4

Results

4.1. Isolation of lactic acid bacteria

The non-motile, non-sporeforming catalase negative and Gram positive cells, denoted as lactic acid bacteria were screened out of the total bacterial isolates on MRS agar. They were further characterized on the basis of cell morphology, gas from glucose and growth in 6.5% (w/v) sodium chloride. Following the taxonomic keys of Sneath *et al.* (1986), lactic acid bacterial isolates were identified up to their generic level. A total of 171 lactic acid bacteria were isolated form 74 samples of different kinds (Table 2; Fig. 1). Lactobacilli constituted 62% of the total lactic acid bacterial isolates and predominated in whey, curd, chana and putrid fish, followed by lactococcal isolates (31%) which occured predominantly in rotten vegetables, putrid meat, silage and cheese. Besides, isolates of leuconostocs were isolated from chhana and rotten vegetables, and pediococci from silage, which together constituted only 7% of the total lactic acid bacterial isolates.

During enrichment of lactic acid bacteria, the plates were incubated in candle jar. But, since the number of pure culture colonies developed on plates incubated microaerophilically (in candle jar) did not differ significantly from those developed on plates incubated aerobically (data not shown), in all subsequent experiments the cultures were grown aerobically.

4.2. Screening and nature of antimicrobial substance

In agar spot test (Fig. 2a), 14% of the total lactic acid bacterial isolates showed antibacterial activity (Table 3). Comparisons of the results in well diffusion assay (Fig.2b) with unadjusted and adjusted pH of the culture supernatants showed that the antibacterial activity of most of the strains was due to organic acid production (Table 4). Out of 24, seven strains were found positive in well diffusion assay when the pH of their cell-free culture supernatants were neutralized. They scored positive even when they were treated with catalase, confirming the nature of antibacterial compounds produced by these seven isolates to be other than acid and hydrogen peroxide. These seven strains tested positive in the reverse-side technique (Tagg and McGiven 1971), establishing the fact that the antibacterial activity was not even due to lytic phages. When the culture supernatants were treated with proteolytic enzymes and the activity assayed by well diffusion method, their antibacterial property was lost (Tables 4 and 5; Fig. 2c). The results of pepsin treatment resembled those of a trypsin one (Fig. 3), confirming the protein or peptide nature of the antibacterial compounds.

4.3. Selection of Bac$^+$ cells from a mixed population

Table 2. Lactic acid bacterial isolates from different sources

Source	No. of isolates			
	Lactobacillus	*Lactococcus*	*Leuconostoc*	*Pediococcus*
Curd (13)	29	3	0	0
Chhana (16)	17	6	4	0
Whey (11)	35	0	0	0
Cheese (7)	5	7	0	0
Silage (5)	1	10	0	6
Rotten vegetables (16)	8	14	2	0
Putrid fish (2)	10	2	0	0
Putrid meat (4)	1	11	0	0
Total (74)	106	53	6	6

Figures in parentheses indicate number of samples analysed.

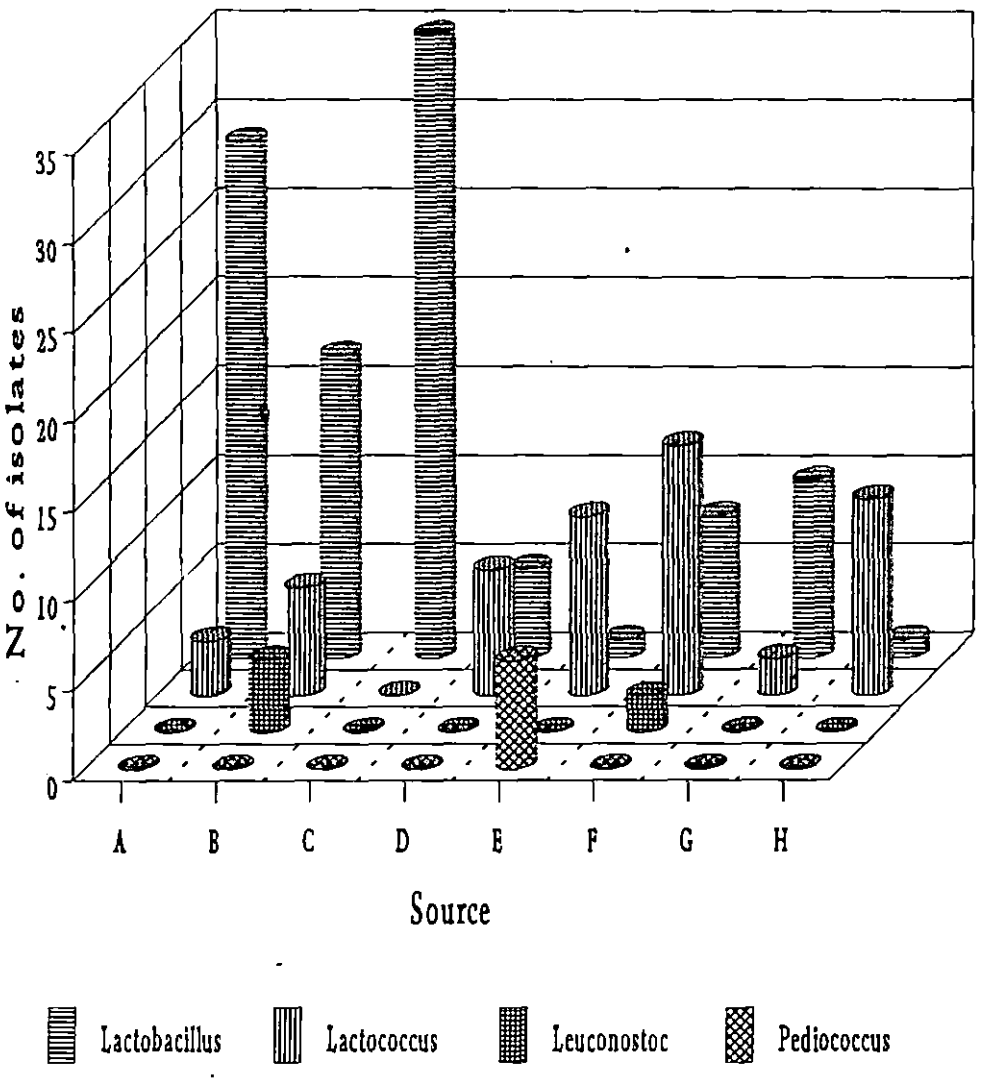

Fig. 1. Lactic acid bacterial isolates from different sources. A, curd; B, chhana, C, whey; D, chhese; E, silage; F, rotten vegetables; G, putrid fish; H, putrid meat.

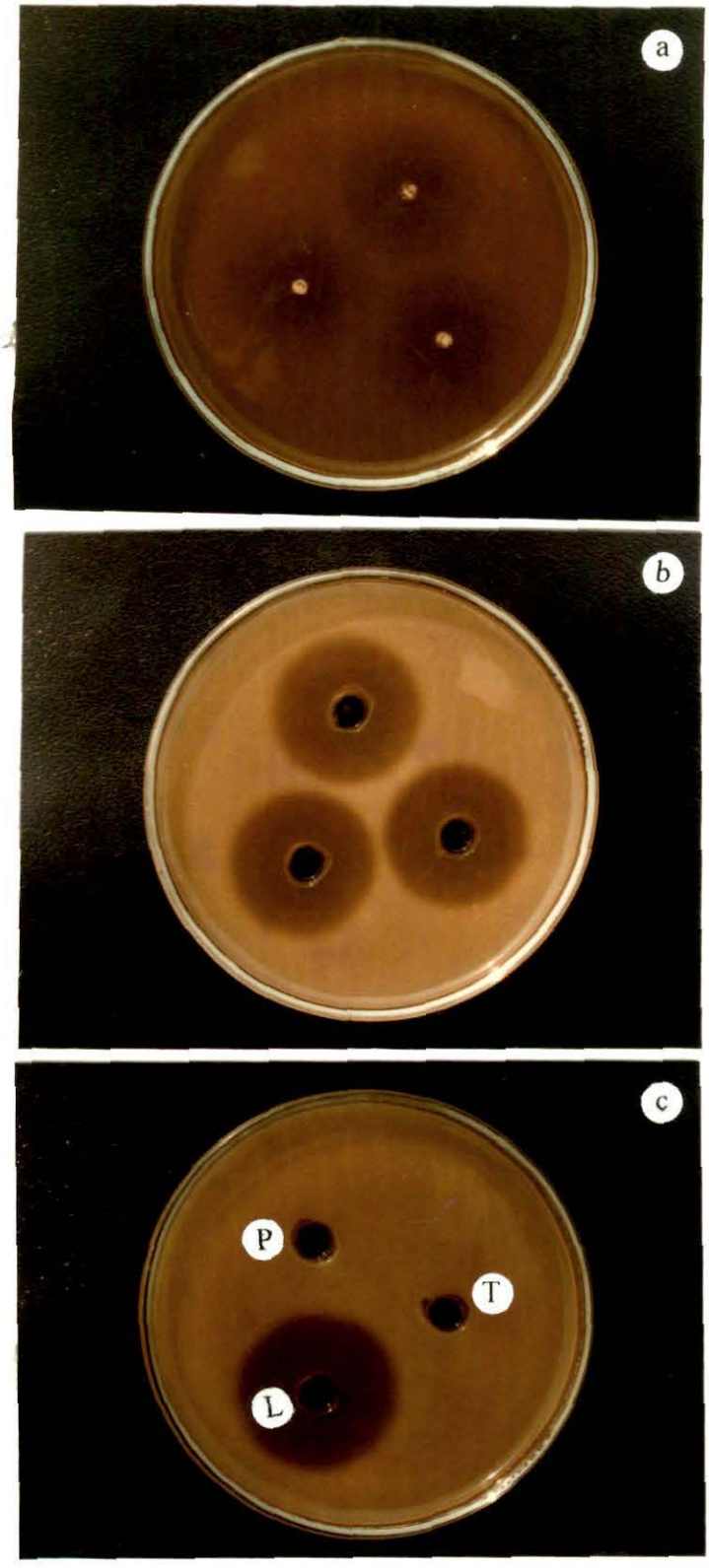

Fig. 2. Testing antimicrobial activities: (a) agar spot test showing growth inhibition by A, B and C; (b) well diffusion assay showing growth inhibition zones caused by neutralized, 10 fold-concentrated and filter-sterilized culture filtrates of A, B, and C; (c) response of neutralized, 10 fold-concentrated and filter-sterilized culture filtrates of C to catalase (L), pepsin (P) and trypsin (T) treatments. Producers: *Lactobacillus casei* W25B (A), W26B (B) and W28 (C). Indicator organism: *Lactobacillus plantarum* GM-R1.

Table 3. Antibacterial activity of lactic acid bacterial isolates determined by agar spot test

Genera	No. of isolates (A)	Positive isolates (B)	Percent frequency (B/A X 100)
Lactobacillus	106	17	16
Lactococcus	53	4	8
Leuconostoc	6	2	33
Pediococcus	6	1	17

Table 4. Nature of antibacterial principles in concentrated culture supernatants

Isolates	Treatment of culture supernatants				
	Unadjusted (pH<3.0)	Neutralized	Neutralized and treated with catalase	Neutralized and treated with protease	Neutralized and subjected to reverse-side technique
Lactobacillus	17	7	7	0	7
Lactococcus	4	0	0	0	0
Leuconostoc	2	0	0	0	0
Pediococcus	1	0	0	0	0

Data indicate the number of strains positive in well diffusion assay.

Table 5. Effect of proteolytic treatment on antibacterial principles of the culture supernatants

Producer (*Lactobacillus*)	Indicator (*Lb. plantarum*)	Width of inhibition zone (mm)*		
		Control	Trypsin treated	Pepsin treated
C34	GMR1	2.62 (0.06)	0.00	0.00
W25B	GMR1	3.80*(0.04)	3.10**	0.00
W25C	GMR1	2.30*(0.04)	2.80**	0.00
W26B	GMR1	3.10 (0.03)	0.00	0.00
W28	GMR1	3.50 (0.13)	0.00	0.00
W30A	GMR1	6.10 (0.28)	0.00	0.00
W30B	MTCC1325	3.60 (0.10)	0.00	0.00

* Data represent the means (with standard error) of triplicate sets in well diffusion assay.
** Values in rows are not significantly different ($P<0.05$).

