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Preliminary Studies on Antimicrobial Activity of *Streptococcus lactis* subsp. *diacetylactis*

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ABSTRACT

Streptococcus lactis subsp. *diacetylactis* is well-known for its ability to inhibit pathogenic and milk spoilage microorganisms. In this study, an attempt was made to standardize the method for estimation of antimicrobial activity of the organism. Fourteen strains of *S. lactis* subsp. *diacetylactis* were examined for production of antimicrobial substances and all strains were found to produce antimicrobial substances. Of the strains, strain DRC_1 produced maximum amounts of antimicrobial substances followed by strains DRC_2 and S_1. Mutants of S_1 (S_1-67/C and S_1-195) produced higher amounts of antimicrobial substances than all other strains tested. The antimicrobial properties of strains S_1-67/C were assessed against 28 bacterial and mold cultures. Cell-free filtrates of S_1-67/C strain were more inhibitory to gram-negative than gram-positive bacteria. This organism also inhibited the growth of *Geotrichum candidum* but failed to inhibit other molds tested in this study.

Streptococcus lactis subsp. *diacetylactis* is known to dominate other lactic streptococci in mixed cheese starter cultures (*7*). Several investigators (*3,8,16*) have reported that this organism inhibits a wide variety of spoilage and pathogenic organisms in a variety of food products, such as butter, vanilla cream-filling, ham sandwiches, bread, soymilk and ground beef. The spent medium as well as washed cells of *S. lactis* subsp. *diacetylactis* inhibit the growth of *Pseudomonas fluorescens, Pseudomonas fragi, Alcaligenes metalcaligenes, Vibrio parahaemolyticus, Pseudomonas aeruginosa* (strains 10145 and ORMS), *Clostridium perfringens, Shigella sonnei, Escherichia coli* and *Salmonella tennessee* (*4,8,9,14*). Some researchers have noted differences in antimicrobial activity of mutant and parent cultures of *S. lactis* subsp. *diacetylactis* (*6,18*). The extent of inhibition of microorganisms by *S. lactis* subsp. *diacetylactis* varies depending on the strain and other factors (*12*).

Several investigators have used different methods for estimating antimicrobial substances produced by lactic acid bacteria (*4,5,15,17*). The purpose of this study was to: (a) standardize a simple bioassay method for the quantitative estimation of antimicrobial substances, (b) select a strain of *S. lactis* subsp. *diacetylactis* exhibiting strong antimicrobial properties, and, (c) determine the spectrum of antimicrobial activity of this organism.

MATERIALS AND METHODS

Sources of cultures

Eight strains of *S. lactis* subsp. *diacetylactis*, including N.I.R.D. 176, 184, 216, 277, 278, 615, 616 and 823 were obtained from the National Institute for Research in Dairying, England. Other isolates of *S. lactis* subsp. *diacetylactis*, including one isolate from butter (strain S_1), two mutants of S_1 (obtained after UV irradiation, namely, S_1-67/C and S_1-195), DRC_1, DRC_2 and DRC_3, *Streptococcus lactis* C-10, *Streptococcus cremoris* C-1, *Streptococcus thermophilus* HST, *Lactobacillus bulgaricus*-LBW, *Lactobacillus acidophilus, Streptococcus liquifaciens, Streptococcus faecalis*-810, *Micrococcus flavus, Staphylococcus aureus* S_6, *Bacillus cereus* D_6, *Bacillus subtilis* K-26, *Clostridium welchi, Pseudomonas fragi, Escherichia coli* F 6/2, *E. coli* F9/2, *E. coli, Geotrichum candidum, Pencillium roqueforti, Aspergillus flavus* and *Aspergillus parasiticus* were obtained from the culture collection of N.D.R.I., Karnal. *Shigella dysenteriae, Shigella flexneri, Salmonella typhi* and *Salmonella paratyphi*-A were obtained from P.G.I. of Medical Sciences, Chandigarh. *Pseudomonas fluorescens* NCIM 2141, *P. fluorescens* NCIM 2390 and *Alcaligenes viscolactis* NCIB-8154 were obtained from N.C.L., Pune.

