CHAPTER 1

STUDIES ON THE RESPONSE OF *STREPTOCOCCUS FAECALIS* TOWARDS PANTOTHENATE AND ITS PRECURSORS

Nutritional requirements of various lactic acid bacteria are well-known (91). They are very fastidious organisms and need different nutrients like various vitamins and various assortments of amino acids and often additional substances such as purine and pyrimidine bases. All the reported lactic acid bacteria essentially require pantothenate for growth and metabolism (91). Toennies et al (49) worked with S. faecalis ATCC NO. 9790 and reported on its growth and lysis under different nutritional conditions regarding pantothenate. In this chapter we report our studies with S. faecalis R ATCC NO. 8043 regarding pantothenate metabolism in growth and lysis.

The work done here deals with S. faecalis R ATCC NO. 8043 in terms of:

a) Response of the organism to β-alanine and DL-pantolactone.
b) Growth and lysis of the organism at different pantothenate levels.
c) Influence of the molarity of the phosphate buffer of the medium upon the growth and lysis of the organism.
d) Growth response of cells pregrown in high- and low- pantothenate levels (and to continue growth) in absence of exogenous pantothenate.

1.1 MATERIALS AND METHODS

1.1.1 Bacterial strain

S. faecalis R ATCC NO. 8043 was kindly supplied by Dr. S. Bannarjee, Scientific Adviser, Dey's Medical Stores, Calcutta.

1.1.2 Growth-conditions, medium and measurement of turbidity

The organisms were grown (in calibrated culture tubes 90 x 150 mm with long glass closures) in a synthetic non-limited complete medium* buffered at pH 6.5 with 0.3 M phosphate buffer (90). Composition of the complete basal medium given below:

Composition of the complete basal medium

The amounts refer to 1 ml of finished culture medium (FCM)

20.0 mg glucose
25.65 mg Na_2HPO_4
16.45 mg $NaH_2PO_4.H_2O$
6.00 mg anhydrous sodium acetate
600 γ $(NH_4)_2SO_4$
442 γ KH_2PO_4
305 γ K_2HPO_4
300 γ L-glutamic acid
200 γ $MgSO_4.7H_2O$
200 γ each of L-arginine, L-cystine, L-histidine, L-hydroxyproline, L-tryptophan, L-tyrosine, L-proline, glycine
200 γ DL-alanine
110 γ L-lysine
100 γ each of L-aspartic acid, L-isoleucine, L-leucine, L-methionine, L- phenylalanine, L-serine, L-threonine, L-valine
35 γ adenine
30 γ uracil
27 γ guanine
10 γ each of NaCl, $FeSO_4.7H_2O$, $MnSO_4.4H_2O$
5.0 γ each of L-asparagine and L-glutamine
1.0 γ nicotinamide
0.40 γ each of pantothenate and pyridoxamine
0.20 γ each of riboflavine and thiamine
0.05 γ folic acid
0.04 γ p-aminobenzoic acid
0.005 γ biotin

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* DL-tryptophane, DL-tyrosine were used instead of the corresponding L-amino acids as they were not available. 1 μg = 1 γ

The basal medium was sterilised by passing it through a Millipore metal filter (0.45 μ pore size). Aqueous solution of pantothenate (Mann Research Labs. Inc., U.S.A.) and β-alanine used in the medium were sterilised by autoclaving at 15 lb. per square inch for 3 min. DL-pantolactone was sterilised by Millipore metal filter as above.

Growth of the culture was measured by determining directly the turbidity of the culture tubes in a colorimeter (Photochem. Colorimeter C-110, red filter, with test tube holder for insertion of the culture tubes). Optical densities were adjusted to AOD (adjusted optical density) values which are proportional to bacterial concentration (1 AOD being equal to 0.8 μg of dry cell).

1.1.3 Preparation of inoculum

The inoculum was grown at 38°C in 6 ml of finished non-limited complete medium with 400 mμg of calcium pantothenate per ml. From an initial bacterial concentration of AOD about 2, an inoculum culture of AOD 240, consisting of active cells in early log phase was grown and chilled. The cells were spun down (2 - 5°C) and supernatant was rejected. The pellet was washed twice with same volume of sterile finished medium without pantothenate and finally re-suspended in the same volume of similar medium. This suspension was used for inoculating the experimental tubes at the rate of one drop per 6 ml of finished medium. In all the experiments reported here 3 ml of the basal medium was diluted to 6 ml with sterile water or sterile test solutions (containing appropriate amounts of the test materials). Haake's unitherm constant ten-

perature stirrer-water bath was used for incubation (38°C).