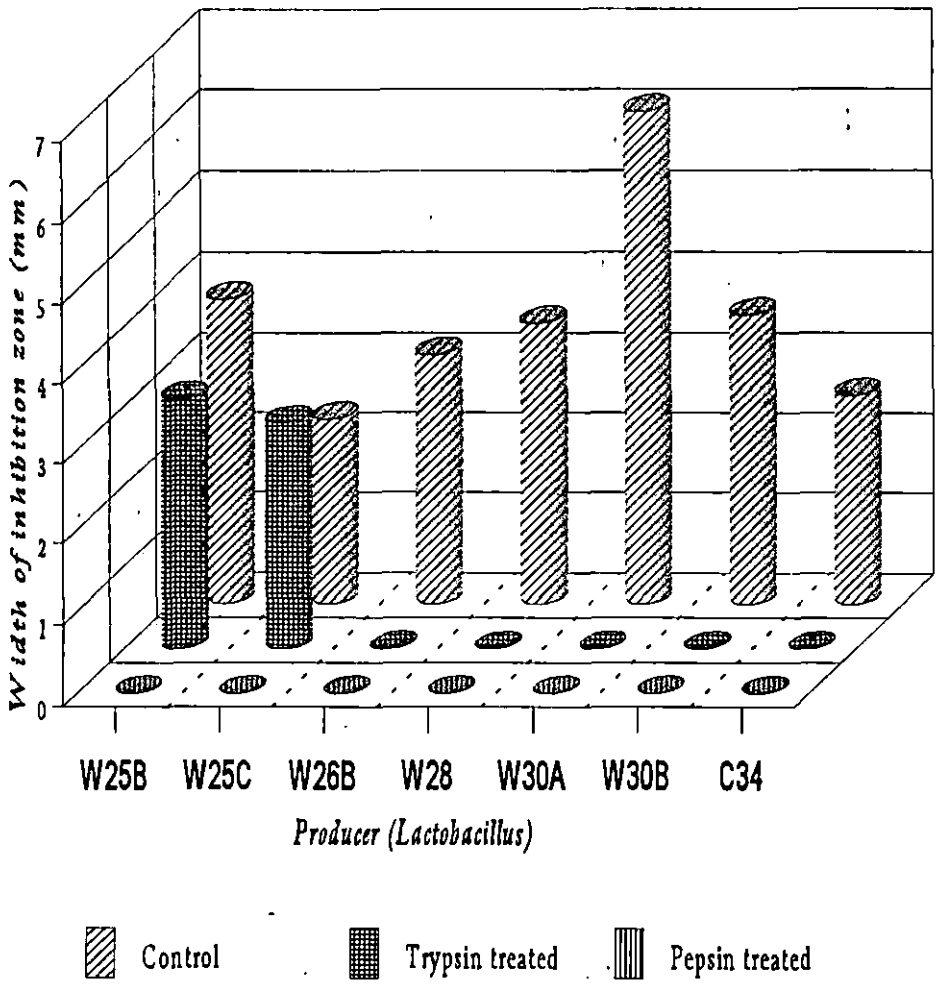

Fig. 3. Effect of proteolytic treatment on antibacterial principles of cell-free culture supernatants, evidenced in well diffusion assay

In the course of study with bacteriocins, the inhibitory activity was found to be gradually diminished and finally disappeared in case of all the producers. This finding led us to often search for the spontaneous loss of bacteriocin-producing bacteria within the populations of positive isolates. In order to regain the original bacteriocin titre, the colonies of Bac$^-$ variants were eliminated by replica plating technique. The picked up positive colonies from the replica plates resumed the original titres. However, activity could be revived in only three of the seven positive isolates.

4.4. Identification of selected Bac$^+$ strains

Characteristics of the three selected *Lactobacillus* isolates were analysed following the keys of Kandler and Weiss (1986). All the three isolates exhibited similar characteristic features. They were rod with square ends, with 2-4 cells in chains (Fig. 4). Their size was 0.7 μm X 2.2-2.4 μm. They were Gram positive and able to grow at 15°C. The isolates were negative for production of catalase and gas from glucose, growth in 6.5% NaCl and at 45°C, liquefaction of gelatin, production of indole, reduction of nitrate and hydrolysis of starch, lipid and casein. They were positive for the utilization of sugars including ribose, glucose, mannose, galactose, sucrose, lactose, maltose, cellobiose, mannitol and sorbitol, but negative for the utilization of arabinose, xylose, fructose, rhamnose, melibiose, raffinose, salicin and starch. These characters were in full agreement with those of co-culture of a reference strain, *Lactobacillus casei* subsp. *casei* ATCC 393. Based on these criteria, the three selected isolates were identified as *Lactobacillus casei*.

4.5. Optimization of bacteriocin production

Bacteriocin titres in all these experiments were determined by well diffusion assay, against *Lactobacillus plantarum* GMR1 as the indicator strain.

4.5.1. Type of medium

Five different media, used frequently for the growth of lactobacilli, were tried to search for the most suitable one for bacteriocin production (Table 6). Among these, SD-MRS broth as well as MRS broth were found to be the best media for this purpose (Fig. 5). Since, the mean bacteriocin titres in SD-MRS broth were higher than those in MRS broth, in all subsequent experiments SD-MRS broth was selected for all

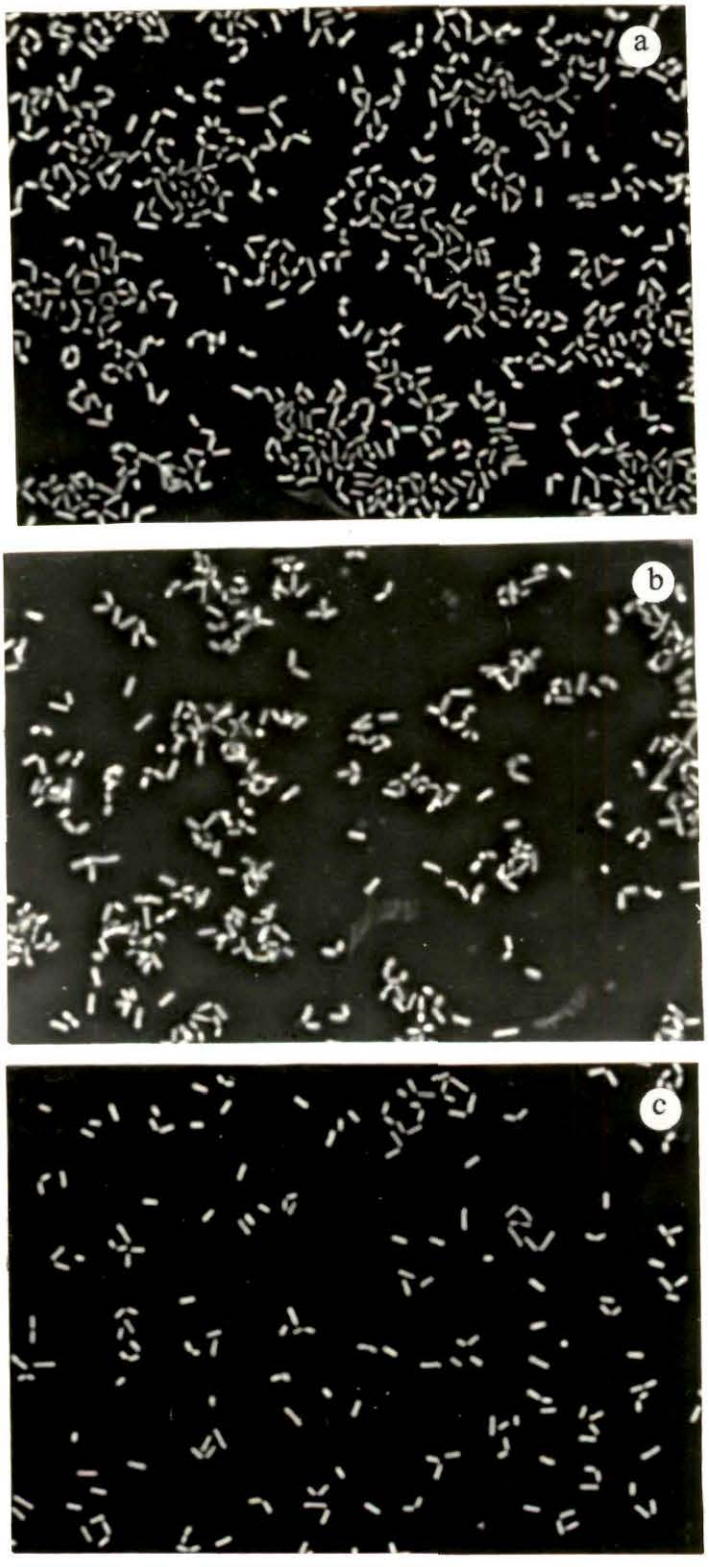

Fig. 4. Phase-contrast micrographs (x 1300) of cells of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 6. Bacteriocin titres in different broth cultures

Producer (*Lactobacillus casei*)	Width of inhibition zone (mm)*				
	MRS broth	MRS-0.2 broth	APT broth	Tomato juice medium	SD-MRS broth
W25B	2.00[a] (0.11)	2.00[a](0.11)	0.35[b] (0.07)	1.00[a] (0.11)	3.90[a] (0.04)
W26B	1.71[a] (0.14)	2.00[a](0.16)	2.00[a] (0.06)	0.00[b]	3.10[a] (0.04)
W28	2.58[a] (0.19)	1.04[b](0.04)	0.00[c]	0.00[c]	3.90[a] (0.04)

*Data represent the means (with standard error) of triplicate sets in well diffusion asay. Values bearing different superscripts in each row differ significantly ($P<0.05$).

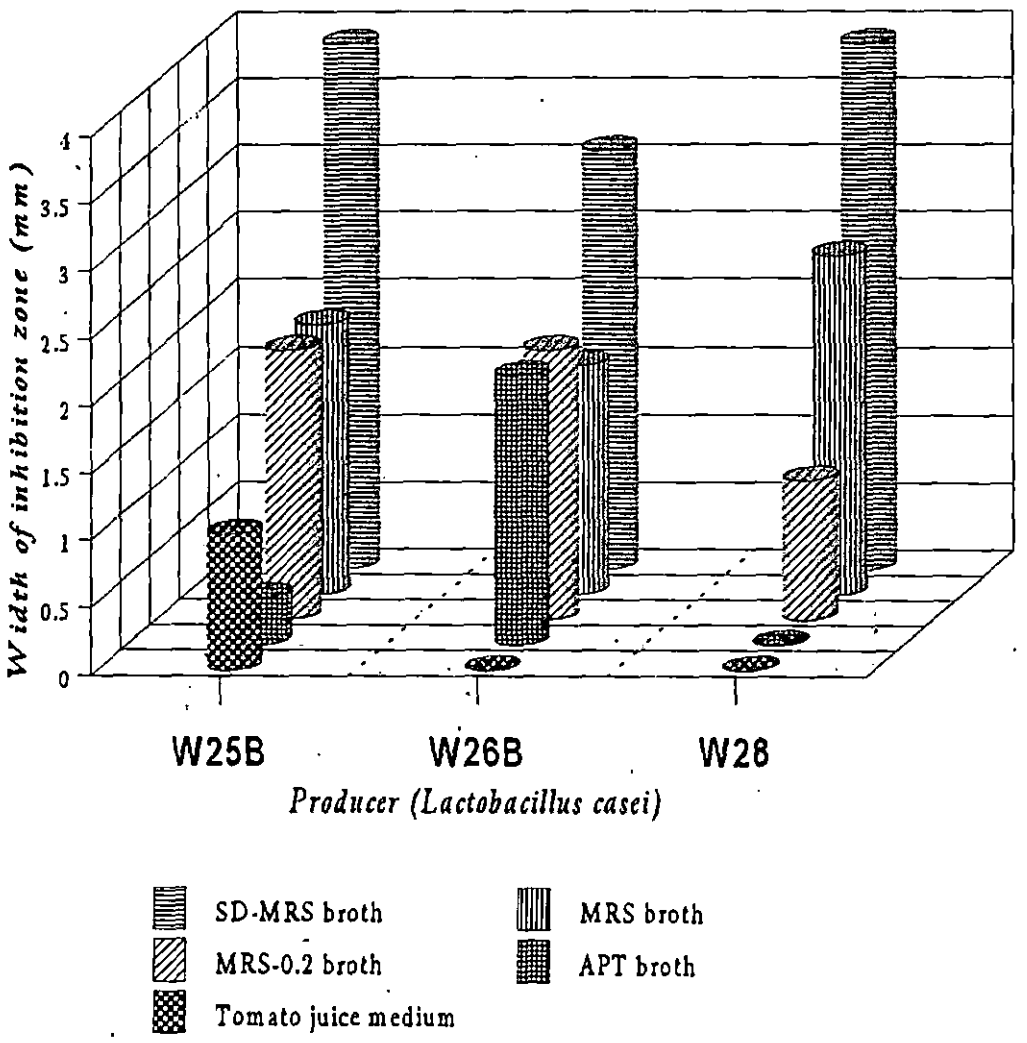

Fig. 5. Bacteriocin titres in different broth cultures, determined by well diffusion assay.

subsequent experiments.

