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Nutritional Factors Affecting Growth and Production of Antimicrobial Substances by *Streptococcus lactis* subsp. *diacetylactis* S_1-67/C

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ABSTRACT

The present study pertains to the effect of nutritional factors on the growth and production of antimicrobial substances (AS) by *Streptococcus lactis* subsp. *diacetylactis* S_1-67/C. Among nine media tested, yeast extract dextrose broth supported good growth and maximum production of AS. Addition of beef extract and yeast extract at 1.0 and 0.6% levels, respectively, increased growth as well as production of AS. Of ten carbohydrates examined, maximum production of AS was achieved with 1% glucose followed by fructose, 4% molasses, lactose, sucrose, galactose, mannitol, maltose and 2% molasses. Xylose inhibited production of AS, although it stimulated growth of the organism. Peptone, tryptone and tryptose (each at the 1.5% level) significantly stimulated production of AS. Other nitrogen sources, including soytone, casein hydrolysate and proteose peptone, retarded production of inhibitory substances. Among the amino acids, L-leucine, DL-methionine and L-glutamic acid were most essential for growth and production of AS, whereas L-lysine, L-proline, DL-serine, DL-asparatic acid, L-arginine-HCl and DL-tryptophan were stimulatory. Other amino acids such as DL-ornithine, L-cysteine-HCl and DL-citrulline slightly stimulated AS production. In the presence of cynocobalmin, niacin, folic acid, calcium pantothenate and riboflavin, *S. lactis* subsp. *diacetylactis* S_1-67/C produced maximum amounts of inhibitory substances. Omission of individual mineral salts from the basal medium did not affect production of AS by the organism.

Streptococcus lactis subsp. *diacetylactis* is known to inhibit a wide variety of food spoilage and pathogenic microorganisms, particularly the gram-negative types (*2,5,10*). Several workers have studied the effect of various nutrients on growth of the organism (*11,12*). Ferreira et al. (*4*) noted that the addition of liver extract concentrate to microinoculum broth stimulated the production of antimicrobial substances by *S. lactis* subsp. *diacetylactis*. Branen et al. (*2*) found that addition of several additives or increasing the whey protein concentration from 5 to 10% in a whey-based medium did not increase antimicrobial activity. However, very little information is with regard to the effect of various nutrients on production of antimicrobial substances (AS) by this organism.

Earlier studies indicated that *S. lactis* subsp. *diacetylactis* S_1-67/C produces considerably higher amounts of AS (*10*). The present study was undertaken to determine the optimum conditions for growth and production of AS by *S. lactis* subsp. *diacetylactis* S_1-67/C.

MATERIALS AND METHODS

Source and maintainance of cultures

Source and maintainance of *S. lactis* subsp. *diacetylactis* S_1-67/C and *Pseudomonas fragi* were described earlier (*10*).

Media

The following nine media were tested: (a) reconstituted skim milk [11% S-N-F (wt/vol) in distilled water]; (b) enriched skim milk containing 11% nonfat dried milk, 0.5% yeast extract, 0.5% dextrose and 0.5% peptone; (c) citric acid whey fortified with 0.5% yeast extract, which was prepared from dried skim milk reconstitted to 11% in distilled water. (Curd was developed by acidification of the milk to pH 4.6 with 1N citric acid. Curd was removed by filtration through cheesecloth and 0.5% yeast extract was added to the resultant whey. The pH of the fortified whey was adjusted to 6.8 with 1N NaOH); (d) rennet whey fortified with 0.5% yeast extract, which was prepared in the same way as citric acid whey except that rennet was used instead of citric acid; (e) citrate broth-1 was prepared according to Harvey and Collins (*6*). (It contained sodium citrate•3 H_2O, 0.5%; lactose (Oxoid), 2%; Bacto peptone (Difco), 1%; yeast extract (Oxoid), 1.5%; KH_2PO_4, 0.05%; $MgSO_4$•7H_2O, 0.02%; and sodium acetate, 0.2%, with the pH adjusted to 6.8); (f) citrate broth-2 (*12*) which contained tryptone (Difco), 1%; glucose, 1%; sodium dihydrate, 2%; yeast extract (Oxoid), 0.5%; dibasic potassium sulfate, 0.1%; $MgSO_4$•7 H_2O, 0.1% with the pH adjusted to 6.8; (g) citrate broth-3 which was the N L medium of Pack et al. (*8*); (h) yeast dextrose broth which contained peptone, 2%; glucose, 1%; sodium chloride, 0.5%; yeast extract, 0.6%; beef extract, 1%; and ammonium citrate, 0.5%, with the pH adjusted to 6.8; and (i) Elliker broth (*3*).

