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Production of Lactococcal Bacteriocin using Repeated-Batch and Continuous Cultures

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Repeated-batch and continuous cultures of *Lactococcus* sp. 1112-1 were carried out for bacteriocin production using a glucose-casein medium. Repeated-batch culture did not efficiently enhanced the bacteriocin production. Continuous production was possible at the dilution rate of 0.4 h^{-1}. Maximum specific production rate (Q_p), bacteriocin production and biomass at the dilution rate were 347,136 IU/g/h, 2,121 IU/ml and 2.45 g/L, respectively.

It has been well known that lactic acid bacteria play a very important role in the fermentation of foods such as vegetable, meat, dairy and bakery products. They inhibit other spoliage and pathogenic microorganisms by producing various inhibitory substances. These include acid, peroxide, diaceytl (7) and bacteriocins, an antimicrobial substance, etc. (5). Many lactic bacteriocins have been reported. There are two classes of bacteriocins (9). One is the group that is inhibitory to narrow range of target organisms and another is the group that inhibits a broad spectrum of organism. The former includes lactacin and lactocin and the latter includes nisin and pediocin. These bacteriocins draw attenition as they can be used as so-called biopreservatives in food systems (2, 10) and in developing a possible food-grade vector system (6, 8).

Research on bacteriocins has been focused on the expression, production of bacteriocins and mode of action, characterization of plasmid encoding production and immunity, their use as vehicles for gene transfer, identification and description of novel bacteriocins and taxonomic characterization of bacteria based on their production or susceptibility (8). In this laboratory, a bacteriocin-producing bacterium was isolated and identified as *Lactococcus* sp.. This strain showed strong inhibition against *Lactobacillus plantarum* (16) and had wide inhibition spectra (14). The bacteriocin was also suggested as a possible preservative for food (16). In this study, Repeated-batch and continuous cultures were introduced as a means of enhancing the productivity of the lactococcal bacterocin.

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MATERIALS AND METHODS

Organism, Medium and Apparatus

Lactococcus sp. 1112-1 that was isolated and identified in this laboratory was used for this experiment. The fermentation medium was glucose-casein broth (14). The medium contained the following components per liter of distilled water: glucose 20 g; casein acid hydrolyzate 15 g; yeast extract 5 g; ammonium citrate dibasic 2 g; sodium acetate 3 g; magnesium sulfate 0.1 g; potassium phosphate dibasic 2 g; manganese sulfate 0.05 g. The pH was controlled to 6.2 with 3 N KOH. Fermentation was performed in a bench-top fermentor (working volume, 350 ml; NBS C-32) and multigen fermentor (working volume 1 L).

Analytical Methods

Bacteriocin activity was assayed by the agar diffusion method described by Tramer and Fowler (12). The target organism was *Lactobacillus plantarum* ATCC 8014. The lawn-plate was prepared by dispensing 20 ml of sterile MRS agar previously mixed with overnight culture of *L. plantarum* ATCC 8014. Calibration curve was prepared by plotting the diameters (cm) of zone of inhibition against the concentrations of bacteriocin. Nisaplin (Applin & Barret Ind. Co.) was used as a reference compound. Glucose was analyzed by YSI Industrial Analyzer (Yellow Spring Inst. U.S.A.). Biomass was determined from calibration curve of dry weight and optical density at 600 nm.

RESULTS AND DISCUSSION

Effects of Yeast Extract Concentration and the Age of Inoculum

In batch culture, poor medium formulation causes nutrient limitation and thus wash-out at a lower dilution rate in continuous culture. Nutrient fortification in this case enables the operation to occur at a higher dilution rate. *Lactococcus* sp. 1112-1 is a lactic acid bacterium that requires the presence of riboflavin and pantothenic acid for the growth and the bacteriocin production (14). To increase the activity of cell during fermentation, yeast extract was added and optimum concentration was decided (Table 1). *Lactococcus* sp. 1112-1 did not grow in a culture without yeast extract. Yeast extract increased the cell and bacteriocin productions after 12 hours. 5 g/L of yeast extract was found to be enough for this batch process. Bacteriocin production yield was highest at 5 g/L of yeast extract. The growth yield ($Y_{X/S}$) decreased slightly according to the level of yeast extract concentration. This was apparently due to the comparatively higher conversion of most of glucose consumed to other metabolites such as lactic acid.