4.5.2. pH of medium

Table 7 and Fig. 6 show the growth and production of inhibitory substance in SD-MRS broth adjusted initially to different pH values. *Lactobacillus casei* W25B showed maximum growth at pH 6, but maximum bacteriocin titre at pH 6.0-6.4. In *Lb. casei* W26B maximum growth was at pH 6.0-6.4, but highest bacteriocin titre was at pH 5.5-6.2. In *Lb. casei* W28 maximum growth as well as bacteriocin production occurred at pH 5.0 - 6.4. When the initial pH of the medium was raised to 8.0 no activity was detected. However, absence of activity was not due to lack of producer growth, since confluent growth occured at all the initial pH levels (5.0-8.0).

4.5.3. Temperature of incubation

Table 8 and Fig. 7 show the viable count and the bacteriocin production ~~after~~ at different temperatures. The maximum growth as well as bacteriocin production in W25B, W26B and W28 were at 30-32°C.

4.5.4. Period of incubation

Tables 9 and 10 and Figs 8 and 9 show the effect of incubation period on growth and bacteriocin production. In *Lb casei* W25B, viable count increased rapidly to reach its maximum in 1-2 d and the maximum bacteriocin titre was achieved between 1-3 d of incubation. Further incubation had an adverse effect on growth as well as bacteriocin titre. *Lactobacillus casei* W26B showed maximum growth after 1 d of incubation, while the maximum bacteriocin titre was obtained between 1 to 4 d of incubation. While the growth of *Lb. casei* W28 was maximum in 1-2 d of incubation, the maximum bacteriocin titre was obtained between 1 and 4 d of incubation.

4.6. Characterization of bacteriocins

4.6.1. Thermostability

The bacteriocins were found totally stable at 98°C for 2 h, the maximum temperature-time treatment applied. Treatment at 121°C for 15 min also did not result in any loss

Table 7. The effect of initial pH of SD-MRS broth on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

pH	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
5.0	335.0^{d} (0.4)	3.12^{c} (0.06)	322.0^{c} (0.5)	3.96^{ab} (0.04)	343.3^{a} (1.8)	5.25^{a} (0.16)
5.5	337.0^{d} (0.4)	3.46^{b} (0.04)	335.0^{b} (0.7)	4.17^{a} (0.05)	356.0^{a} (8.3)	5.33^{a} (0.08)
6.0	395.5^{a} (2.9)	4.17^{a} (0.05)	377.0^{a} (0.5)	4.42^{a} (0.05)	392.0^{a} (2.1)	5.96^{a} (0.12)
6.2	388.0^{b} (5.0)	3.92^{a} (0.05)	375.5^{a} (0.6)	4.37^{a} (0.06)	391.0^{a} (2.4)	6.08^{a} (0.14)
6.4	350.0^{c} (1.0)	3.83^{a} (0.12)	374.0^{a} (0.9)	4.12^{ab} (0.06)	389.0^{a} (1.8)	5.96^{a} (0.12)
7.0	224.2^{e} (0.9)	3.17^{c} (0.05)	205.0^{d} (1.7)	3.17^{b} (0.12)	224.0^{b} (0.6)	3.00^{b} (0.09)
8.0	3.7^{f} (0.1)	0.00^{d}	2.8^{e} (0.1)	0.00^{c}	3.7^{c} (0.1)	0.00^{c}

* Incubated at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

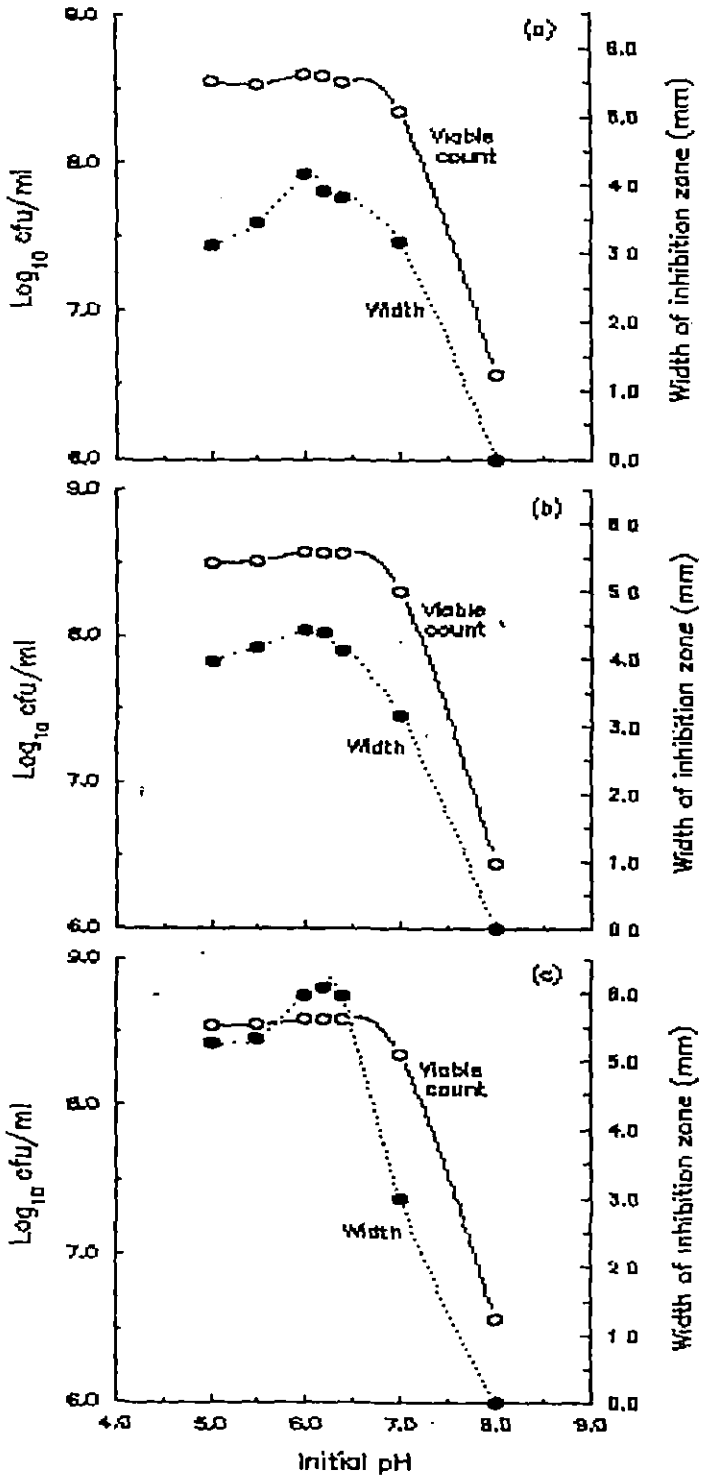

Fig. 6. The effect of initial pH of SD-MRS broth on viable count and bacteriocin titre (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B and W28 (c).

Table 8. The effect of temperature of incubation on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

Temperature (°C)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
20.0	36.0^{d} (1.4)	0.00^{e}	44.0^{e} (0.6)	0.00^{d}	36.0^{d} (1.1)	0.00^{d}
25.0	211.8^{c} (3.2)	1.33^{d} (0.05)	118.0^{d} (0.4)	1.12^{c} (0.06)	212.0^{c} (3.2)	2.16^{c} (0.05)
27.5	302.0^{b} (4.9)	2.33^{c} (0.05)	254.0^{c} (0.5)	3.17^{b} (0.05)	302.0^{b} (4.5)	4.16^{b} (0.05)
30.0	332.0^{ab} (8.8)	3.87^{ab} (0.06)	402.0^{a} (2.5)	4.29^{a} (0.10)	345.3^{a} (8.3)	5.62^{a} (0.08)
32.0	388.0^{a} (3.6)	4.54^{a} (0.14)	409.0^{a} (0.9)	4.33^{a} (0.05)	388.0^{a} (3.6)	5.87^{a} (0.12)
35.0	211.0^{c} (1.8)	3.67^{b} (0.05)	308.0^{b} (1.0)	3.83^{ab} (0.08)	211.0^{c} (2.1)	4.37^{b} (0.06)

* Grown in SD-MRS broth for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

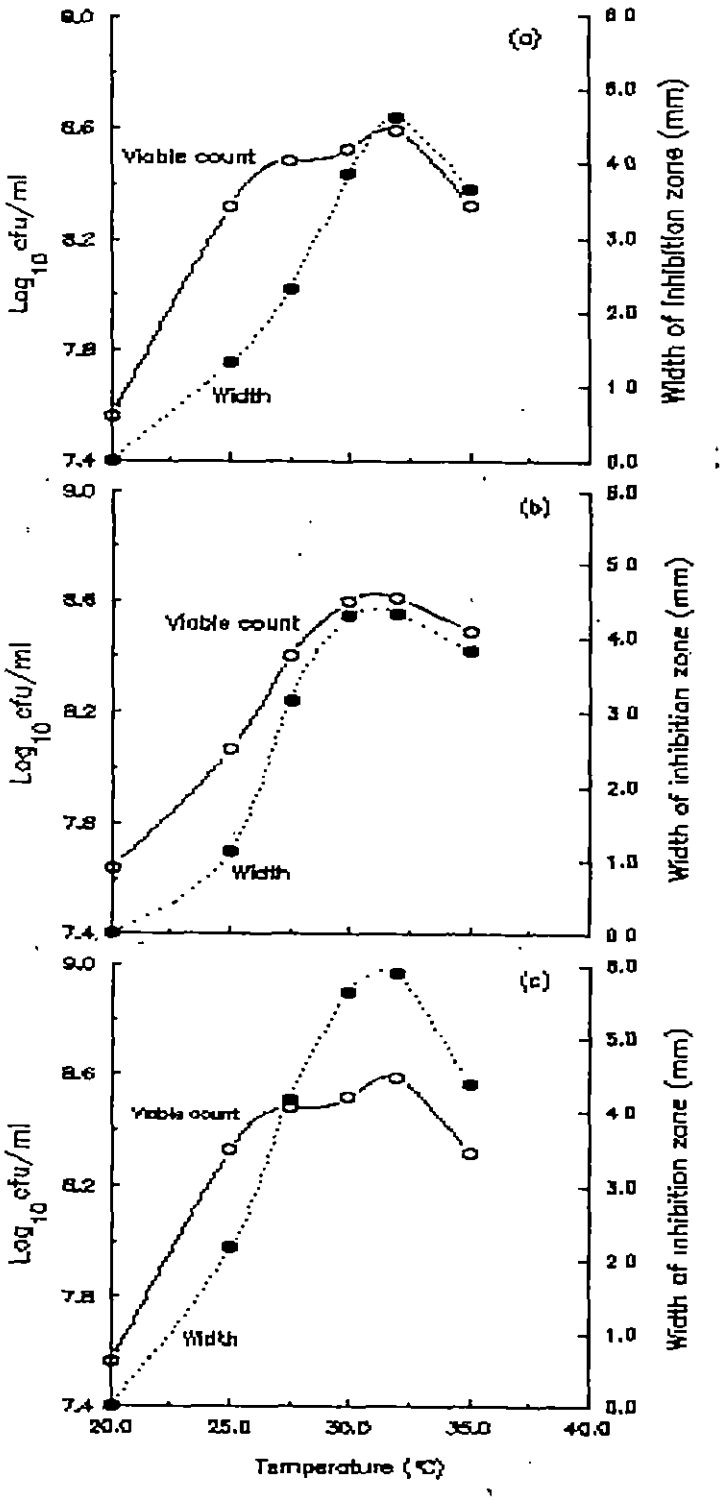

Fig. 7. The effect of temperature of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 9. The influence of incubation period on growth and bacteriocin production of the three isolated strains of *Lactobacillus casei**

