
P. aeruginosa	0 / 8
Salmonella spp.	0 / 2
Staphylococcus aureus	1 / 14
Streptococcus caprinus	2 / 2
Streptococcus sp.	2 / 2

*Number of test microorganisms sensitive to bacteriocin / total number of microorganism tested.

3.2 Identification of isolate ST22Ch

Isolate ST22Ch is rods-shaped visualized by AFM (data not shown) and based on sugar fermentation reactions (not shown), 99.8 % related to *Lactobacillus curvatus*. Amplification of genomic DNA with genus-specific primers produced a 226 bp fragment, which corresponded in size to that of *Lactobacillus sakei* NCFB 2714^T (not shown). The 16s rDNA amplified from isolate ST22Ch revealed 98% homology to the 16s rDNA sequence of *L. sakei*. Isolate ST22Ch is thus regarded a strains of *L. sakei*.

3.3 Isolation of bacteriocin ST22Ch

According to tricine-SDS PAGE, bacteriocins ST22Ch is approximately 2.8 – 3.4 kDa in size (not shown). This is within the size range of most bacteriocins reported for the genus *Lactobacillus* (De Vuyst and Vandamme, 1994) and sakacin G, a 3.8 kDa.

3.4 Production of bacteriocin ST22Ch

The cell density of *Lb. sakei* ST22Ch increased from 0.1 to 5.7 (OD_{600}) during 36 h of growth at 30 °C (Figure 1). The pH decreased from 6.40 to around 4.0, over the same period (Figure 1). Production of bacteriocin ST22Ch increased from 200 AU mL^{-1} after 4 h of growth to 1600 AU mL^{-1} during the following 19 h and stabilize for next 5 h and decrease to 800 AU/mL in next 3 h and to 400 for the rest of the fermentation period (Figure 1). Optimal production of bacteriocin ST22Ch was recorded during stationary growth, which may suggest that the peptide is a secondary metabolite. Similar results were reported for plantaricin ST31 (Todorov et al., 1999) and bacteriocin ST8KF (Powell et al., 2007).

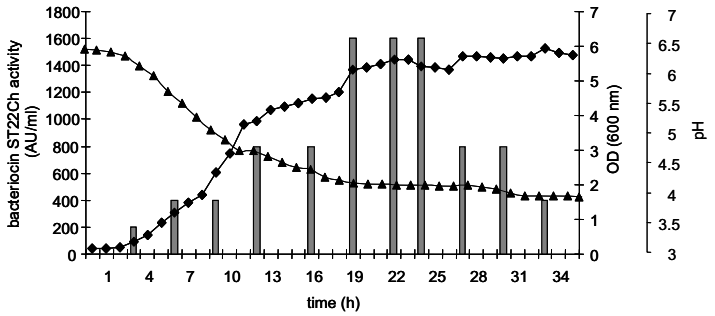

Figure 1: Growth of Lactobacillus sakei ST22Ch and bacteriocin ST22Ch production in MRS broth (Merck). Symbols: (♦) = growth, (▲) = change in pH, (■) = bacteriocin production. Incubation was at 30 °C

3.5 Effect of medium components on bacteriocin ST22Ch production

Bacteriocin ST22Ch was produced at 1600 AU mL^{-1} when strain ST22Ch was grown in MRS broth supplemented with 10, 20 or 30 g L^{-1} glucose (Table 2). The same level of activity was recorded when glucose was replaced by 20 g L^{-1} fructose, lactose or saccharose (Table 2). Decrease in bacteriocin ST22Ch activity was recorded in presence of 20 g L^{-1} maltose or 20 g L^{-1} gluconate. This results suggesting that the glucose moiety of sucrose is favored for production (Table 2). No activity were recorded when the cells were grown in the presence of the same concentration of 20 g/L mannose, 5 g/L glucose or 50 g/L glucose, even the cell growth was similar to this obtain in presence of 20 g/L glucose, yielding highest activity of 1600 AU/mL. Our results show that bacteriocin ST22Ch production is stimulated by glucose. Similar results have been reported for plantaricin ST31 (Todorov et al., 2000).

Of all nitrogen sources tested (Table 2), yeast extract yielded two time increased activity (3200 AU mL^{-1}) compared with control (tryptone, meat extract and yeast extract) (1600 AU mL^{-1}) for bacteriocin ST22Ch. A combination of Tryptone and Meat Extract (12.5:7.5 g L^{-1}), Meat Extract and Yeast Extract (10.0:10.0 g/L) or tryptone as single organic nitrogen source yielded 1600 AU mL^{-1} (Table 2), suggesting that combination of yeast extract required for optimal bacteriocin ST22Ch production.

MRS medium, supplemented with 1.0 g L^{-1} up to 5.0 g L^{-1} glycerol not effect bacteriocin ST22Ch (1600 AU mL^{-1}) production. Previously was found that glycerol have a negative effect on production of plantaricin ST31, in which case glycerol at 2.0 g L^{-1} and higher resulted in lower activity (Todorov et al., 2000). Glycerol is not used as a carbon source and the decrease in bacteriocin production may be due to changes in osmotic stress.