All lactic cultures were maintained in litmus milk by routine weekly transfer. Pathogenic bacterial cultures were maintained on brain heart infusion agar slants, mold cultures were maintained on potato dextrose agar slants and all other cultures were maintained on nutrient agar slants.

Media

The following media were used in this study: (a) Elliker broth (*11*), (b) lactic agar, prepared by adding 1.5% agar (Difco) to Elliker broth, (c) nutrient broth (beef extract, 3 g, peptone, 2.5 g, sodium chloride, 5 g, and distilled water, 1000 ml), (d) nutrient agar, prepared by adding 1.5% agar (Difco) to nutrient broth, (e) brain heart infusion agar, (f) potato dextrose agar, and (g) litmus milk (11% reconstituted skim milk + 1% of a 4% aqueous litmus solution). All the above media, except litmus milk, were autoclaved at 15 psi for 20 min. Litmus milk was steamed for 30 min on three consecutive days.

Fermentation

A 16-h culture of *S. lactis* subsp. *diacetylactis* was inoculated (1% by volume) into 100-ml conical flasks containing 50 ml of Elliker broth and incubated for 24 h at 30°C. The cell-free filtrate was obtained by centrifuging 24-h cultures at $15,000 \times g$ for 15 min. The pH of the cell-free

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TABLE 1. *Screening of* S. lactis *subsp.* diacetylactis *for antimicrobial activity.*

Strain	pH[a]	Diameter of zone of inhibition (mm)[b]		Antimicrobial activity[e] (Units/ml)[b]
		P. fragi[c]	*S. aureus* S_6[d]	
DRC_1	4.23	12.7±0.1	12.3±0.1	13.5±0.4
DRC_2	4.23	12.5±0.1	12.2±0.2	12.0±0.3
DRC_3	4.19	11.7±0.1	11.6±0.2	11.0±0.3
S_1	4.23	12.0±0.1	12.0±0.2	12.0±0.3
S_1-67/C	4.15	13.4±0.1	13.2±0.2	16.0±0.3
S_1-195	4.14	12.8±0.1	12.6±0.2	13.5±0.3
N.I.R.D- 176	4.20	11.4±0.1	11.3±0.2	10.0±0.3
N.I.R.D- 184	4.06	11.7±0.1	11.5±0.1	11.0±0.3
N.I.R.D- 276	4.41	9.8±.1	9.7±0.1	6.0±0.4
N.I.R.D- 277	4.25	11.1±0.1	11.2±0.2	9.0±0.4
N.I.R.D- 278	4.48	9.7±0.1	10.0±0.2	6.0±0.2
N.I.R.D- 615	4.26	11.1±0.1	11.1±0.2	9.0±0.2
N.I.R.D- 616	4.21	11.6±0.1	11.3±0.2	10.5±0.3
N.I.R.D- 823	4.25	12.1±0.2	11.7±0.1	12.0±0.2

[a]Average of three separate trials (each in duplicate).
[b]Means with standard deviation of three separate trials (each in duplicate).
[c]Incubated at 20°C for 16 to 24 h.
[d]Incubated at 37°C for 24 h.
[e]*P. fragi* used as test organism.

filtrate was adjusted to 4.5 and the filtrate was autoclaved at 15 psi for 20 min.

Cup (well) assay method

The procedure recommended in British standard methods (*5*), with a slight modification, was followed. The assay medium was melted, tempered to 45 ± 2°C and 2% Tween 80 (maintained at 45°C) was added and thoroughly mixed. To this medium, 16-h washed cells of *P. fragi* were added to give a final inoculum of 5 to 8×10^5 cells/ml of nutrient agar. Ten ml of the inoculated medium were transferred to petri plates and allowed to solidify. The petri plates were placed in a refrigerator for 1 h to facilitate the boring of wells in the medium. Five wells (6 mm diam.) were made in each petri plate using a sterile steel borer.

Sterile culture filtrate (0.05 ml) was added to each well and the petri plates were left undisturbed for 1 h to facilitate diffusion of culture filtrate into the medium. The petri plates were then incubated at 20°C for 16 to 24 h and the diameter of inhibitory zones was measured and expressed as units/ml. Control assays were done using sterile Elliker broth (pH adjusted to 4.5) instead of culture filtrates.