The age of the inoculum was reported as a very important factor in fermentation to produce microbial metabolites when the viability or activity of starter culture was taken into consideration (4), even though the inoculum size also affects the process (13). To decide the optimum cultivation time for a starter culture, several batch cultures were carried out (Table 2). The age of inoculum did not affect the biomass production by *Lactoccus* sp. 1112-1, but it did increase the production of bacteriocin according to the cultivation time of the inoculum. The product yield as well as specific productivity increased with culture time. However, it was not necessary to grow the culture more than 12 hours. This was regarded as a relatively young culture in the fermentation process. Chesseman and Berridge (1) used an 8 hour-old starter culture after repeated 24-hour-activation for nisin production by *Streptococcus lactis*. Egorov *et al.* (4) reported that the induction period of bacteriocin production could be reduced when they used a young culture.

Table 1. Effect of yeast extract concentration on bacteriocin production by *Lactoccus* sp. 1112-1.

Conc. (g/L)	Biomass (g/L)	Activity (IU/ml)	$Y_{P/S}$ (IU/g)	$Y_{X/S}$	Growth rate (h^{-1})
0	0.14	105	–	–	–
3	1.44	1704	263369	0.22	0.8859
5	1.75	3418	377680	0.19	0.8437
8	2.05	2711	207422	0.16	0.8612
10	2.71	2421	140348	0.16	0.8721

Table 2. Effect of age of seed culture on bacteriocin production by *Lactococcus* sp. 1112-1.

Time (h)	Biomass (g/L)	Activity (IU/ml)	$Y_{P/S}$ (IU/g)	$Y_{X/S}$	Q_p (IU/g/h)
4	1.793	1810	101061	0.10	84123
8	1.860	1881	136999	0.14	84274
12	1.743	3725	277985	0.13	178093
16	1.732	3725	314081	0.15	179224
24	1.764	3725	302846	0.14	175973

Medium: glucose, 20 g; casein acid hydrolysate, 15 g; yeast extract, 5 g; ammonium citrate dibasic, 2 g; sodium acetate, 3 g; magnesium sulfate, 0.1 g; potassium phosphate dibasic, 2 g; manganese sulfate, 0.05 g; Tween 80, 1 g/L, pH 6.2, Temp.; 35℃, Incubation time; 12 hours.

Repeated-batch Culture

Bacteriocin 1112-1 also inhibits the producer (data not shown). Once bacteriocin is accumulated, it will affect the growth of the producer and bacteriocin production. Therefore, in the experiment discussed here, one tenth fraction of the culture broth was replaced every 12 hour with the fresh medium (10 folds strength) and the glucose concentration was adjusted to 20 g/L (Fig. 1). No more than a 2nd running cycle of the operation was possible. The repeated-batch operation did not efficiently enhance the bacteriocin production. The glucose consu-

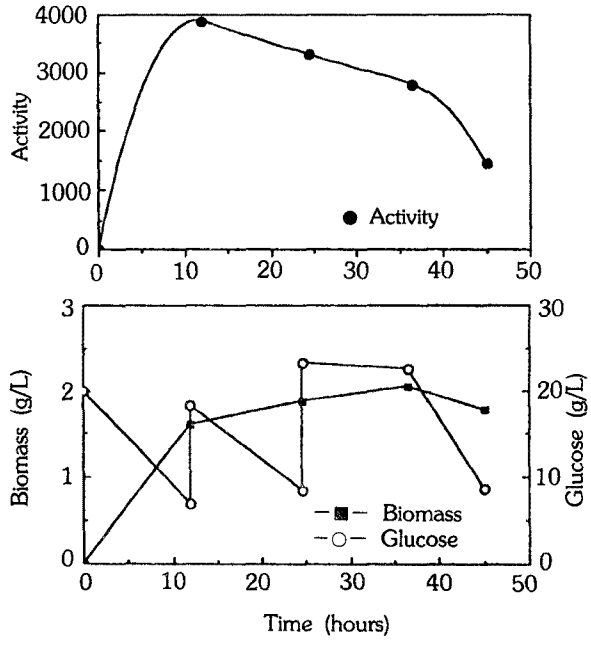

Fig. 1. Repeated-batch fermentation of *Lactococcus* sp. 1112-1 for the production of bacteriocin 1112-1.

mption in the culture noticeably decreased after repeated operation and the loss of bacteriocin activity was recognized. It was concluded that the remaining bacteriocin activity was still high enough to inhibit the producing organism and that the replacement volume of the culture broth be increased to enable further operation. This was a very similar result to the result for *Streptococcus lactis* IFO 12007 (13).