Little is known about the influence of potassium ions on the production of bacteriocins. Levels of 2.0 g L^{-1} KH_2PO_4 are optimal for the production of bacteriocins ST22Ch (1600 AU mL^{-1}) Replacement of KH_2PO_4 with 5.0 or 20.0 K_2HPO_4 not effecting bacteriocin ST22Ch production (Table 2). The changes in activity cannot be due to pH changes caused by higher potassium levels, since all media were adjusted to pH 6.5 before inoculation.

Presence of cyanocobalamin, thiamine or DL-6.8-thiocit acid (0.002 g L^{-1}) not effecting bacteriocin ST22Ch production. Presence of L-ascorbic acid has a negative effect on bacteriocin ST22Ch production (Table 2).

Table 2: Effect of carbohydrates, nitrogen, potassium, glycerol, vitamins and tri-ammonium sulphate on bacteriocin ST22Ch production

Component	Concentration ($g\ L^{-1}$)	pH	OD	Bacteriocin ST22CH activity ($AU\ mL^{-1}$)
Glucose	5.0	4.72	3.29	0
-"-	10.0	4.19	4.27	1600
-"-	20.0	4.02	4.68	1600
-"-	30.0	4.00	4.29	1600
-"-	50.0	3.99	3.31	0
Fructose	20.0	4.19	3.54	1600
Lactose	20.0	4.36	4.20	1600
Mannose	20.0	4.13	3.50	0
Maltose	20.0	4.39	4.31	800
Saccharose	20.0	4.14	5.47	1600
Gluconate	20.0	5.13	4.2	800
Tryptone	20.0	4.15	2.87	1600
Meat Extract	20.0	4.10	0.99	800
Yeast Extract	20.0	4.09	4.03	3200
Tryptone and Meat Extract	12.5 and 7.5	4.16	4.94	1600
Tryptone and Yeast Extract	12.5 and 7.5	4.02	3.37	800
Meat Extract and Yeast Extract	10 and 10	4.07	3.22	1600
Glycerol	1.0	4.02	3.77	1600
-"-	2.0	4.01	2.97	1600
-"-	5.0	4.00	3.35	1600
-"-	10.0	4.01	5.34	800
-"-	20.0	4.03	2.94	0
KH_2PO_4	2.0	3.99	3.27	800
-"-	5.0	4.00	3.15	800
-"-	10.0	3.99	3.73	1600
-"-	20.0	4.04	4.21	1600
K_2HPO_4	2.0	4.11	4.05	1600
-"-	5.0	4.11	4.05	1600
-"-	10.0	4.27	3.90	800
-"-	20.0	4.99	3.44	1600
Cyanocobalamin (B12)	0.002	3.97	4.15	1600
Thiamine (B1)	0.002	3.98	4.20	1600
L-ascorbic acid (C)	0.002	4.02	3.79	800
DL-6,8-thioctic acid	0.002	3.98	3.15	1600
Tri-ammonium citrate	Free	4.01	4.15	1600
-"-	5.0	4.02	4.68	1600
-"-	10.0	5.81	4.70	3200
pH 4.5		3.74	0.99	400
pH 5.0		3.86	1.35	1600
pH 5.5		3.92	4.60	3200
pH 6.0		3.94	4.96	3200
pH 6.5		4.02	4.68	1600
$MgSO_4$	Free	3.96	3.75	3200
$MnSO_4$	Free	4.40	2.14	800
Tween 80	Free	4.13	5.60	1600
-"-	1.0	4.02	4.68	1600
-"-	2	4.15	4.39	3200
-"-	5	4.17	4.69	6400

Bacteriocin ST22Ch production was stimulated in the presence 10.0 g L^{-1} tri-ammonium citrate (Table 2), compared to growth in absence or 5.0 g L^{-1} (Table 2). Similar effect of tri-ammonium citrate was found for bacteriocin ST8KF production (Powel et al., 2007). Bacteriocin ST22Ch production was stimulated in exclusion of $MgSO_4$ from the MRS medium. However, exclusion of $MnSO_4$ from MRS medium has a negative effect on the bacteriocin ST22Ch production (Table 2). Bacteriocin ST8KF production was reduced in the absence of $MgSO_4$, while no bacteriocin production was observed in the absence of $MnSO_4$ (Powell et al., 2007). Therefore, $MgSO_4$ are required for bacteriocin ST22Ch production. Was recorded that exclusion of Tween 80 from the MRS media formula have no negative effect on bacteriocin ST22Ch production (Table 2). Increasing of the amount of Tween 80 in the MRS broth up to 5.0 g L^{-1} has a stimulating effect of bacteriocin ST22Ch production. Bacteriocin ST22Ch was produced at same levels in MRS broth with an initial pH of 5.5 to 6.0 (3200 AU mL^{-1}) (Table 2).

4. Conclusions

Bacteriocin ST22Ch have a narrow spectrum of activity, are heat resistant and stable between pH 2.0 and 10.0 and is produced at higher levels during the stationary phase of fermentation in the presence of 2 % (w/v) D-glucose. Different levels of bacteriocin ST22Ch was produced in presence of combination of tryptone, meat extract and yeast extract. This results suggesting that this bacteriocin may be produced at high levels during the all phases of meat production. Regarding antibacterial spectrum of activity of strain ST22Ch they may be used in a mixed starter culture for fermentation of the meat products. Further research on there technological properties and the production of specific flavor compounds is in progress.

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