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A Bacteriocin Production on Soya Nutri Nuggets Extract Medium by *Lactococcus lactis* Subsp. *Lactis* CCSUB202

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Abstract: A new medium soya nutri nuggets extract medium with different glucose concentration for lactic acid bacteria is presented. In this we used soya nutri nuggets extract, which is supported good growth for lactic acid bacteria isolated from milk and milk products and glucose, which affects cell growth and bacteriocin biosynthesis. For many of the strains tested, bacteriocin production in Soya Nutri Nuggets Extract Medium (SNNEM) were similar to those achieved in MRS medium. This medium allowed growth to continue to higher densities than four other defined media. SNNEM is compared with MRS and other previously published medium for lactic acid bacteria.

Key words: Bacteriocin, soya, fermenter, lactic acid bacteria, *Latococcus*, media

INTRODUCTION

Lactic Acid Bacteria (LAB) are anaerobic Gram-positive bacteria with a GRAS (Generally Regarded as Safe) status. They are also food grade bacteria and therefore, they can be used for the delivery of proteins of interest in foodstuff or in the digestive tract. Lactic acid bacteria is widely distributed in milk and milk products as a part of the naturally accurring microflora and as deliberately added starter cultures (Hammes and Knauf, 1994) their most important habitat is in untreated milk, fermented milk and cheeses. *Lactococcus lactis* subsp. *lactis*, either in pure form or associated with other micro-organisms is the mesophilic strain most commonly used as a starter culture for lactic products, thus they fulfill an irreplaceable role in ensuring the structure, taste, conservation and healthfulness of these products (Salminen and Von Wright, 1993; Boonmee *et al*., 2003; Ziadi *et al*., 2005; Do-Won *et al*., 2006).

One of the important steps in the study of bacteriocins is their production. The composition of culture medium and culture conditions such as temperature, pH and time of incubation have profound effect on the production of bacteriocins. In general, conditions that provide high cell density favour high bacteriocin concentration.

It is necessary to study the physiological conditions most suited for their production. For examination of nutritional requirements a defined must be used as such a medium (i) provides reproducibility of chemical composition, (ii) avoids an unnecessary excess of nutrients, allowing the determination of nutrients limitation, (iii) meets the experimentally determined nutritional requirements of several strains and (iv) supports growth at a reasonably high rate (Foucaud *et al*., 1997).

Lactococcus are extremely fastidious organism with numerous growth requirements. They are usually grown in a complex medium such as MRS (De Man *et al*., 1960). However, such media are too

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expensive for an economical production process. Recently, there has been increased interest in the production of bacteriocin from soya permeate since certain LAB strains can grow and produce appreciable amounts of bacteriocin in this medium, which has a low cost (De Vuyst and Vandamme, 1994).

The purpose of our investigation was therefore to develop an inexpensive basal medium from soya nutri nuggets extract to produce a high bacteriocin titre using *Lactococcus lactis* CCSUB202 and evaluate the effect of aeration and controlled pH on bacteriocin production using soya nutri nuggets extract medium.

MATERIALS AND METHODS

Bacterial Strains and Media

The bacteriocin producing strain used in this study was *Lactococcus lactis* CCSUB202. Bacteriocin bioactivity was tested quantitatively with *Lactococcus lactis* subsp. *lactis* MTCC 3038 as a test organism. Strains were maintained on MRS slant bottles at 4°C for shorter duration of 1 to 2 months. For longer duration, the cultures were stored frozen at -20°C in 30% sterile skimmed milk in small vials of 2 mL.

Soya Nutri Nuggets Extract Medium (SNNEM) was supplemented with various nutrients such soya nutri nuggets extract (1000 mL), glucose (40 g), sodium acetate (3.0 g), KH_2PO_4 (2.0 g), $MgSO_4$ (0.2 g), Tween-80 (1 mL). The medium was inoculated and incubated at 37°C for 24 h to study their effect on bacteriocin production in the developed medium.

Optimization of physical factors (pH, Temperature and Incubation period) for bacteriocin production on soya nutri nuggets extract medium.

Fermentation

A 24 h old culture of actively growing cells was always used as the inoculum; the inoculum size was always 10% (v/v). The fermentation was carried out in a 2 L bench fermenter (LAB FORS AG CH-4103 Bottmingen/Switzerland) with pH, temperature, dissolved oxygen, agitation and aeration control, using 1 L of medium composition, aeration and control pH on bacteriocin production has been evaluated. The samples were withdrawn at regular intervals to determine the concentration of total protein concentration, pH and bacteriocin activity.