Continuous Culture

Continuous culture was carried out in Bioflo C-32 reactor with a 350 ml vessel and by feeding the fresh glucose-casein medium. A sample was taken after reaching a steady-state (5~6 residence time). This system enabled the operation at 0.4 h^{-1}, and then the system at a higher dilution rate, the culture was washed out (Table 3). Maximum biomass concentration and bacteriocin activity were 2.45 g/L and 2,121 IU/ml, respectively. Maximum specific productivity (Q_p) and volumetric activity were 347,136 IU/g/h and 848,400 IU/L/h; that is twice as much as in batch culture (16). The value of the energy for cell maintenance can be derived from the equation:

$$Q_{glu} = \frac{\mu}{Y_{glu}^{max}} + m_{glu}$$

where Y_{glu}^{max} is the maximum molar growth yield for growth (g·cell/mole·glucose), m_{glu} is the maintenance coefficient (mmole glucose/g-dry cell/h) and μ is the specific growth rate. To derive the maintenance coefficient, the specific glucose uptake rate (Q_s) was plotted against the dilution rate (Fig. 2). The maintenance demand for glucose and maximum growth yield were 545 mg (3 mmole) glucose/g dry cell/h and 188 mg dry cell/g glucose (33.8 g/mole). The maintenance energy here for *Lactococcus* sp. 1112-1 is high value comparing other microorganisms such as *Lactobacillus casei* (0.135 g/g/h) (3), and *Saccharomyces cerevisiae* (0.018 g/g/h)

Table 3. Continuous culture of *Lactococcus* sp. 1112-1 for bacteriocin production.

Dilution rate (h^{-1})	Biomass (g/L)	Activity (IU/ml)	$Y_{P/S}$ (IU/g)	$Y_{X/S}$	Q_p (IU/g/h)	VP (IU/L/h)
0.12	1.98	2121	120170	0.11	128610	254520
0.21	2.06	2121	120102	0.12	216428	445410
0.25	2.46	2121	120238	0.14	215812	530250
0.30	2.39	2121	120270	0.14	266346	636300
0.40	2.45	2121	122238	0.14	347136	848400
0.46	2.13	1455	112442	0.17	316275	673665
0.49	0.37	233	353030	0.56	210245	114170

Culture was done in Bioflo C-30 bench-top fermentor (working volume: 350 ml)

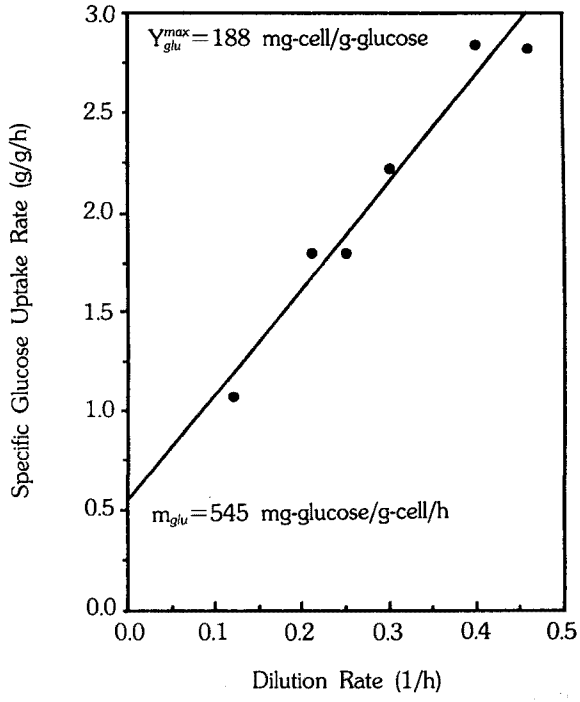

Fig. 2. Maintenance demand of glucose and maximum growth yield of *Lactococcus* sp. 1112-1.

(11). However, m_{glu} for *Escherichia coli* was 0.558 g/g/h in a glucose-limited culture (11).

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