All media were prepared using distilled water and dispensed into Erlenmeyer flasks in 50-ml quantities. Reconstituted skim milk and enriched skim milk were sterilized by steaming for 30 min for 3 successive days, after which they were determined to be sterile by plating. All other media were sterilized in an autoclave at 15 psig for 20 min.

Of this media, yeast extract dextrose broth was selected as the basal medium because it allowed the organism to produce the maximum amount of AS. The effect of different growth-promoting ingredients, such as yeast extract, beef extract, carbohydrates, nitrogen supplements and

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TABLE 1. *Effect of medium on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis *S_1-67/C*[a].

Medium	Growth (× 10 CFU/ml)	Antimicrobial substances (Units/ml)
Reconstituted skim milk (11% S-N-F)	54.0 ± 3.8	9.0 ± 0.4
Enriched skim milk	46.0 ± 2.8	10.5 ± 0.4
Elliker broth	25.0 ± 4.2	17.0 ± 0.4
Citric acid whey + yeast extract	167.0 ± 5.4	3.5 ± 0.4
Rennet whey + yeast extract	66.0 ± 3.9	6.0 ± 0.2
Citrate broth-1	107.0 ± 3.5	14.0 ± 0.2
Citrate broth-2	7.7 ± 0.8	9.0 ± 0.2
Citrate broth-3	82.0 ± 2.8	6.0 ± 0.4
Yeast extract dextrose broth	9.6 ± 0.5	20.0 ± 0.6

[a]Growth and production of antimicrobial substances were assessed after 72 h of incubation at 25°C using *Pseudomonas fragi* as the test organism. Values reported are means with the standard deviation of three trials (each in duplicate).

sodium chloride (0 to 2% wt/vol) was determined by incorporating each singly into the basal medium, but keeping all other variables constant.

The effect of individual amino acids, vitamins, purine and pyramidine bases, and mineral salts on growth and production of AS was also studied by following the single omission technique in synthetic medium (*11*).

Fermentation

Sterile media were inoculated with 1% by volume of an active culture of *S. lactis* subsp. *diacetylactis* S_1-67/C and incubated for 72 to 120 h at 25°C. Fermented liquors were assessed for growth and production of AS as described earlier (*10*).

Measurement of growth

Growth in yeast extract dextrose broth and in synthetic medium was measured by determining optical density using a photoelectric colorimeter at 520 and 450 nm (maximum absorption in synthetic medium occurred at 450 nm), respectively.

The number of colony-forming units (CFU)/ml was determined by pour plate method using lactic agar (*3*).

All data presented were means with standard deviation of three separate trials (each in duplicate).

RESULTS AND DISCUSSION

Results of the effect of different media on growth and production of AS by *S. lactis* subsp. *dicetylactis* S_1-67/C are shown in Table 1. Of the nine media, yeast extract dextrose broth allowed maximum production of AS (20.0 ± 0.6 units/ml) followed by Elliker broth (17.0 ± 0.4 units/ml), citrate broth-1 (14.0 ± 0.2 units/ml), enriched skim milk (10.5 ± 0.4 units/ml), reconstituted skim milk and citrate broth-2 (9.0 ± 0.4 units/ml). Production of AS was least in citric acid whey fortified with yeast extract; however, this medium yielded the greatest amount of growth followed by citrate broth-1, citrate broth-2, reconstituted skim milk, enriched skim milk and Elliker broth. Elliker broth allowed good growth and production of AS; however, the uninoculated broth (pH adjusted to 4.5) had some inhibitory activity (*2*). Since *S. lactis* subsp. *diacetylactis* S_1-67/C produced the greatest amount of AS in yeast extract broth and because the uninoculated pH-adjusted (4.5) broth had no antimicrobial activity when compared to other media, this medium was selected as the basal medium for further studies.

Results on the effect of supplementation of different concentrations of beef extract in the basal medium are shown in Table 2. Increasing the concentration of beef extract from 0 to 1% stimulated the production of AS from 11 to 20 units/ml. Any additional increase in the level of beef extract did not improve the production of AS.

TABLE 2. *Effect of beef extract on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis *S_1-67/C.*[a]

Concentration of beef extract (%)	Growth (O.D. at 520 nm)	Antimicrobial substances (Units/ml)
0.0	0.27 ± 0.01	11.0 ± 0.3
0.5	0.35 ± 0.02	14.0 ± 0.6
1.0	0.48 ± 0.01	20.0 ± 0.6
1.5	0.48 ± 0.01	20.0 ± 0.6
2.0	0.50 ± 0.01	20.5 ± 0.5

[a]See footnote "a" of Table 1.