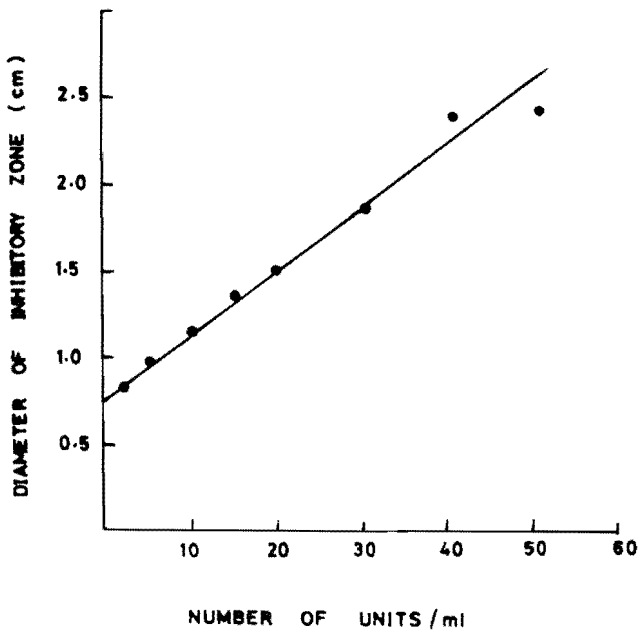

Figure 1. *Standard curve for estimation of antimicrobial activity of* S. lactis *subsp.* diacetylactis S_1*-67/C using* P. fragi *as the test organism. Y=8.0776 + 0.3461 X (Y=Diameter of inhibitory zone in mm and X=units/ml).*

Definition of unit

A unit is defined as the amount of inhibitory activity required to produce an inhibitory zone of 1.5 cm in diameter when tested by the well assay method (Agar diffusion method) using 5 to 8×10^5 cells of *P. fragi*/ml. Units/ml $= \frac{\text{diameter of inhibitory zone (mm)} - 8.0776}{0.3461}$.

Standard curve of antimicrobial activity

The cell-free filtrate with 20 units/ml concentration of antimicrobial activity was concentrated to give 30, 40, 50 and 60 units/ml and also diluted with distilled water to 1, 3, 6, 10 and 15 units/ml. The pH of the diluted as well as concentrated cell-free filtrate was adjusted to 4.5 and assayed using *P. fragi* as the test organism. The standard curve was drawn by plotting the diameter of the inhibitory zone on the 'y' axis and the units/ml on the 'x' axis (Fig. 1).

Screening of strains of S. lactis *subsp.* diacetylactis

The sterile cell-free filtrate of each strains of *S. lactis* subsp. *diacetylactis* was assayed against *S. aureus* S_6 and *P. fragi* using the above described procedure.

Spectrum of antimicrobial activity of S. lactis *subsp.* diacetylactis S_1*-67/C*

The spectrum of inhibitory activity of *S. lactis* subsp. *diacetylactis* S_1-67/C was determined using 28 milk spoilage and pathogenic microorganisms. All data presented are means with standard deviation of three separate trials (each in duplicate).

RESULTS AND DISCUSSION

All the 14 strains of *S. lactis* subsp. *diacetylactis* examined showed antimicrobial activity against both *S. aureus* S_6 and *P. fragi*, and the extent of inhibition was greater against the latter (Table 1). These results confirm the earlier observations of Angelo et al. (*2*) who reported that a strain of this organism exhibited greater inhibitory activ-

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TABLE 2. *Antimicrobial activity of* S. lactis *subsp.* diacetylactis *S_1-67/C against lactic acid bacteria.*

Strain	Diameter of zone of inhibition (mm)[a]
S. lactis C-10[b]	12.0±0.2
S. cremoris C-1[b]	10.0±0.3
S. lactis subsp. *diacetylactis* DRC_1[b]	11.0±0.3
S. lactis subsp. *diacetylactis* S_1-67/C[b]	0.0
S. thermophilus HST[c]	13.0±0.2
Streptococcus sp. LF-40[b]	10.0±0.2
L. bulgaricus[d]	0.0
L. acidophilus 'R'[d]	0.0

[a]Means with standard deviation of three separate trials (each in duplicate).
[b]Incubated at 30°C for 24 h.
[c]Incubated at 37°C for 24 h.
[d]Incubated at 40°C for 24 h.