1.2 RESULTS AND DISCUSSION

It would be evident from the data in Table 1.1 that the organism under study could not utilize (even after 3 days of incubation) β-alanine, DL-pantolactone alone or their mixtures for sparing the need of pantothenate for the organism. This, presumably, was due to absence of necessary cellular enzymes for utilization of either of these substrates alone or in combination for biosynthesis of the vitamin. This proved that the organism used is a very stable pantothenate auxotroph and needs the vitamin essentially for its growth and metabolism.

Fig. 1.1 shows characteristic data in regard to the response of the organism to pantothenate at different levels. With decreasing pantothenate level (below 8 mug/ml) the generation rate showed decreasing tendency. With the increasing concentrations of pantothenate generation rate tended to be higher and attained a plateau. Increase of pantothenate concentrations in the medium elevated the peak-growth and the half-times of lysis (50 percent reduction of initial turbidity) rate tended to decrease.

In all the cases peak-growth depended on the level of exogenous pantothenate. Termination of growth (upto about 20 mug/ml) in each case was followed by lysis. These observations confirmed the results obtained with S. faecalis ATCC NO. 9790 (49).at various limiting pantothenate levels. It is further to be noted that the rate of half-times of lysis

LEGEND OF FIG. 1.1

Growth of Streptococcus faecalis R ATCC NO. 8043 on differen pantothenate concentrations. The tubes were inoculated with a calcul OD of 2 and incubated at 38° C, AOD readings being taken periodicall The curves represent from right to left, the responses to 2, 4, 8, 1 20, 50, 100 and 200 mμg of calcium pantothenate per ml. The cultures were run simultaneously but for clarity individual points are not sh and the curves have been separated by spaces on the time scale. The minutes shown on the ascending and descending branches show approxim duplication times and half-times of lysis ($t_{\frac{1}{2}}$ time for AOD to drop by percent) respectively.