TABLE 3. *Effect of yeast extract on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis *S_1-67/C.*[a]

Concentration of yeast extract (%)	Growth (O.D. at 520 nm)	Antimicrobial substances (Units/ml)
0.0	0.19 ± 0.01	11.0 ± 0.4
0.3	0.29 ± 0.01	13.0 ± 0.6
0.6	0.48 ± 0.01	20.0 ± 0.4
1.0	0.49 ± 0.02	20.5 ± 0.5
2.0	0.55 ± 0.01	20.5 ± 0.4

[a]See footnote "a" of Table 1.

Growth of *S. lactis* subsp. *diacetylactis* S_1-67/C was stimulated by 0.3 to 2% yeast extract. Production of AS was stimulated from 11 to 20 units/ml by addition of 0.6% yeast extract (Table 3). Any further increase in the level of yeast extract resulted in a negligible change in production of AS.

Results of the effect of different concentrations of glucose on growth and production of AS by *S. lactis* subsp. *Diacetylactis* S_1-67/C are presented in Table 4. Addition of up to 1% glucose stimulated growth and production of AS (5.0 ± 0.6 to 22.0 ± 0.4 units/ml). Of the carbohydrates tested for AS production (Table 5), 1% glucose promoted maximum production followed by fructose, 4% molasses, lactose, sucrose and galactose. Xylose stimulated

TABLE 4. *Effect of glucose on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis S_1-67/C[a].

Concentration of glucose (%)	Growth (O.D. at 520 nm)	Antimicrobial substances (Units/ml)
0.0	0.20 ± 0.01	5.0 ± 0.6
0.5	0.43 ± 0.01	18.0 ± 0.4
1.0	0.48 ± 0.01	22.0 ± 0.4
1.5	0.48 ± 0.01	20.5 ± 0.4
2.0	0.48 ± 0.01	20.5 ± 0.4

[a]See footnote "a" of Table 1.

maximum growth (O.D 0.65 ± 0.01), although it completely inhibited production of AS. This may be due to the conversion of inhibitory substances into other metabolites or the organism may have failed to convert other high molecular weight compounds into low molecular weight cationic inhibitory substances in the presence of xylose. The precise reason for this failure of the organism is not clearly known. Similarly, maltose, mannitol, galactose and 2% molasses enhanced growth.

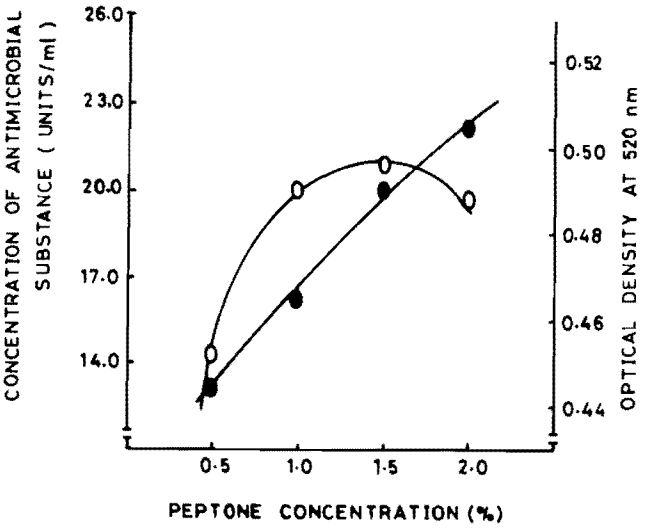

Figure 1. *Effect of peptone on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis S_1-67/C *using* Pseudomonas fragi *as the test organism; growth (○——○) and production of antimicrobial substances (●——●).*

Increasing the concentration of peptone from 0.5 to 1.5% resulted in a corresponding increase of both growth and production of AS (14.0 ± 0.4 to 21.0 ± 0.6 units/ml). Any additional increase in peptone concentration resulted in slight reduction in production of AS (Fig. 1). Among the nitrogen sources, peptone, tryptose and tryptone (each at 1.5%) enhanced the production of AS (Table 6), whereas other nitrogen sources, like soytone, casein hydrolysate and proteose peptone, retarded the production of AS.

Omission of sodium chloride from the basal medium resulted in poor growth and decreased the production of AS. The optimum concentration of NaCl for maximum growth and production of AS was 0.5%. Any additional increase (0.5% to 2%) or decrease (0.5% to 0.1%) in the NaCl concentration suppressed growth and production of AS (data not presented).

TABLE 5. *Effect of carbohydrates on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis S_1-67/C[a].