Incubation period (d)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
0	46.0^{f} (1.4)	0.00^{d}	36.0^{f} (1.5)	0.00^{d}	38.0^{e} (1.6)	0.00^{d}
1	489.0^{a} (2.2)	3.96^{a} (0.04)	399.0^{a} (1.2)	4.04^{a} (0.04)	406.0^{a} (2.4)	4.08^{a} (0.08)
2	462.0^{ab} (2.7)	3.92^{a} (0.05)	379.0^{b} (1.9)	3.92^{a} (0.08)	366.7^{ab} (7.5)	4.29^{a} (0.08)
3	426.0^{b} (3.4)	3.25^{ab} (0.11)	315.3^{c} (1.1)	3.83^{a} (0.05)	318.0^{b} (1.4)	4.08^{a} (0.08)
4	383.0^{c} (5.6)	2.83^{bc} (0.05)	303.0^{c} (0.4)	3.37^{ab} (0.08)	218.0^{c} (3.2)	4.04^{a} (0.04)
5	225.0^{d} (2.5)	2.62^{bc} (0.08)	249.0^{d} (1.2)	2.33^{b} (0.16)	120.0^{d} (3.7)	3.08^{b} (0.08)
6	109.0^{e} (2.1)	1.96^{c} (0.04)	114.0^{e} (0.9)	1.62^{c} (0.08)	105.0^{d} (0.4)	2.62^{c} (0.06)

* Grown in SD-MRS broth at 32°C. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

†Determined by well diffusion assay.

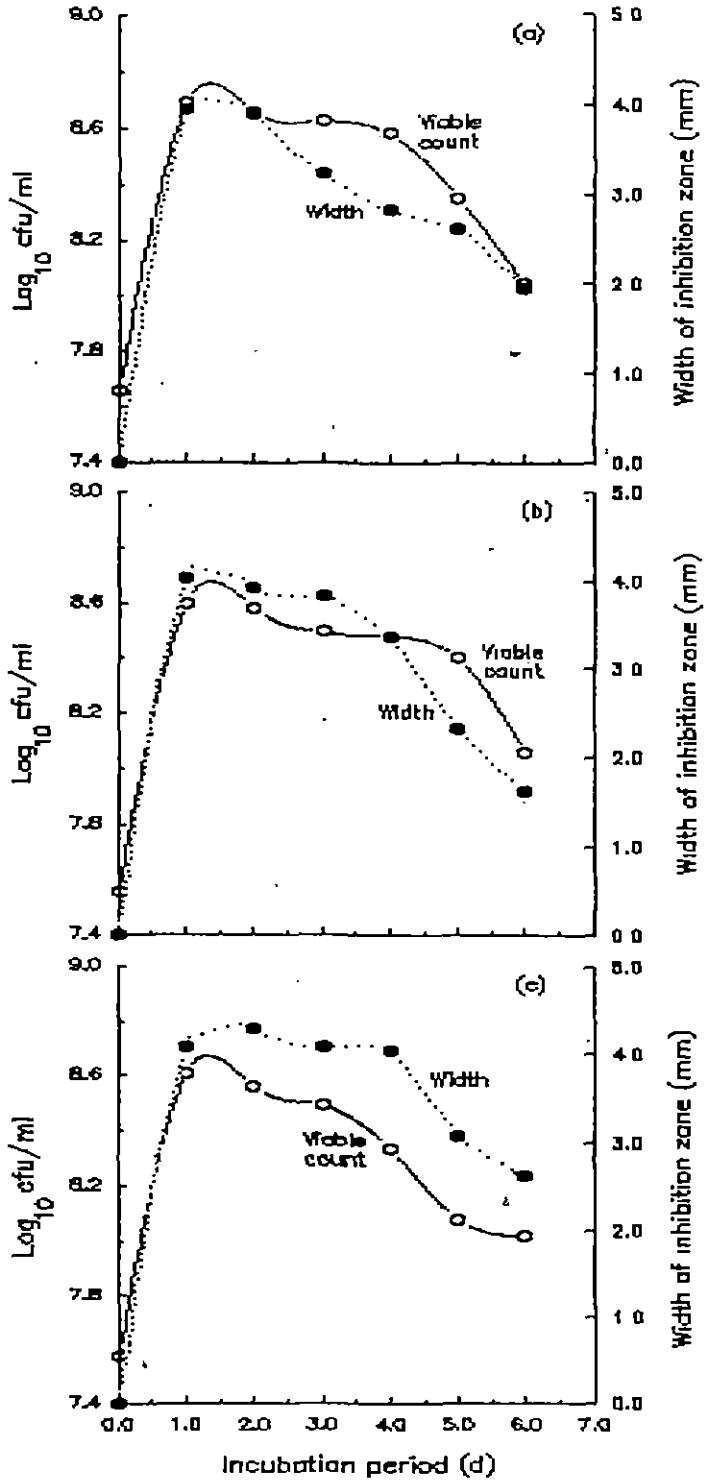

Fig. 8. The effect of period of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

Table 10. Growth and bacteriocin production of the three isolated strains of *Lactobacillus casei* during 36-h incubation period*

Incubation period (h)	W25B		W26B		W28	
	Million cfu/ml	Width of inhibition zone (mm)†	Million cfu/ml	Width of inhibition zone (mm)	Million cfu/ml	Width of inhibition zone (mm)
0	35.3^{d} (1.9)	0.00^{d}	40.0^{d} (3.3)	0.00^{d}	42.2^{e} (1.7)	0.00^{d}
6	116.0^{c} (2.6)	0.00^{d}	112.2^{c} (3.3)	0.00^{d}	126.0^{d} (5.9)	0.00^{d}
12	295.0^{b} (5.4)	1.78^{c} (0.16)	281.0^{b} (5.8)	1.30^{c} (0.03)	301.0^{c} (3.6)	1.83^{c} (0.08)
18	377.8^{a} (3.2)	2.40^{b} (0.04)	371.0^{a} (6.0)	2.30^{b} (0.04)	396.0^{b} (5.7)	2.60^{b} (0.12)
24	397.8^{a} (4.7)	4.62^{a} (0.13)	386.0^{a} (2.8)	4.40^{a} (0.06)	431.3^{a} (8.8)	5.90^{a} (0.09)
30	395.0^{a} (4.0)	4.60^{a} (0.13)	382.0^{a} (2.9)	4.40^{a} (0.10)	427.8^{a} (2.0)	5.90^{a} (0.09)
36	380.0^{a} (2.5)	4.93^{a} (0.07)	376.0^{a} (4.3)	4.60^{a} (0.11)	418.0^{a} (2.5)	6.00^{a} (0.11)

* Grown in SD-MRS broth at 32°C. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
†Determined by well diffusion assay.

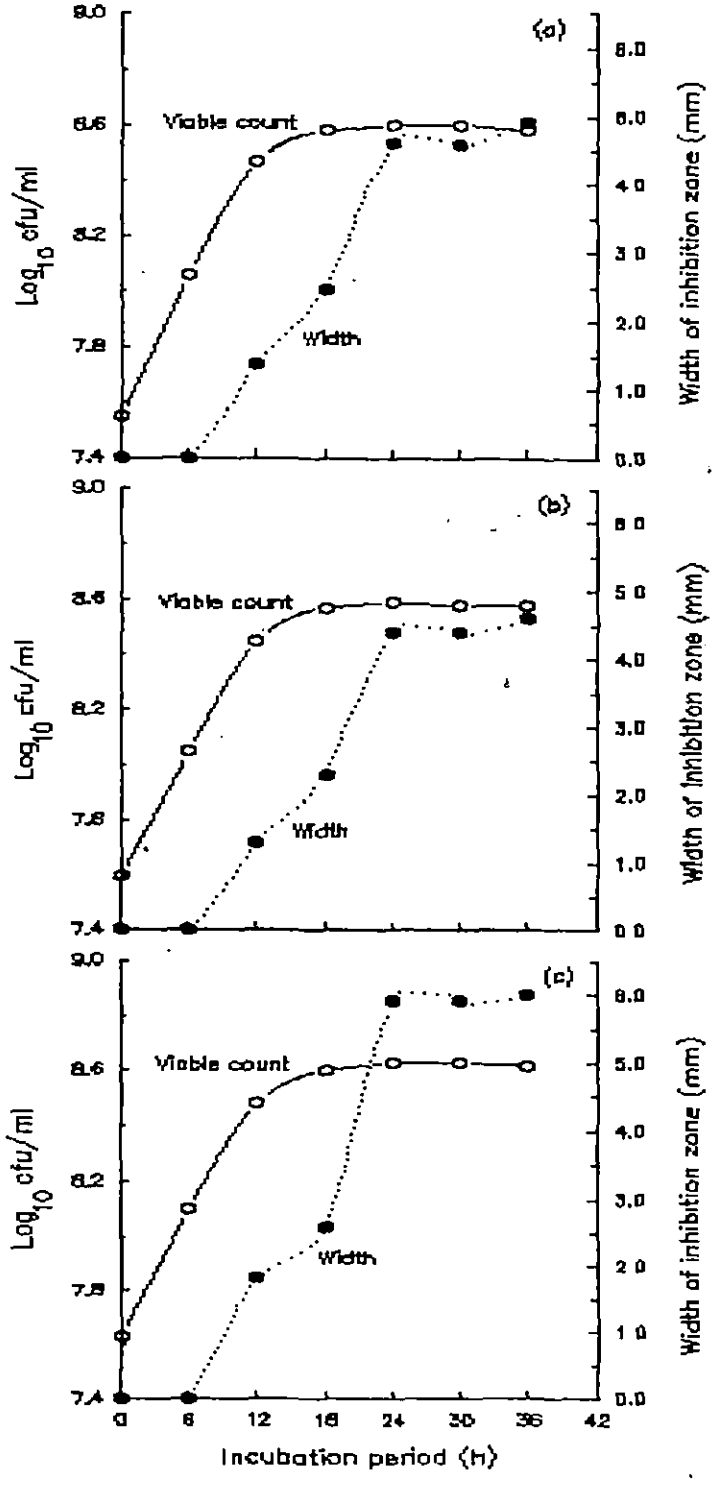

Fig. 9. The effect of period of incubation on growth and bacteriocin production (determined by well diffusion assay) of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c).

of activity (Table 11).

4.6.2. pH stability

While the bacteriocin activity present in the crude extract of *Lb. casei* W25B and W28 was fully stable at pH 2-8, the same in *Lb. casei* W26B was at pH 2-9 (Table 12; Fig. 10)

4.6.3. Storage stability

The effect of storage on bacteriocin activity is presented in Tables 13A-C and Fig. 11. On storing the culture broth at 37°C, a rapid loss of activity was recorded with time. Strains W25B, W26B and W28 lost complete activity at a storage period of 30, 20 and 60 d, respectively. On storing the cell-free culture supernatant at 37°C again a loss of activity with time was observed, but the complete loss of activity was after a longer period of time, when compared to culture broth stored at the same temperature. There was no significant loss of activity when the cell-free culture supernatant was stored at -85 to 4°C over a period up to 60 d.