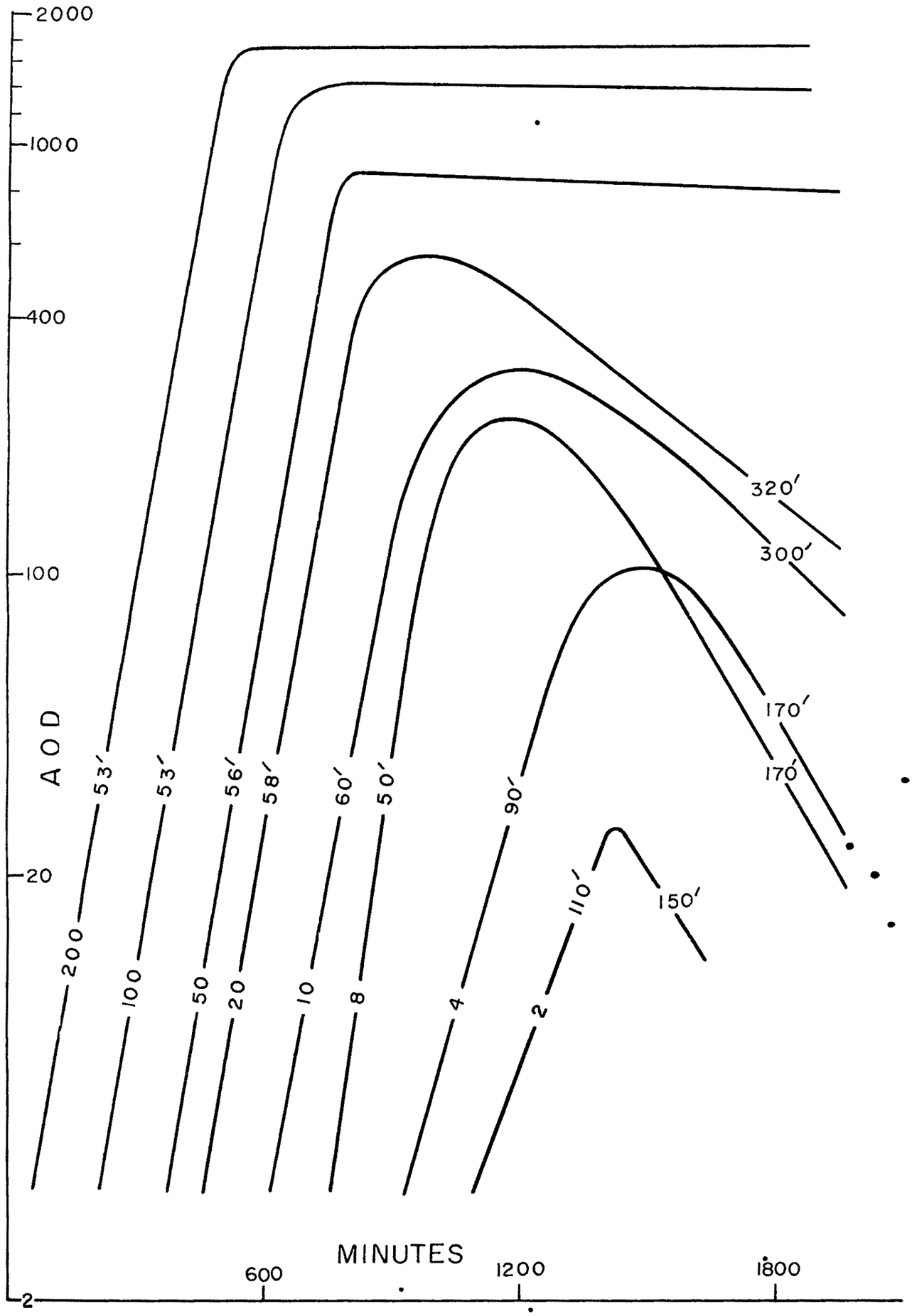
2000
1000
400
100
20
2
AOD
MINUTES
600
1200
1800
53′
53′
56′
58′
60′
50′
90′
110′
150′
320′
300′
170′
170′
200
100
50
20
10
8
4
2

FIG. 1·1

observed in the present set of experiment (20 mµg/ml pantothenate level and below) was appreciably slower than that reported by Toennies et al (49) with S. faecalis ATCC NO. 9790. The difference could be due to faster doubling rate of S. faecalis ATCC NO. 9790 (generation time varied from 32 to 35 min.).

Generally, depletion of nutrients which are structural components of cell wall leads to lysis, whereas depletion of other nutrients is followed by postexponential synthesis of wall and membrane (90, 92). Accordingly, if upon depletion of pantothenate the cofactors derived from pantothenate which are present at that time do not seem capable of continuing production of the acyl compounds needed for the integumental structures, one may conclude that (i) these cofactors are in turnover and suffer net degradation as soon as exponential growth ceases, or (ii) cofactors involved in critical acyl transfers are themselves essential components of integumental structure, or both.

Studies were undertaken to see whether the pantothenate-dependent growth and lysis were dependent on the molarity of phosphate buffer of the medium as reported in the case of lysine lysis by Shockman et al (93). Results of these experiments are shown in Table 1.2 and Fig. 1.2.

It is evident from Fig. 1.2 that the peak-growth was dependent on the molarity of the buffer. Optimum-growth was obtained between 0.15 and 0.30 molarity. Rates of lysis unlike the rate of growth of the organism was significantly affected by molarity of the buffer (Table 1.2) and this reached the maximum value at 0.3 molarity. At lower and higher strengths lysis rate tended to be slower. Obtaining of lower

Table 1.2

Effects of molarity of the phosphate buffer upon the lysis rate of Streptococcus faecalis R ATCC NO. 8043*

Molarity** (M)	Lysis time*** (hr.)
1 : Control****	—
2 : 1 + 0.15	14.5
3 : 1 + 0.20	9.0
4 : 1 + 0.25	7.5
5 : 1 + 0.30	6.0
6 : 1 + 0.40	10.0
7 : 1 + 0.50	13.0
8 : 1 + 0.75	18.0

* Grown in the synthetic medium with 20 mμg of pantothenate per ml. Growth rates in this medium with buffer of different molarities were the same (duplication time being about 60 min.).