Carbohydrate	Growth (O.D. at 520 nm)	Antimicrobial substances (Units/ml)
Glucose, 1%	0.43 ± 0.01	22.0 ± 0.7
Lactose, 1%	0.46 ± 0.01	19.5 ± 0.6
Galactose, 1%	0.58 ± 0.01	18.5 ± 0.4
Fructose, 1%	0.44 ± 0.01	21.0 ± 0.6
Sucrose, 1%	0.44 ± 0.01	19.0 ± 0.4
Xylose, 1%	0.65 ± 0.01	0.0
Mannitol, 1%	0.48 ± 0.01	17.5 ± 0.4
Maltose, 1%	0.48 ± 0.01	14.0 ± 0.5
Molasses, 2%	0.48 ± 0.01	12.0 ± 0.4
Molasses, 4%	0.52 ± 0.01	20.0 ± 0.4

[a]See footnote "a" of Table 1.

TABLE 6. *Effect of nitrogen source on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis S_1-67/C[a].

Nitrogen source (at 1.5% level)	Growth (O.D. at 520 nm)	Antimicrobial substances (Units/ml)
Peptone	0.48 ± 0.01	20.0 ± 0.4
Proteose peptone	0.52 ± 0.01	17.5 ± 0.4
Tryptone	0.52 ± 0.01	20.5 ± 0.5
Tryptose	0.43 ± 0.01	20.0 ± 0.4
Soytone	0.47 ± 0.01	19.0 ± 0.4
Casein hydrolysate	0.44 ± 0.01	18.0 ± 0.4

[a]See footnote "a" of Table 1.

Growth and production of AS were greater in a non-synthetic medium (yeast extract dextrose broth) than a synthetic medium containing the amino acids present in aseptically drawn milk (*11*). The latter failed to provide growth factors, such as peptides or peptide-like substances, which play an important part in the nutrition of lactic acid bacteria (*1*).

Of the amino acids evaluated, L-leucine, DL-methionine and L-glutamic acid were essential for both growth and production of AS (Table 7). Omitting L-histidine resulted in poor growth (O.D. 0.17 ± 0.01) and the production of AS decreased from 17.5 ± 0.6 to 14.0 ± 0.4 units/ml. The present findings are in partial agreement with those of Reiter and Oram (*11*) who have shown that apart from the four amino acids mentioned above, DL-valine and DL-isoleucine were also essential for growth of all streptococci.

Although some amino acids, such as L-valine, DL-citrulline, DL-threonine, DL-ornithine and L-cysteine-HCl, were not required for growth of *S. lactis* subsp. *diacetylactis* S_1-67/C, single omission of these amino acids resulted in decreased production of AS. Single omission of amino acids such as DL-phenylalanine, L-glycine, DL-alanine

TABLE 7. *Effect of amino acids on growth and production of antimicrobial substances by* S. lactis *subsp.* dicetylactis *S_1-67/C*[a].

Amino acid omitted	Growth (O.D. at 450 nm)	Antimicrobial substances (Units/ml)
L-Proline	0.36 ± 0.01	16.0 ± 0.7
L-Tyrosine	0.39 ± 0.01	13.0 ± 0.8
L-Leucine	0.15 ± 0.01	0.0
DL-Valine	0.41 ± 0.01	12.0 ± 0.4
L-Histidine	0.17 ± 0.01	14.0 ± 0.4
DL-Phenylalanine	0.36 ± 0.01	20.0 ± 0.6
DL-Alanine	0.35 ± 0.01	18.5 ± 0.5
L-Lysine	0.35 ± 0.01	13.0 ± 0.4
DL-Serine	0.35 ± 0.01	12.0 ± 0.4
DL-Methionine	0.16 ± 0.01	3.5 ± 0.3
DL-Asparatic acid	0.36 ± 0.01	14.0 ± 0.4
L-Arginine-HCl	0.36 ± 0.01	10.5 ± 0.4
DL-Threonine	0.40 ± 0.01	12.0 ± 0.4
L-Glycine	0.39 ± 0.01	18.5 ± 0.4
DL-Isoleucine	0.37 ± 0.01	17.5 ± 0.4
L-Glutamic acid	0.10 ± 0.01	0.0
DL-Ornithine	0.40 ± 0.01	16.0 ± 0.7
L-Tryptophane	0.35 ± 0.01	14.0 ± 0.4
L-Cysteine-HCl	0.60 ± 0.01	12.0 ± 0.4
DL-Citrulline	0.40 ± 0.01	16.0 ± 0.5
All amino acids	0.09 ± 0.01	0.0
Synthetic medium	0.40 ± 0.01	17.5 ± 0.6
Yeast extract dextrose broth[b]	0.51 ± 0.01	21.0 ± 0.6

[a]Growth and antimicrobial substance production were assessed after 120 h of incubation at 25°C using *P. fragi* as the test organism. Values reported are means with the standard deviation of three trials (each in duplicate).
[b]Growth measured at O.D. 520 nm.