4.6.4. Stability against organic solvents

Various organic solvents were tested for their effect on bacteriocin stability (Table 14). Treatment with benzene, propane-2-ol, iso-amyl alcohol, diethyl ether, acetone, formaldehyde, n-hexane and chloroform did not result in any loss of bacteriocin activity in all the three producer strains. However, when treated with n-butanol, the activity was reduced and a part of the activity was recovered from the organic phase.

4.7. Purification of bacteriocin

4.7.1. Dialysis

Bacteriocin activity in cell-free culture supernatants was not retained after exhaustive dialysis against 50 mM (pH 7.2) phosphate buffer using membrane tubings with molecular exclusion limits of 12 kDa. However, when dialysis was done using membrane tubings with molecular exclusion limit of 3.5 kDa, activity was detected in the retentate. This

Table 11. Temperature stability of the bacteriocins in crude extracts of the three isolated strains of *Lactobacillus casei*

Treatment of crude extract		Width of inhibition zone (mm)*		
Temperature (°C)	Time (min)	W25B	W26B	W28
Control		4.04 (0.04)	4.29 (0.04)	4.79 (0.04)
70	60	3.75 (0.13)	4.17 (0.05)	4.58 (0.14)
80	60	3.75 (0.11)	4.17 (0.05)	4.54 (0.15)
90	60	3.62 (0.06)	4.17 (0.05)	4.58 (0.08)
98	120	3.79 (0.04)	4.17 (0.05)	4.42 (0.05)
121	15	3.42 (0.05)	4.12 (0.08)	4.62 (0.08)

* Grown in SD-MRS broth at 32° C for 24 h. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values in each column are not significantly different ($P<0.05$).

Table 12. pH stability of the bacteriocins in crude extracts of the three isolated strains of *Lactobacillus casei*

Adjusted pH of the crude extract*	Width of inhibition zone (mm)†		
	W25B	W26B	W28
2	6.17^{ab} (0.08)	6.29^{a} (0.08)	8.92^{a} (0.05)
3	6.17^{ab} (0.08)	6.33^{a} (0.08)	8.96^{a} (0.04)
4	6.17^{ab} (0.08)	6.33^{a} (0.08)	9.04^{a} (0.04)
5	6.21^{ab} (0.10)	6.50^{a} (0.16)	8.88^{a} (0.08)
6	6.17^{ab} (0.08)	6.87^{a} (0.12)	8.87^{a} (0.06)
7	6.25^{a} (0.09)	6.87^{a} (0.12)	8.83^{a} (0.05)
8	6.12^{ab} (0.06)	6.29^{a} (0.14)	8.83^{a} (0.10)
9	4.79^{b} (0.04)	5.12^{a} (0.06)	7.62^{b} (0.12)
10	3.17^{c} (0.08)	2.12^{b} (0.08)	6.12^{c} (0.06)
11	1.96^{c} (0.04)	1.21^{b} (0.14)	3.62^{d} (0.06)

* Incubated at the pH for 24 h at 4°C.

† Grown in SD-MRS broth at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

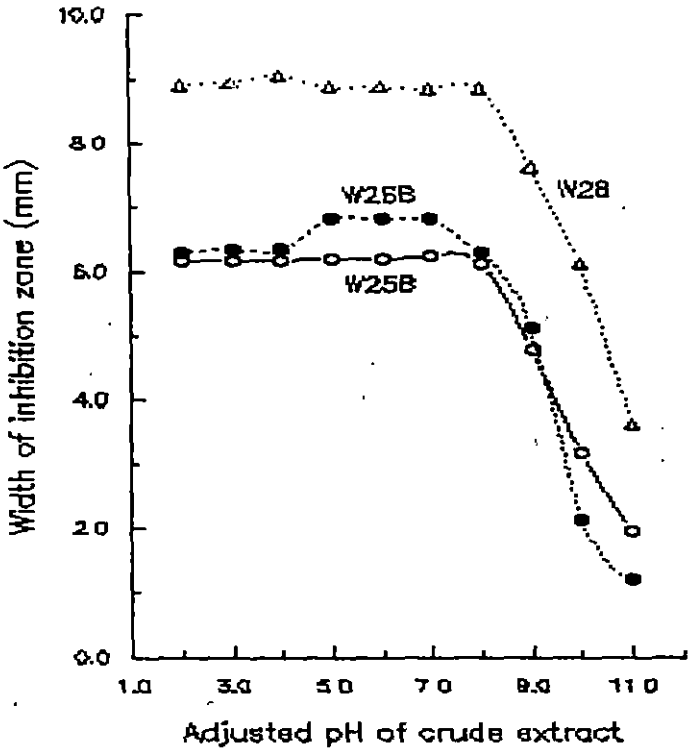

Fig. 10. The effect of pH on bacteriocin activity of the three isolated strains of *Lactobacillus casei*, as determined by well diffusion assay.

Table 13A. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W25B

Days	Width of inhibition zone (mm)*				
	Culture broth	Cell-free culture supernatant			
	37°C	37°C	4°C	-20°C	-85°C
1	6.42[a] (0.05)	6.46[a] (0.42)	6.46[a] (0.04)	6.45[a] (0.04)	6.42[a] (0.05)
5	5.12[b] (0.06)	5.92[b] (0.05)	6.46[a] (0.04)	6.54[a] (0.04)	6.96[a] (0.04)
10	3.62[c] (0.06)	4.87[c] (0.06)	6.33[a] (0.05)	6.46[a] (0.04)	6.92[a] (0.05)
20	1.54[d] (0.10)	3.72[d] (0.06)	6.33[a] (0.05)	6.62[a] (0.06)	6.46[a] (0.04)
30	0.00[e]	0.00[e]	6.33[a] (0.05)	6.67[a] (0.05)	6.67[a] (0.05)
40	0.00[e]	0.00[e]	6.17[a] (0.05)	6.46[a] (0.04)	6.46[a] (0.04)
50	0.00[e]	0.00[e]	6.17[a] (0.05)	6.46[a] (0.04)	6.42[a] (0.05)
60	0.00[e]	0.00[e]	6.42[a] (0.05)	6.67[a] (0.05)	6.67[a] (0.05)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 13B. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W26B

Days	Width of inhibition zone (mm)*				
	Culture broth	Crude extract			
	37°C	37°C	4°C	-20°C	-85°C
1	6.50[a] (0.16)	6.67[a] (0.05)	6.71[a] (0.04)	6.67[a] (0.05)	6.67[a] (0.05)
5	4.83[b] (0.05)	5.37[b] (0.06)	6.71[a] (0.04)	6.62[a] (0.06)	6.58[a] (0.08)
10	2.17[c] (0.05)	4.12[c] (0.06)	6.62[a] (0.06)	6.62[a] (0.06)	6.62[a] (0.12)
20	0.00[d]	2.87[d] (0.06)	6.67[a] (0.05)	6.71[a] (0.04)	6.62[a] (0.17)
30	0.00[d]	1.54[e] (0.08)	6.67[a] (0.05)	6.62[a] (0.06)	6.67[a] (0.05)
40	0.00[d]	0.00[f]	6.67[a] (0.05)	6.62[a] (0.06)	6.67[a] (0.08)
50	0.00[d]	0.00[f]	6.62[a] (0.06)	6.62[a] (0.06)	6.46[a] (0.08)
60	0.00[d]	0.00[f]	6.62[a] (0.06)	6.54[a] (0.12)	6.46[a] (0.12)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 13C. The influence of time-temperature treatments on the stability of bacteriocin of *Lactobacillus casei* W28

Days	Width of inhibition zone (mm)*				
	Culture broth	Crude extract			
	37°C	37°C	4°C	-20°C	-85°C
1	8.92[a] (0.08)	8.96[a] (0.04)	8.87[a] (0.06)	8.96[a] (0.04)	8.96[a] (0.04)
5	7.37[b] (0.06)	8.92[a] (0.05)	8.83[a] (0.05)	8.96[a] (0.04)	8.96[a] (0.04)
10	5.12[c] (0.06)	7.62[b] (0.12)	8.79[a] (0.08)	8.92[a] (0.08)	8.96[a] (0.04)
20	4.83[c] (0.05)	7.04[b] (0.04)	8.79[a] (0.08)	8.92[a] (0.08)	8.92[a] (0.05)
30	4.12[cd] (0.06)	6.92[b] (0.08)	8.46[a] (0.04)	8.92[a] (0.08)	8.92[a] (0.08)
40	3.67[d] (0.12)	6.67[b] (0.08)	8.54[a] (0.04)	8.87[a] (0.06)	8.92[a] (0.08)
50	1.83[e] (0.05)	5.00[c] (0.06)	8.54[a] (0.04)	8.87[a] (0.06)	8.92[a] (0.12)
60	0.00[f]	4.08[c] (0.05)	8.62[a] (0.06)	8.83[a] (0.05)	8.92[a] (0.12)

* Grown in SD-MRS broth for 24 h at 32°C. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

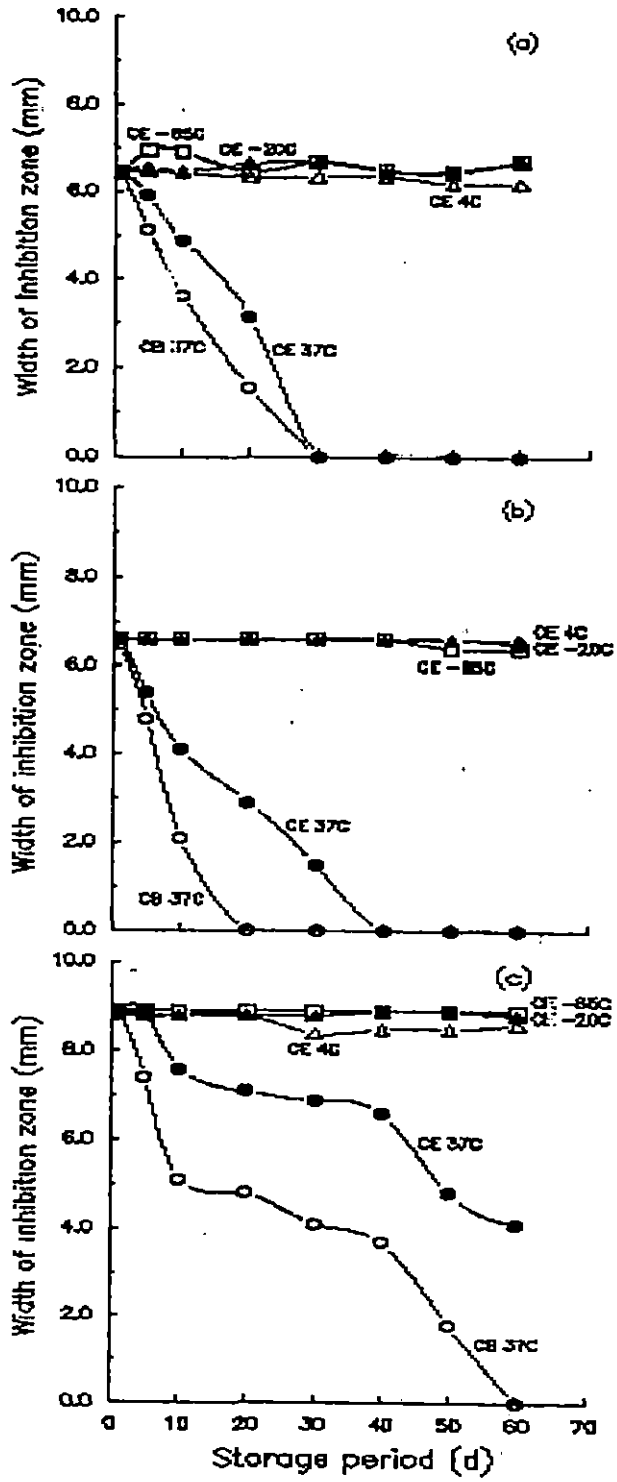

Fig. 11. The effect of time-temperature treatment on the stability of bacteriocins of *Lactobacillus casei* W25B (a), W26B (b) and W28 (c), as determined by well diffusion assay. CB, culture broth; CE, crude extract (cell-free culture supernatant).