** Sterile phosphate buffer 2.4 M obtained by mixing 1.152 moles of NaH_2PO_4 plus 1.248 moles of Na_2HPO_4 in 1000 ml. Appropriate portions of this buffer were used.

*** Times for AOD to drop by 50 percent after peak-growth.

**** Control: its buffer concentration was 0.005 M (as K-phosphate). This tube showed insignificant growth evidently due to very low phosphate concentrations.

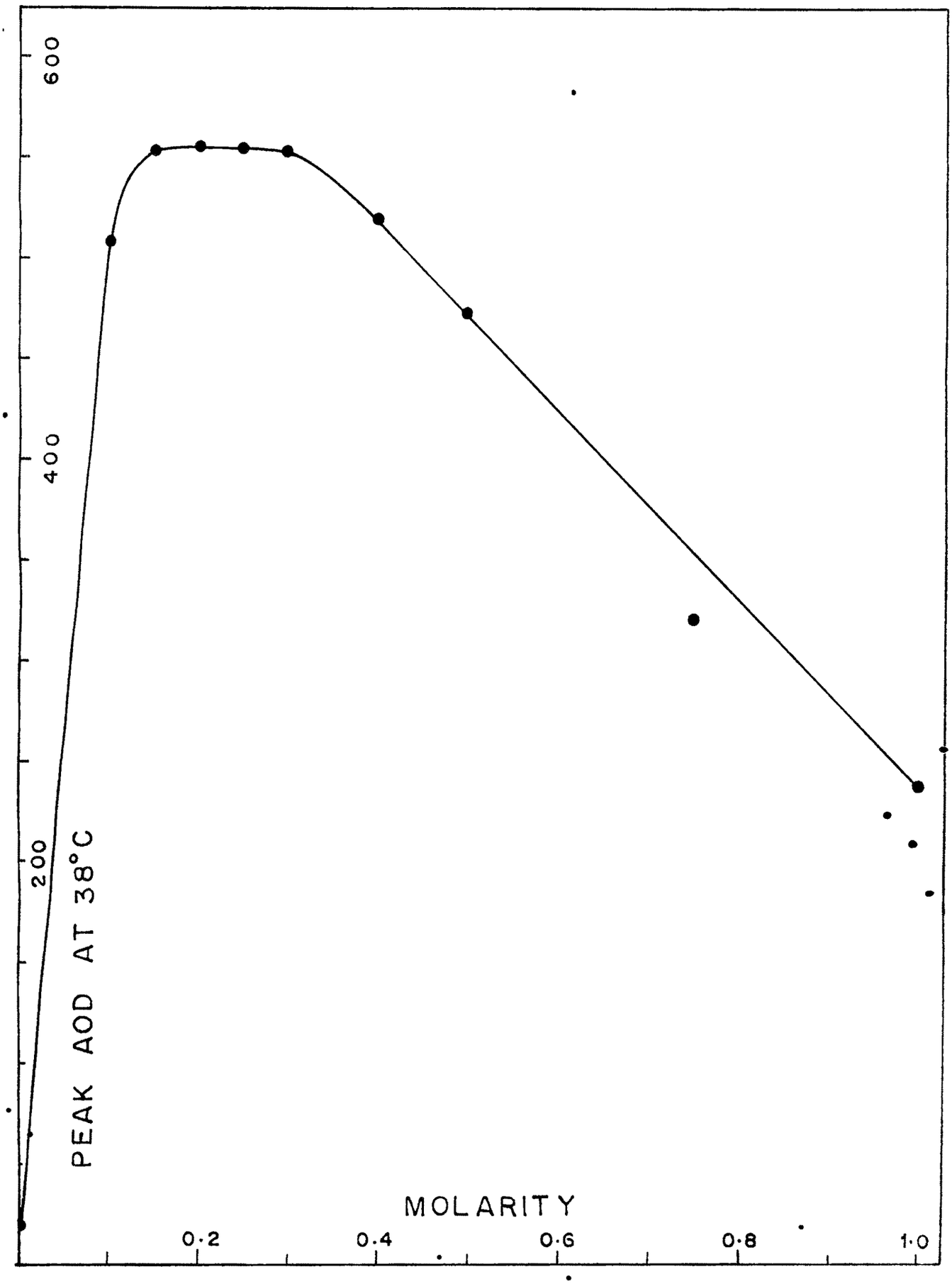

FIG. 1·2

peak-growth and slower lysis rate at lower molarity was presumably due to higher concentration of free H^+ ions (7) as reported by Shockman et al (93). Lower peak-growth and slower rate of lysis at higher molarity of buffer could be due to increased ionic concentration of the medium.

Table 1.3 shows the results of our studies relating to the growth capacity of high-pantothenate (400 mug/ml) S. faecalis R 8043 in a medium free of pantothenate but otherwise complete (Fig. 1.3). It appears from the data of Table 1.3 that with harvesting levels upto about 1000 AOD secondary growth responses were exponential and 9 - 12 times the initial AOD levels.

As there was no exogenous pantothenate in the medium, growth response in this medium very probably was due to the storage pantothenate and/or pantothenate containing factors like CoA, ACP and etc.

Table 1.4 shows that the results of our studies relating to the growth capacity of low-pantothenate (20 mug/ml) pregrown S. faecalis cells in the medium free of pantothenate but otherwise complete. It appears from the data of Table 1.4 that the secondary peak-growths of low-pantothenate pregrown cells (Fig. 1.4) were remarkably lower than those obtained with high-pantothenate pregrown cells (Table 1.3 and Fig. 1.3). With the higher harvesting AOD levels of low-pantothenate pregrown culture, (Table 1.4 and Fig. 1.4) the storage of pantothenate containing factors seemed to be decreasing. This was evident from obtention of their lower peak-growths in the pantothenate-free secondary medium.

It is further to be noted that the generation rate of the seco-