Growth Determination

Growth (biomass) was measured by determination of turbidity after withdrawn the sample take O.D. (optical density) by spectrophotometer (Systronics UV-VIS double-beam spectrophotometer 2201) at 650 nm.

Protein Estimation

Protein concentration was determined by the method of Lowry *et al*. (1951) using Bovine Serum Albumin (BSA) as standard.

Bacteriocin Activity Determination

Inhibitory activity titres against the indicator bacteria were determined by agar diffusion well assay with slightly modification. Fresh culture of bacteriocin sensitive strain (*Lactococcus lactis* subsp. *lactis* MTCC3038) was inoculated in 40 mL of sterilized MRS broth and incubated at 37°C for 24 h. One hundred microliter of broth culture was spread on fresh MRS medium plates. Wells were cut with sterile cork borer (4 mm in diameter).

Fifty microliter of CFF (cell free filtrate) which was serially diluted 1:2, 1:4,1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1: 512 (Two fold dilution) was placed in each well with the help of micropipette using sterilized tips. The plates were then incubated at 37°C for 24 h, without inversion. After incubation the plates were observed for clear circular zones of inhibition around the wells. The diameter of zone of inhibition was measured in mm with the help of standard scale (Zone measured scale, Hi-media). The highest dilution that gave a well defined zone of inhibition of growth was used to calculate AU mL^{-1} (Highest dilution that showed a distinct zone of inhibition X 20 (1000 µL/50 µL).

AU mL^{-1} = highest dilution that showed clear well-defined zone of inhibition x 1000 µL/Volume (µL) used in the well.

RESULTS AND DISCUSSION

Mass Production of Bacteriocin in SNNEM

Bacteriocin production in SNNEM by batch fermenter (LAB FORS AG CH-4103 Bottmingen/Switzerland) with a capacity of 2 L was used in present study.

The relationship between absorbance and bacteriocin activity by *Lactococcus lactis* subsp. *lactis* CCSUB202 as influenced by temperature and pH values was assessed. Table 4 represent fermentation at a controlled temperature 37°C and at constant pH 7.5 after optimized the physical parameters Table 1-3.

Table 4 shows that the bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSUB202 in a batch fermenter was slightly increased as compared to fermentation in shaker culture. In this experiment the highest bacteriocin activity was reached at the end of the exponential growth phase 5280 AU mL^{-1} of medium (Table 4) when absorbance had also reached a maximum value in

Table 1: Bacteriocin production from *Lactococcus lactis* subsp. *lactis* CCSUB202 in SNNEM broth at 37°C at different pH

Initial pH	Time (h)	Final pH of spent medium	Absorbance (O.D. 600 nm)	Activity unit (AU mL^{-1})
5.5	0	5.50	0.004	00
	8	4.80	0.821	640
	16	4.54	1.312	960
	24	3.83	1.932	1280
	32	3.74	2.121	1280
	40	3.65	2.132	1040
6.0	0	6.00	0.030	00
	8	4.92	0.911	640
	16	4.67	1.346	1600
	24	4.02	1.912	2560
	32	3.92	2.110	2560
	40	3.74	2.121	1920
6.5	0	6.51	0.040	00
	8	4.86	0.921	3200
	16	4.52	1.347	3840
	24	4.17	2.213	5120
	32	3.98	2.231	5120
	40	3.76	2.243	4860
7.0	0	7.00	0.030	00
	8	5.02	0.921	3200
	16	4.62	1.321	3840
	24	4.22	2.476	5120
	32	4.12	2.512	5120
	40	3.87	2.542	4880
7.5	0	7.50	0.005	00
	8	5.13	1.042	3200
	16	4.71	2.714	5120
	24	4.32	3.012	5120
	32	4.14	3.032	5120
	40	3.98	3.045	4860

Table 1: Continued

Initial pH	Time (h)	Final pH of spent medium	Absorbance (O.D. 600 nm)	Activity unit (AU mL^{-1})
8.0	0	7.99	0.002	00
	8	5.21	0.742	3200
	16	4.62	1.343	3840
	24	4.41	2.321	5120
	32	4.32	2.342	5120
	40	4.12	2.534	3840
8.5	0	8.50	0.003	00
	8	5.36	0.751	3200
	16	4.82	1.434	3840
	24	4.56	2.142	5120
	32	4.42	2.213	5120
	40	4.23	2.234	4860
9.0	0	9.00	0.004	00
	8	5.48	0.641	3200
	16	4.92	1.213	3840
	24	4.64	1.873	5120
	32	4.54	1.921	5120
	40	4.42	2.024	3840