Table 14. Solubility of bacteriocins in different organic solvents

Organic solvent	Width of inhibition zone (mm)*					
	W25B		W26B		W28	
	Aqueous phase	Organic phase	Aqueous phase	Organic phase	Aqueous phase	Organic phase
Control	5.10 [a] (0.04)	0.00	4.10 [a] (0.04)	0.00	6.07 [a] (0.23)	0.00
Benzene	4.62 [a] (0.11)	0.00	4.00 [a] (0.09)	0.00	5.62 [a] (0.17)	0.00
Propane-2-ol	4.25 [a] (0.09)	0.00	3.83 [a] (0.05)	0.00	6.04 [a] (0.19)	0.00
iso-Amyl alcohol	4.75 [a] (0.09)	0.00	3.62 [a] (0.06)	0.00	5.92 [a] (0.26)	0.00
n-Butanol	2.08 [b] (0.05)	2.38 [a] (0.45)	1.04 [b] (0.04)	2.29 [b] (0.08)	2.12 [b] (0.06)	3.67 [a] (0.10)
Diethyl ether	4.71 [a] (0.08)	0.00	3.75 [a] (0.09)	0.00	5.92 [a] (0.23)	0.00
Acetone	5.00 [a] (0.09)	0.00	3.79 [a] (0.12)	0.00	6.29 [a] (0.22)	0.00
Formaldehyde	4.71 [a] (0.08)	0.00	3.75 [a] (0.09)	0.00	5.92 [a] (0.19)	0.00
n-Hexane	4.75 [a] (0.10)	0.00	3.83 [a] (0.10)	0.00	6.17 [a] (0.23)	0.00
Chloroform	5.03 [a] (0.26)	0.00	3.70 [a] (0.16)	0.00	6.00 [a] (0.18)	0.00

* Grown in SD-MRS broth at 32°C for 24 h. Data represent the means (with standard error) of triplicate sets in well diffusion assay. Values bearing different superscripts in each column differ significantly ($P<0.05$).

activity was lost on proteolytic treatments. To check the possibility of adsorption of the bacteriocin to dialysis membrane, the membranes were cut after dialysis (0.5 cm X 2.0 cm) and placed on MRS agar plates exposing the inner side up and overlaid with seeded MRS soft agar. No zone of inhibition around the membrane was observed after incubation.

4.7.2. Gel filtration

Gel filtration through Sephadex G-25 was the first step involved in purification of W-28 bacteriocin (Fig. 12). Activity was eluted with 50 mM (pH 7.2) sodium phosphate buffer containing 0.6% sodium chloride in the last 12 ml. Following purification, the total activity recovered was 24%, and the specific activity increased from 2.28 AU/mg before gel filtration to 148.15 AU/mg. The sample was 65-fold purified after filtration (Table 15).

4.7.3. Anion-exchange and desalting column chromatographies

The purified sample after gel filtration was subjected to anion-exchange chromatography (Fig. 13). The bacteriocin was adsorbed strongly at pH 8 to DEAE cellulose (Sigma, USA as the column material, and was released only in the flow through fractions by applying 0.5 M sodium chloride in the elution buffer. Active fractions were run through Exocellulose GF-5 desalting columns to remove sodium chloride (Fig. 14), and freeze-dried. Under these conditions, an extensive removal of other contaminants was achieved. Activity was eluted in the last 4 ml fractions, which were combined and found to contain only 0.06 mg/ml protein (11% of the sample protein was recovered from the active fraction). However, loss of activity was also observed (4% recovery). The specific activity increased to 666.67 AU/mg and 292-fold purification was achieved (Table 15). When the above purification step was repeated under the same conditions, but at pH 7.2, adsorption did not occur. Exposure of active samples to high sodium chloride concentrations showed the W28 bacteriocin to be stable.

4.8. Mode of action

Mode of action studies were done for all the three selected producer strains which showed similar action (Tables 16A-C, 17A-C, 18A-C; Figs. 15-17). After addition of cell-free culture supernatant, a strong decrease in the number of viable cells of the indicator organism was observed within 1.5 h, and 100% killing was observed after 4.5 h. The killing effect was dependent on the stage of the indicator cells; it was most

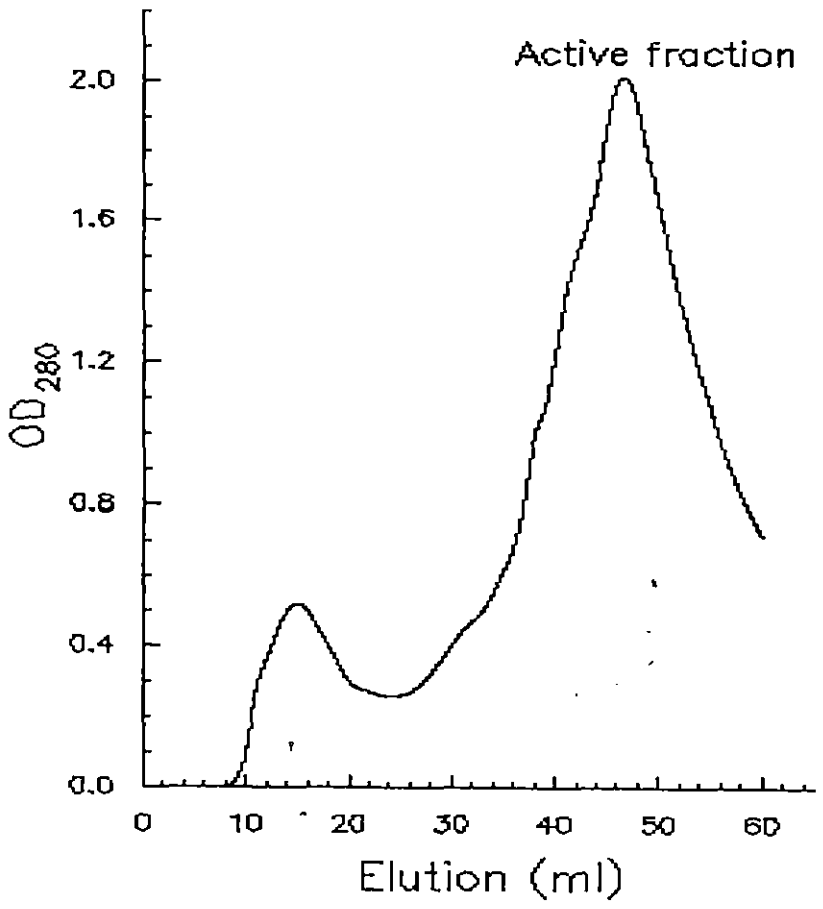

Fig. 12. Elution of *Lactobacillus casei* W28 bacteriocin from Sephadex G-25. A 2 ml-volume of culture supernatant was applied to Sephadex G-25 column equilibrated with 50 mM (pH 7.2) phosphate buffer. Activity was eluted with 0.6% (w/v) NaCl in the buffer. Fractions (1 ml) were collected and assayed for bacteriocin activity.

Table 15. Purification of *Lactobacillus casei* W28 bacteriocin

Sample*	Volume (ml) (A)	Activity (AU/ml) (B)	Total activity (C=A x B)	Protein concentration (mg/ml) (D)	Specific activity (AU/mg) (E=B/D)	Activity recovered (%) (C_2/C_1x100)	Fold purification (E_2/E_1)
1	100	40	4000	17.53	2.28	100.0	1.0
2	12	80	960	0.54	148.15	24.0	65.0
3	4	40	160	0.06	666.67	4.0	292.4

*1, 10-fold concentrated culture supernatant (pH 6.5); 2, G-25-purified sample; 3, DEAE cellulose and GF-5 Exocellulose-purified sample.

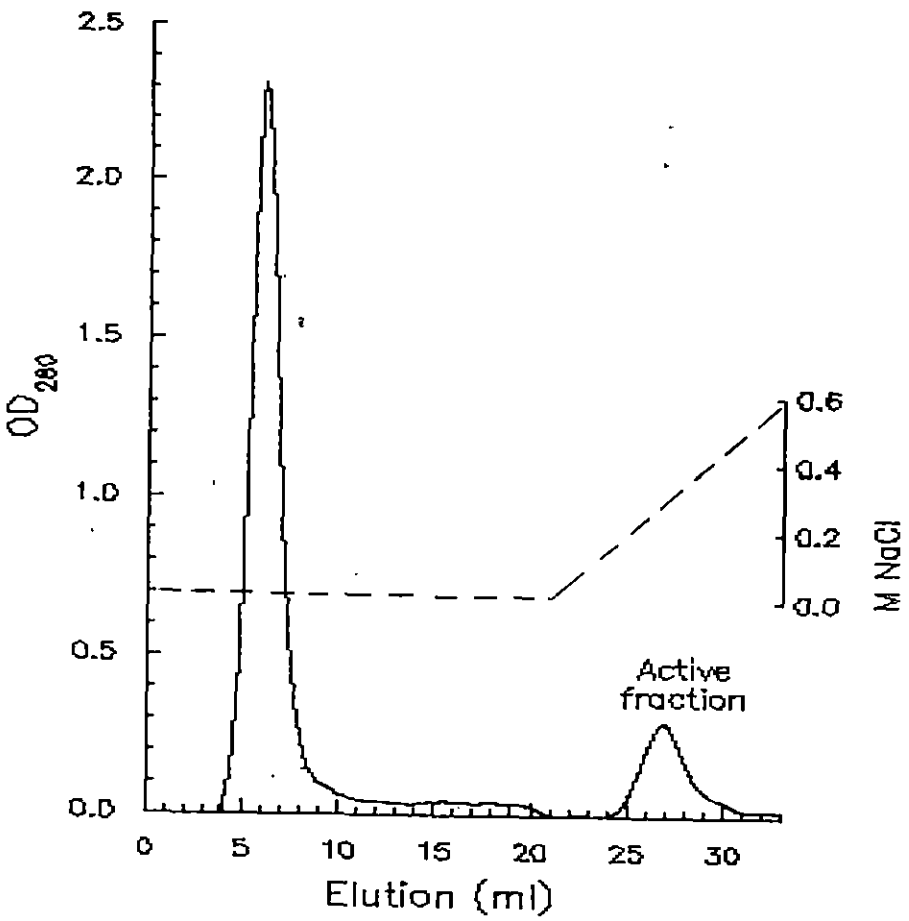

Fig. 13. Elution of *Lactobacillus casei* W28 bacteriocin from DEAE cellulose. Cell-free culture supernatants (12 ml), purified through G-25 Sephadex, were applied to DEAE cellulose column equilibrated with 20 mM (pH 8.0) phosphate buffer. The column was washed until absorbance at 280 nm returned to zero. Bacteriocin was eluted with a linear gradient of 0-0.6 M NaCl in 20 mM Tris. Fractions (1 ml) were collected and assayed for bacteriocin activity.