Table 1.3 *

Secondary growth** response of Streptococcus faecalis R ATCC NO. 8043 in a complete medium containing no exogenous pantothenate at 38° C.

Harvesting AOD from primary culture	Initial AOD in secondary medium	Peak-growth*** (in secondary medium)	Peak AOD / Initial AOD (approx.)
125	37	440	12
250	44	520	12
480	46	450	10
900	37	246	9

* Computed from the data obtained in the growth experiment (Fig. 1.3).

** Primary culture was grown in finished complete medium containing 400 mμg/ml pantothenate and harvested at various AOD levels and washed two times (at 2° - 5° C) with the medium free of pantothenate but otherwise complete and appropriately diluted with the same medium to adjust the AOD levels to around 40.

*** After peak-growth lysis followed gradually.

Table 1.4 *

Secondary growth** response of Streptococcus faecalis R ATCC NO. 8043 in a complete medium containing no exogenous pantothenate at 38° C.

Harvesting AOD from Primary culture	Initial AOD in secondary medium	Peak-growth*** (in secondary medium)	Peak-growth / Initial AOD
72	50	205	4.1
120	40	180	4.5
260	51	162	3.2
510	35	91	2.6

* Computed from data obtained in the growth experiment (Fig. 1.4)

** Primary culture was grown in finished complete medium containing 20 mµg/ml pantothenate and harvested at various AOD levels and washed two times (at 2° - 5° C) with the medium free of pantothenate but otherwise complete and appropriately diluted with the same medium to adjust the AOD levels to around 40.

*** After peak-growth lysis followed gradually.

LEGEND OF FIG. 1.3

Capacity of S. faecalis R ATCC NO. 8043 cells pregrown on hig pantothenate (400 mμg/ml) level to continue growth in absence of pan othenate. Conditions of the experiment have been stated under table Numbers on the growth curves denote generation times. Generation tim of primary culture was 51 min. The cultures were run simultaneously, but for clarity the curves have been separated by spaces correspondi to 50 min. on the time scale.