TABLE 8. *Effect of vitamins on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis *S_1-67/C*[a].

Vitamin omitted	Growth (O.D. at 450 nm)	Antimicrobial substances (Units/ml)
Vitamin B_{12}	0.06 ± 0.01	3.5 ± 0.4
PABA	0.43 ± 0.01	18.5 ± 0.4
Ascorbic acid	0.40 ± 0.01	20.0 ± 0.7
Pyridoxal-HCl	0.35 ± 0.01	16.0 ± 0.6
Niacin	0.15 ± 0.01	0.0
Folic acid	0.11 ± 0.01	10.5 ± 0.4
Biotin	0.38 ± 0.01	17.5 ± 0.4
Thiamine-HCl	0.38 ± 0.01	17.5 ± 0.4
Ca-pantothenate	0.06 ± 0.01	0.0
Riboflavin	0.15 ± 0.01	10.5 ± 0.4
All vitamins	0.06 ± 0.01	0.0
Synthetic medium	0.40 ± 0.01	17.0 ± 0.4
Yeast extract dextrose broth[b]	0.50 ± 0.01	20.0 ± 0.6

[a]See footnote "a" of Table 7.
[b]Growth measured at O.D. 520 nm.

and DL-isoleucine resulted in depressed growth and enhanced production of AS. These findings partially agree with earlier reports of Niven (*7*) and Reiter and Oram (*11*) who demonstrated the growth-promoting activity of DL-phenylalanine and DL-isoleucine and inhibitory effect of DL-alanine and DL-glycine on lactic streptococci.

TABLE 9. *Effect of purine and pyrimidine bases on growth and production of antimicrobial substances by* S. lactis *subsp.* diacetylactis *S_1-67/C*[a].

Base omitted	Growth (O.D. at 450 nm)	Antimicrobial substances (Units/ml)
Xanthine	0.34 ± 0.01	16.0 ± 0.6
Adenine	0.35 ± 0.01	20.0 ± 0.6
Uracil	0.35 ± 0.01	18.0 ± 0.4
Guanine	0.40 ± 0.01	17.0 ± 0.5
All bases	0.23 ± 0.01	7.5 ± 0.3
Synthetic medium	0.40 ± 0.01	17.5 ± 0.4
Yeast extract dextrose broth[b]	0.50 ± 0.02	20.0 ± 0.6

[a]See footnote "a" of Table 7.
[b]Growth measured at 520 nm.

Results of some vitamins on growth and production of AS by *S. lactis* subsp. *diacetylactis* S_1-67/C are shown in Table 8. Vitamins such as cynocobalmin, niacin and Ca-pantothenate were essential for both growth and production of AS. Individual omission of folic acid and riboflavin resulted in poor growth and decreased production of AS. These results partially agree with those of Reiter and Oram (*11*) who showed that, except for cynocobalmin and folic acid, the other three vitamins were essential for growth.

The stimulatory effect of pyridoxal-HCl on the growth of *S. lactis* subsp. *diacetylactis* S_1-67/C confirms several earlier reports (*1,11,13*) on the growth-promoting ability of this vitamin on lactic streptococci. The non-essentiality of para-amino-benzoic acid (PABA) and ascorbic acid for growth of streptococci was also observed. Although biotin has been reported to be required for growth of all streptococci (*1*), results of this study show that this organism does not require biotin for either growth or production of AS. Omission of ascorbic acid and PABA from the snythetic medium resulted in enhanced production of AS, indicating that these vitamins exert an inhibitory effect on production of AS.

Results of individual purine and pyrimidine bases on growth and production of AS are shown in Table 9. Omitting adenine and uracil increased the production of AS and depressed growth, indicating that these bases had some stimulatory effect on growth of the organism. Similarly, xanthine exhibited a growth-stimulating effect. Omitting guanine had no effect on growth or production of AS. Omission of all four purine and pyrimidine bases greatly retarded both growth (O.D. 0.23 ± 0.01) and production of AS (7.5 ± 0.3 units/ml). Results of the present study corraborate earlier findings of Niven (*7*) and Yano et al. (*13*) who also observed retardation of growth of lactic streptococci when all four bases were omitted from synthetic medium.

Omission of individual mineral salts, such as $MgCl_2$, $CaCl_2$, $FeCl_2 \cdot H_2O$, $ZnSO_4$, $CuSO_4$ and Na_2MoO_4, did not affect either growth or production of AS, although omis-

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