SNNEM = Soya Nutri Nuggets Extract Medium

Table 2: Bacteriocin production from *Lactococcus lactis* subsp. *lactis* CCSUB202 in SNNEM broth at different incubation temperatures

Incubation temperature (°C)	Time (h)	Final pH of spent medium	Absorbance (O.D. 600 nm)	Activity Unit (AU mL^{-1})
25	0	7.50	0.004	00
	8	5.71	0.912	960
	16	4.34	2.627	3200
	24	3.83	3.012	5120
	32	3.78	3.123	5120
	40	3.64	3.212	4840
30	0	7.50	0.003	00
	8	5.84	1.126	1280
	16	4.42	2.323	3840
	24	3.72	3.121	5120
	32	3.68	3.241	5120
	40	3.54	3.312	5020
37	0	7.50	0.003	00
	8	4.74	1.912	3200
	16	4.12	2.720	5120
	24	3.78	3.212	5120
	32	3.64	3.321	5120
	40	3.58	3.348	5040
40	0	7.50	0.003	00
	8	4.81	1.826	1920
	16	4.01	2.614	3200
	24	3.79	3.029	5120
	32	3.61	3.068	5120
	40	3.52	3.124	3200
45	0	7.50	0.004	00
	8	5.34	0.912	640
	16	4.17	1.214	640
	24	4.17	0.925	320
	32	4.01	1.024	320
	40	3.94	1.076	160
50	0	7.51	0.005	00
	8	5.70	0.812	320
	16	4.67	1.128	320
	24	4.67	1.202	160
	32	4.54	1.201	160
	40	4.42	1.243	120

SNNEM = Soya Nutri Nuggets Extract Medium

Table 3: Bacteriocin production by *Lactococcus lactis* subsp. *lactis* CCSUB202 in SNNEM broth at 37°C at different incubation periods

Incubation period (h)	Final pH of spent medium	Absorbance (O.D. 600 nm)	Activity Unit (AU mL^{-1})
0	7.50	0.006	00
2	5.81	0.306	160
4	5.23	1.618	640
8	4.78	1.718	3200
12	4.42	1.821	3200
16	3.89	2.313	3840
20	3.81	2.341	3840
24	3.83	2.412	5120
28	3.76	2.218	5120
32	3.78	2.017	5120
36	3.79	2.010	5120
40	3.77	2.054	4840

SNNEM = Soya Nutri Nuggets Extract Medium

Table 4: Bacteriocin production in batch fermenter at 150 rpm at constant temperature (37°C) and pH 7.5 in SNNEM

Incubation period (h)	Final pH of spent medium	Absorbance (O.D. 600 nm)	Activity Unit (AU mL^{-1})
0	7.5	0.070	00
2	7.5	0.506	160
4	7.5	1.743	640
8	7.6	2.718	3400
12	7.6	2.792	3400
16	7.5	2.821	5280
20	7.7	2.942	5280
24	7.6	3.121	5280
28	7.6	3.214	4860

SNNEM = Soya Nutri Nuggets Extract Medium

experiment. Bacteriocin activity was already detectable after 4 h of fermentation when 40% of the absorbance had been produced. Table 4 shows that maximum activity was obtained at the 10th h of fermentation (5280 AU mL^{-1}). However, the higher absorbance was achieved in this experiment at 10th h of fermentation Table 4.

Traditionally, optimization of bacteriocin fermentation processes has been performed by physiological and metabolic control of their biosynthesis. Bacteriocin are usually produced in complex media (Biswas *et al*., 1991; De Vuyst and Vandamme, 1992, 1993; Parente and Hill, 1992) under well controlled conditions of temperature and pH (Biswas *et al*., 1991; De Vuyst and Vandamme, 1992; Parente and Ricciardi, 1994; Parente *et al*., 1994; Mortvedt-Abildgaard *et al*., 1995; De Vuyst *et al*., 1996a) seem to play an important role in bacteriocin production.

Similar behaviour was observed by De Vuyst and Vandamme (1992) using *Lactococcus lactis* subsp. *lactis* NIZO22186 and glucose as a carbon source. These experiments achieved 1.27 g L^{-1} of cell mass in the eighth hour of fermentation and nisin activity of approx. 2000 IU mL^{-1} of medium. Several other workers Callewaert *et al*. (1999), De Vuyst *et al*. (1996) and Lejeune *et al*. (1998) also used fermenter for bacteriocin production.

CONCLUSION

Lactococcus lactis subsp. *lactis* CCSUB202 produced bacteriocin in SNNEM (Soya Nutri Nuggetes Extract Medium), which showed higher bacteriocin production than other used medium.

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