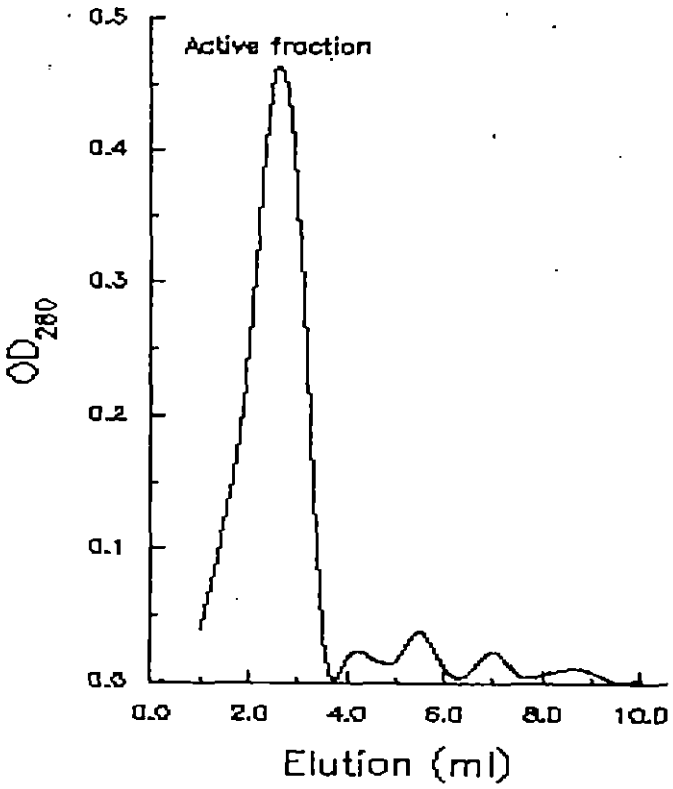

Fig. 14. Elution of *Lactobacillus casei* W28 bacteriocin from Exocellulose G-5 desalting column. Cell-free culture superantant (1.25 ml), purified through Sephadex G-25 and DEAE cellulose, was applied to Exocellulose G-5 equilibrated with 20 mM (pH 8.0) ammonium bicarbonate buffer. Activity was eluted with the same buffer until absorbance at 280 nm returned to zero.

Table 16A. The effect of crude extract of *Lactobacillus casei* W25B on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 ^{c} (0.001)	42.33 ^{e} (1.45)	0.051 ^{a} (0.001)	42.00 ^{a} (0.06)
1.5	0.066 ^{c} (0.001)	101.00 ^{d} (1.73)	0.053 ^{a} (0.001)	2.22 ^{b} (0.05)
3.0	0.086 ^{bc} (0.002)	166.33 ^{c} (2.96)	0.058 ^{a} (0.001)	0.18 ^{c} (0.02)
4.5	0.155 ^{b} (0.001)	217.33 ^{b} (1.45)	0.070 ^{a} (0.006)	0.02 ^{c} (0.00)
6.0	0.259 ^{a} (0.006)	275.33 ^{a} (2.91)	0.070 ^{a} (0.006)	0.02 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 16B. The effect of crude extract of *Lactobacillus casei* W25B on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.050 ^{c} (0.001)	38.67 ^{d} (0.88)	0.050 ^{a} (0.003)	37.67 ^{a} (0.88)
1.5	0.064 ^{c} (0.006)	99.67 ^{c} (5.21)	0.057 ^{a} (0.002)	3.47 ^{b} (0.13)
3.0	0.095 ^{c} (0.001)	171.00 ^{b} (4.73)	0.064 ^{a} (0.000)	0.22 ^{b} (0.00)
4.5	0.166 ^{b} (0.002)	211.00 ^{b} (5.29)	0.082 ^{a} (0.001)	0.02 ^{b} (0.00)
6.0	0.259 ^{a} (0.006)	277.33 ^{a} (2.91)	0.101 ^{a} (0.001)	0.01 ^{b} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 16C. The effect of crude extract of *Lactobacillus casei* W25B on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 [d] (0.001)	43.0 [d] (0.6)	0.051 [cd] (0.001)	40.7 [c] (0.9)
1.5	0.051 [d] (0.001)	45.3 [d] (1.2)	0.050 [d] (0.000)	40.0 [c] (2.5)
3.0	ND [†]	117.3 [c] (3.5)	ND	55.7 [c] (3.8)
4.5	ND	154.0 [b] (0.6)	ND	115.3 [b] (2.3)
6.0	0.060 [c] (0.001)	183.0 [b] (6.2)	0.057 [c] (0.002)	163.3 [b] (0.9)
12.0	0.080 [b] (0.001)	329.0 [a] (4.4)	0.077 [b] (0.001)	310.3 [a] (0.2)
24.0	0.135 [a] (0.001)	ND	0.133 [a] (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
[†] ND, not determined.

Table 17A. The effect of crude extract of *Lactobacillus casei* W26B on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.052 ^{e} (0.001)	42.67 ^{e} (1.45)	0.051 ^{b} (0.001)	41.00 ^{a} (1.00)
1.5	0.067 ^{d} (0.001)	108.33 ^{d} (0.88)	0.053 ^{ab} (0.001)	2.20 ^{b} (0.01)
3.0	0.083 ^{c} (0.002)	166.33 ^{c} (2.40)	0.058 ^{ab} (0.002)	0.19 ^{c} (0.02)
4.5	0.153 ^{b} (0.002)	216.00 ^{b} (2.65)	0.063 ^{ab} (0.001)	0.02 ^{c} (0.00)
6.0	0.260 ^{a} (0.006)	279.33 ^{a} (5.46)	0.070 ^{a} (0.001)	0.01 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 17B. The effect of crude extract of *Lactobacillus casei* W26B on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.045 ^{c} (0.002)	33.00 ^{e} (1.53)	0.044 ^{a} (0.002)	32.00 ^{a} (1.15)
1.5	0.064 ^{c} (0.003)	74.33 ^{d} (5.18)	0.049 ^{a} (0.001)	2.76 ^{b} (0.04)
3.0	0.095 ^{bc} (0.004)	119.33 ^{c} (2.85)	0.065 ^{a} (0.002)	0.18 ^{b} (0.00)
4.5	0.138 ^{b} (0.004)	194.00 ^{b} (3.61)	0.086 ^{a} (0.002)	0.01 ^{b} (0.00)
6.0	0.231 ^{a} (0.005)	242.67 ^{a} (6.44)	0.088 ^{a} (0.003)	0.01 ^{b} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 17C. The effect of crude extract of *Lactobacillus casei* W26B on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 [c] (0.001)	43.3 [d] (1.2)	0.051 [d] (0.001)	42.0 [c] (0.6)
1.5	0.051 [c] (0.001)	45.0 [d] (1.5)	0.051 [d] (0.001)	42.7 [c] (2.2)
3.0	ND †	118.7 [c] (2.2)	ND	54.0 [c] (3.6)
4.5	ND	155.0 [bc] (2.5)	ND	115.0 [b] (3.8)
6.0	0.060 [bc] (0.001)	176.0 [b] (2.3)	0.054 [c] (0.001)	154.0 [b] (6.0)
12.0	0.079 [b] (0.001)	355.0 [a] (1.7)	0.078 [b] (0.002)	295.7 [a] (2.6)
24.0	0.134 [a] (0.001)	ND	0.134 [a] (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
† ND, not determined.

Table 18A. The effect of crude extract of *Lactobacillus casei* W28 on growth of 6 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.052 ^{c} (0.001)	42.00 ^{e} (1.00)	0.052 ^{a} (0.001)	43.00 ^{a} (1.00)
1.5	0.063 ^{c} (0.001)	122.00 ^{d} (1.86)	0.053 ^{a} (0.002)	2.20 ^{b} (0.06)
3.0	0.082 ^{bc} (0.001)	170.00 ^{c} (1.00)	0.061 ^{a} (0.002)	0.19 ^{c} (0.01)
4.5	0.150 ^{b} (0.001)	218.00 ^{b} (1.53)	0.066 ^{a} (0.002)	0.02 ^{c} (0.00)
6.0	0.252 ^{a} (0.001)	287.00 ^{a} (2.52)	0.073 ^{a} (0.002)	0.01 ^{c} (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 18B. The effect of crude extract of *Lactobacillus casei* W28 on growth of 12 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.043 [b] (0.001)	34.00 [c] (0.58)	0.044 [a] (0.002)	33.33 [a] (0.67)
1.5	0.065 [b] (0.003)	52.33 [c] (2.03)	0.048 [a] (0.003)	2.62 [b] (0.22)
3.0	0.022 [b] (0.003)	103.67 [b] (6.94)	0.053 [a] (0.003)	0.23 [b] (0.01)
4.5	0.129 [a] (0.001)	175.00 [a] (5.57)	0.086 [a] (0.001)	0.02 [b] (0.00)
6.0	0.131 [a] (0.001)	223.33 [a] (5.92)	0.095 [a] (0.004)	0.01 [b] (0.00)

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).

Table 18C. The effect of crude extract of *Lactobacillus casei* W28 on growth of 22 h-old indicator cells (*Lactobacillus plantarum* GMR1)*

Incubation period (h)	Control		Treated	
	Growth (OD_{660})	Million cfu/ml	Growth (OD_{660})	Million cfu/ml
0	0.051 ^{b} (0.001)	44.0 ^{de} (1.2)	0.050 ^{d} (0.001)	42.0 ^{c} (1.2)
1.5	0.050 ^{b} (0.001)	36.0 ^{e} (2.3)	0.051 ^{d} (0.001)	46.0 ^{c} (2.3)
3.0	ND †	49.7 ^{d} (2.9)	ND	53.3 ^{c} (1.7)
4.5	ND	120.7 ^{c} (4.6)	ND	123.0 ^{b} (5.0)
6.0	0.062 ^{b} (0.001)	161.0 ^{b} (1.5)	0.059 ^{c} (0.000)	161.0 ^{b} (1.5)
12.0	0.080 ^{b} (0.001)	303.0 ^{a} (2.3)	0.075 ^{b} (0.002)	302.0 ^{a} (2.5)
24.0	0.136 ^{a} (0.001)	ND	0.133 ^{a} (0.001)	ND

* Data represent the means (with standard error) of triplicate sets. Values bearing different superscripts in each column differ significantly ($P<0.05$).
† ND, not determined.

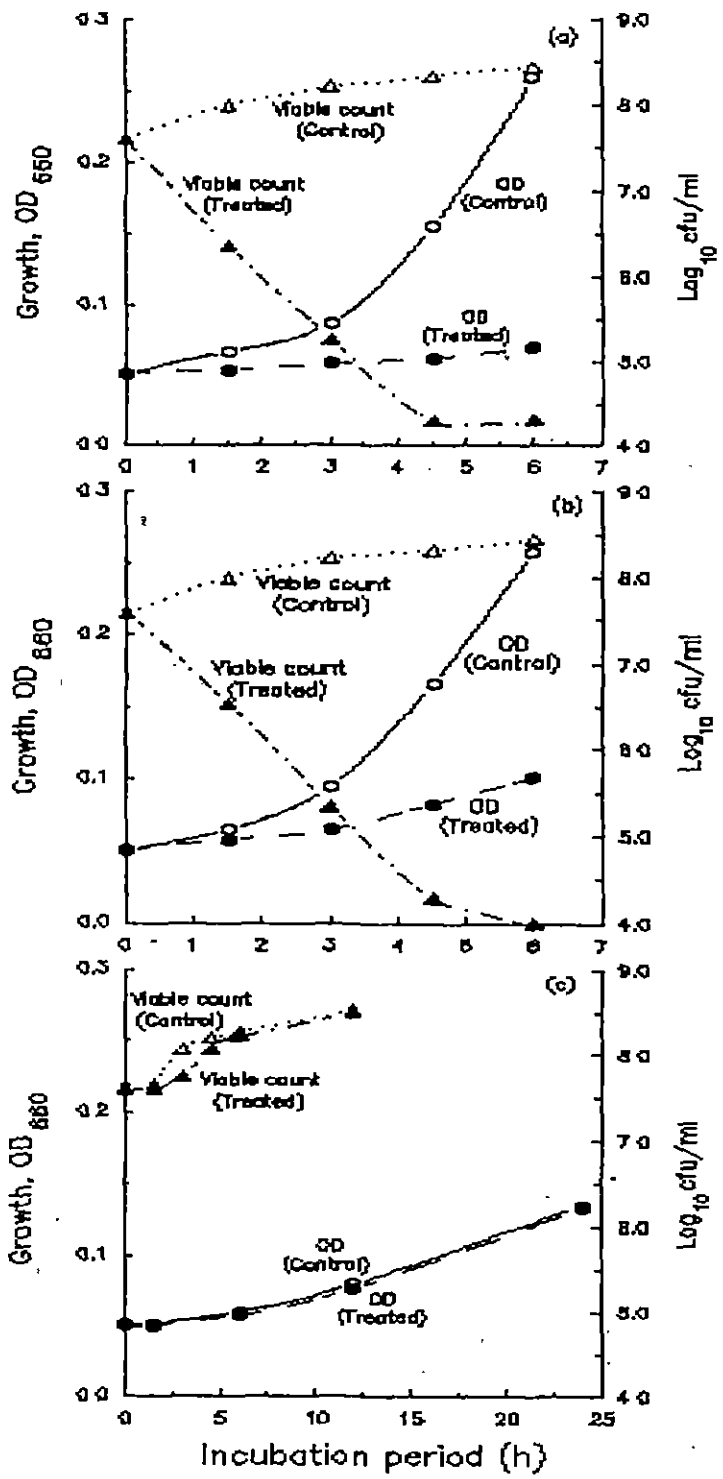

Fig. 15. The effect of crude extract of *Lactobacillus casei* W25B on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