TABLE 3. *Antimicrobial activity of* S. lactis *subsp.* diacetylactis *S_1-67/C against milk spoilage and pathogenic bacteria.*

Culture	Diameter of zone of inhibition (mm)[a]
Gram-positive bacteria	
S. liquefaciens 108[b]	15.0±0.5
S. faecalis PAH 810[b]	15.6±0.2
M. flavus[b]	16.8±0.3
S. aureus S_6[b]	13.2±0.3
B. cereus D_6[b]	13.2±0.2
B. subtilis K-26[b]	12.6±0.3
C. welchii[b]	17.3±0.2
Gram-negative bacteria	
S. dysenteriae[b]	16.0±0.2
S. flexneri[b]	14.7±0.2
S. typhi[b]	13.5±0.4
S. paratyphi A[b]	16.0±0.4
P. fragi[c]	15.0±0.4
P. fluorescens NCIM-2141[c]	13.0±0.3
P. fluorescens NCIM-2390[c]	18.0±0.4
E. coli F 6/2[b]	13.3±0.2
E. coli F 9/2[b]	13.7±0.2
E. coli[b]	14.0±0.4
A. viscolactis NCIB-8154[c]	20.0±0.4

[a]Means with standard deviation of three separate trials (each in duplicate).
[b]Incubated at 37°C for 24 h.
[c]Incubated at 20°C for 16 to 24 h.

ity against *P. fluorescens* than against *S. aureus*. Like others (*4,15*), we observed that, among the standard strains, strain DRC_1 had the greatest antimicrobial activity (13.5 units/ml) followed by DRC_2 (12 units/ml). The antimicrobial activity of strain S_1 was identical to that of DRC_2 and mutants of S_1 had appreciably greater antimicrobial activity than the parent strain. These observations agree with those of Sidorova et al. (*18*) who reported that mutants of *S. lactis* subsp. *diacetylactis* 639/1 exhibited greater activity than the parent strain.

Of the mutants, *S. lactis* subsp. *diacetylactis* S_1-67/C produced the greatest antimicrobial activity, although this culture did not reduce the pH of the fermented broth as much as other strains. Similar results regarding the relationship between production of acid and the antimicrobial activity of *S. lactis* subsp. *diacetylactis* have been reported earlier (*1*). Because of its strong antimicrobial activity, strain S_1-67/C was selected for further studies.

S. lactis subsp. *diacetylactis* S_1-67/C was active against all lactic streptococci except itself (Table 2). However, lactobacilli were not inhibited by this organism. Results of this study are in agreement with those of Delaney et al. (*10*), who showed that the antimicrobial substances produced by *S. lactis* subsp. *diacetylactis* were inhibitory to 45 strains of lactic streptococci but not to itself.

The antimicrobial activity of strain S_1-67/C was generally greater against the gram-negative than gram-positive organisms tested (Table 3). This observation confirms an earlier report (*13*). *Alcaligenes viscolactis* was most sensitive, whereas *Bacillus subtilis* K-26 was least sensitive of the organisms tested.

Strain S_1-67/C inhibited *G. candidum* but failed to inhibit other molds (Table 4). The inhibitory activity of this organism against *G. candidum* was also reported by Magak'yan and Chuprina (XX Int. Dairy Congr. 1978., p. 539).

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TABLE 4. *Antimicrobial activity of* S. lactis *subsp.* diacetylactis S_1-67/C *against molds.*

Culture	Diameter of zone of inhibition (mm)[a]
G. candidum[b]	13.0±0.2
P. roqueforti[b]	0.0
A. flavus[b]	0.0
A. parasiticus[b]	0.0

[a]Means with standard deviation of three separate trials (each in duplicate).
[b]Assayed at 21°C for 48 to 72 h.

All strains of *S. lactis* subsp. *diacetylactis* showed antimicrobial activity against both gram-positive and gram-negative organisms. The cell-free filtrate of strain S_1-67/C inhibited a wide variety of microorganisms including *G. candidum*. Therefore, the use of this organism would appear to have great potential for controlling the growth of food spoilage and pathogenic microorganisms in foods. Studies in this regard are in progress.

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