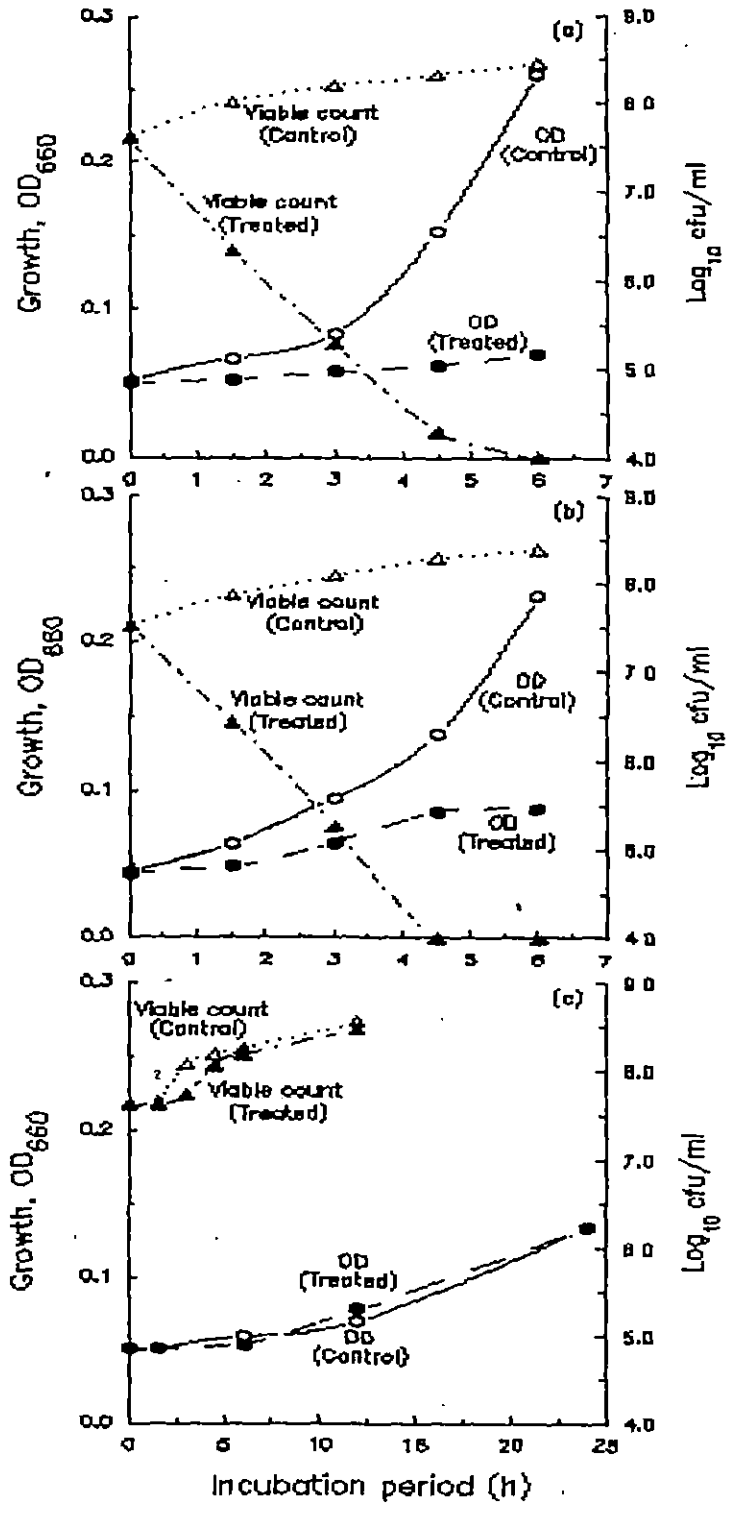

Fig. 16. The effect of crude extract of *Lactobacillus casei* W26B on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

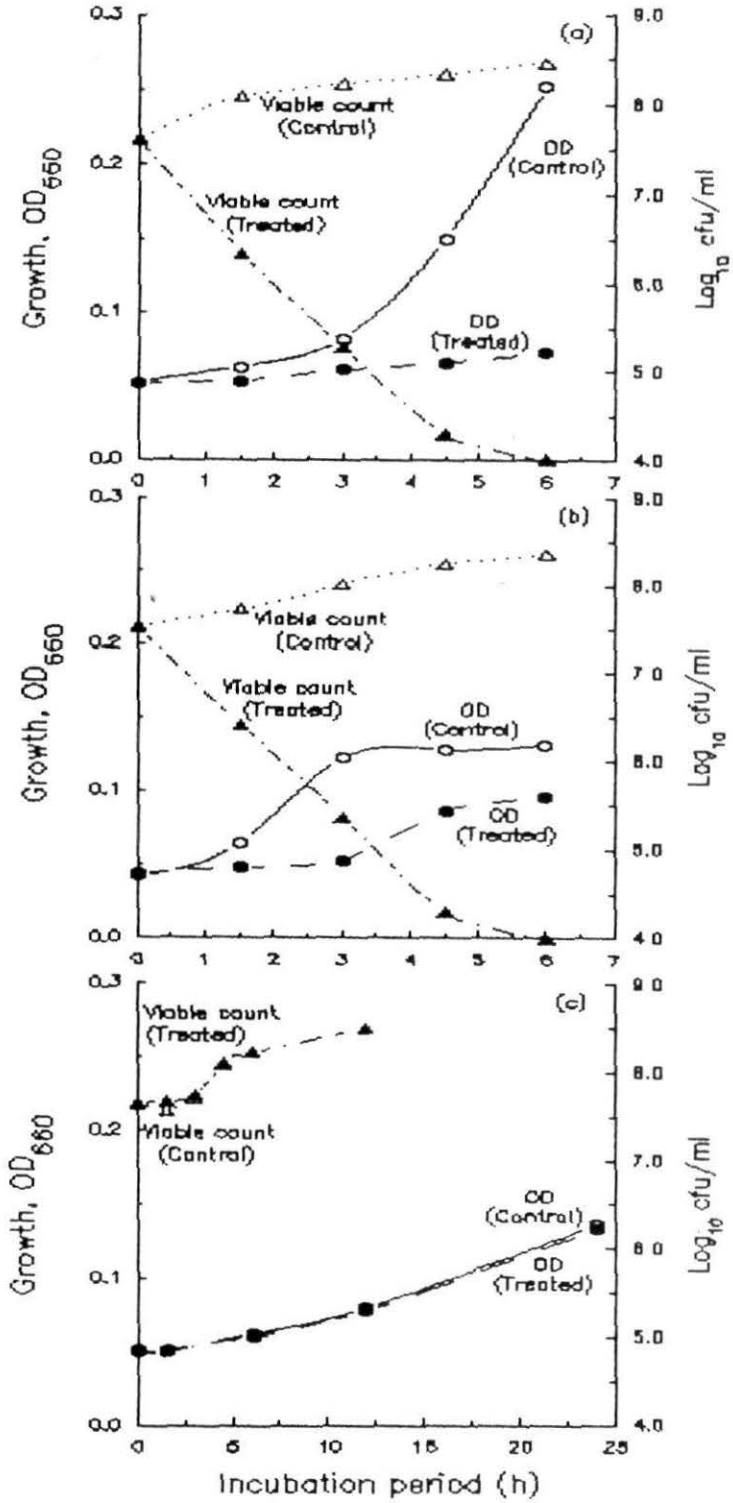

Fig. 17. The effect of crude extract of *Lactobacillus casei* W28 on growth of *Lactobacillus plantarum* GM-R1, aged 6 h (a), 12 h (b) and 22 h (c).

pronounced on logarithmic phased cells, and no action was observed on stationary phased cells.

4.9. Inhibitory spectra

Inhibitory spectra of the crude bacteriocins of the three selected strains were examined against the test organisms listed in Table 19. The bacteriocins from the strains W25B and W28 were inhibitory to four, and W26B to five of the 10 lactic acid bacteria tested. Activity was restricted to Gram positive bacteria, including *Bacillus subtilis, Bacillus cereus, Enterococcus faecium, Staphylococcus aureus* and a few actinomycetes.

Table 19. Antimicrobial spectrum of bacteriocins from the three isolated strains of *Lactobacillus casei*

Indicator strains	Source	Sensitivity† to bacteriocin activity of		
		W25B	W26B	W28
Lactic acid bacteria:				
Lactobacillus acidophilus 447	MTCC	-	-	-
Lactobacillus casei 1423	MTCC	+	+	+
Lactobacillus casei ssp. *rhamnosus*1408	MTCC		-	- -
Lactobacillus fermentum LS-MS12	NBU	-	-	-
Lactobacillus lactis 1484	MTCC	-	-	-
Lactobacillus maltaromicus 108	MTCC	-	-	-
Lactobacillus plantarum GM-R1	NBU	+	+	+
Lactobacillus plantarum LM-R1	NBU	+	+	-
Lactobacillus plantarum LS-R1	NBU	-	+	+
Lactobacillus plantarum 1325	MTCC	+	+	+
Gram positive eubacteria:				
Bacillus cereus 07M1	NBU	(+)	(+)	+
Bacillus licheniformis 04M1	NBU	-	-	+
Bacillus mycoides 06M1	NBU	-	-	-
Bacillus subtilis DK-W1	NBU	+	+	+
Bacillus subtilis var. natto	NBU	+	+	+
Bacillus subtilis 026M1	NBU	(+)	+	+
Enterococcus faecium DK-C1	NBU	(+)	(+)	+
Staphylococcus aureus 96	MTCC	+	+	+
Actinomycetes:				
Amycolata autotrophica 031M6	NBU	-	-	-
Microbispora rosea 033M6	NBU	-	-	-
Nocardia asteroides 034M6	NBU	-	-	-
Rhodococcus rhodochrous 030M6	NBU	-	-	-

Contd....

Indicator strains	Source	Sensitivity† to bacteriocin activity of		
		W25B	W26B	W28
Streptomyces coelicolor 028M6	NBU	+	+	+
Saccharopolyspora hirsuta 032M6	NBU	-	-	-
Streptosporangium roseum 029M6	NBU	(+)	(+)	+
Gram negative bacteria:				
Escherichia coli 118	MTCC	-	-	-
Escherichia coli K-12	NBU	-	-	-
Myxococcus fulvus 49305	ATCC	-	-	-
Paracoccus denitrificans 035M1	NBU	-	-	-
Pseudomonas putida 102	MTCC	-	-	-
Salmonella sp. 05M1	NBU	-	-	-
Salmonella typhimurium 98	MTCC	-	-	-
Yeast :				
Candida parasilopsis 1744	MTCC	-	-	-
Geotrichum candidum 1735	MTCC	-	-	-
Saccharomyces cerevisiae 173	MTCC	-	-	-

*MTCC, Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India; NBU, Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri, India; ATCC, American Type Culture Collection, Maryland, USA.

† Symbols for degree of inhibition in well diffusion assay: + large inhibition zone (width, ≥ 3.0 mm); (+), small inhibition zone (width, <3.0 mm); -, no inhibition zone.