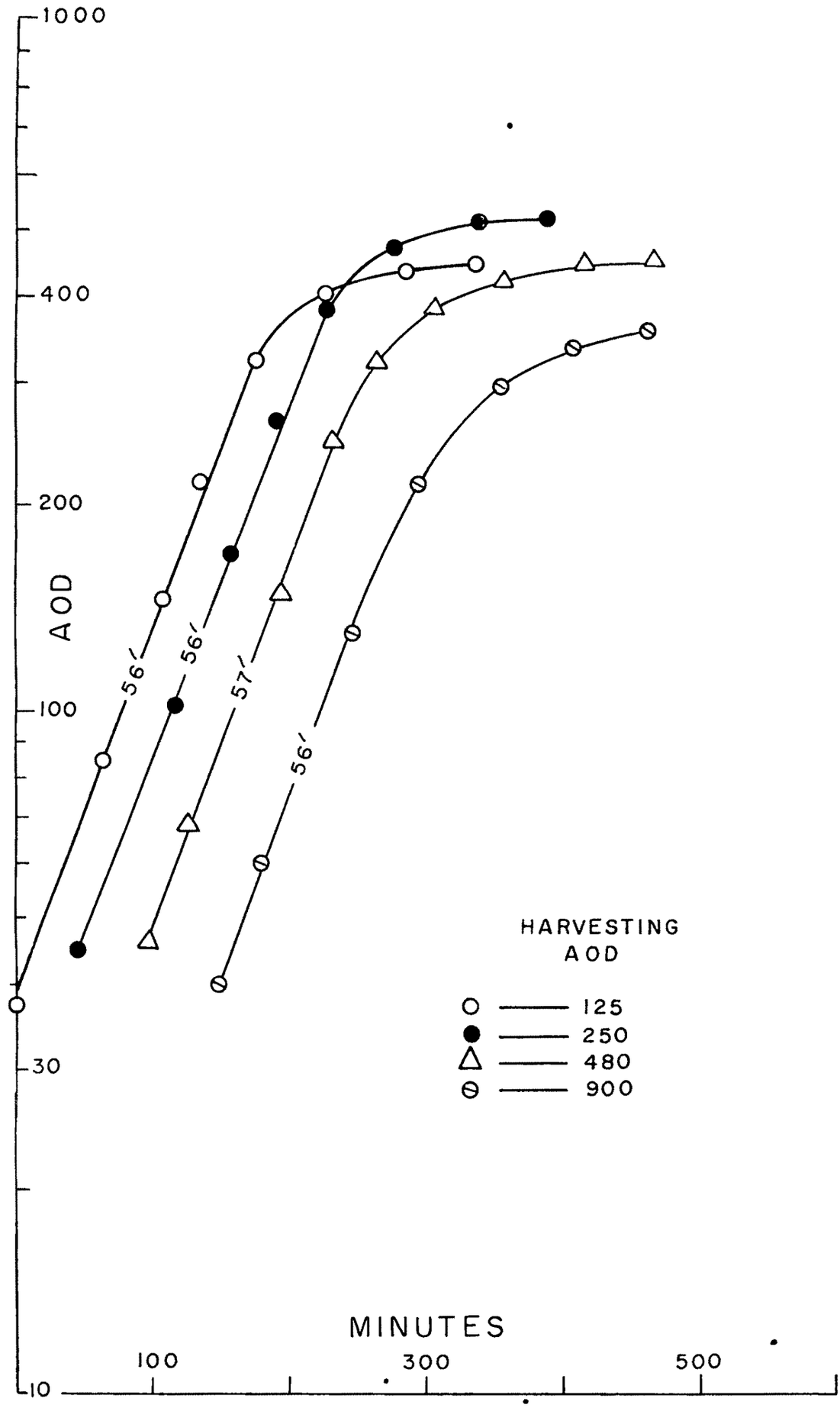
1000
400
200
100
30
10
AOD
56′
56′
57′
56′
HARVESTING
AOD
125
250
480
900
MINUTES
100
300
500

FIG. 1·3

LEGEND OF FIG. 1.4

Capacity of *S. faecalis* R ATCC NO. 8043 cells pregrown on lo pantothenate (20 mμg/ml) level to continue growth in absence of pan thenate. Conditions of the experiment have been stated under Table Numbers on the growth curves denote generation times. Generation ti of the inoculum culture was 55 min. The cultures were run simultane but for clarity the curves have been separated by spaces correspond to 50 min. on the time scale.

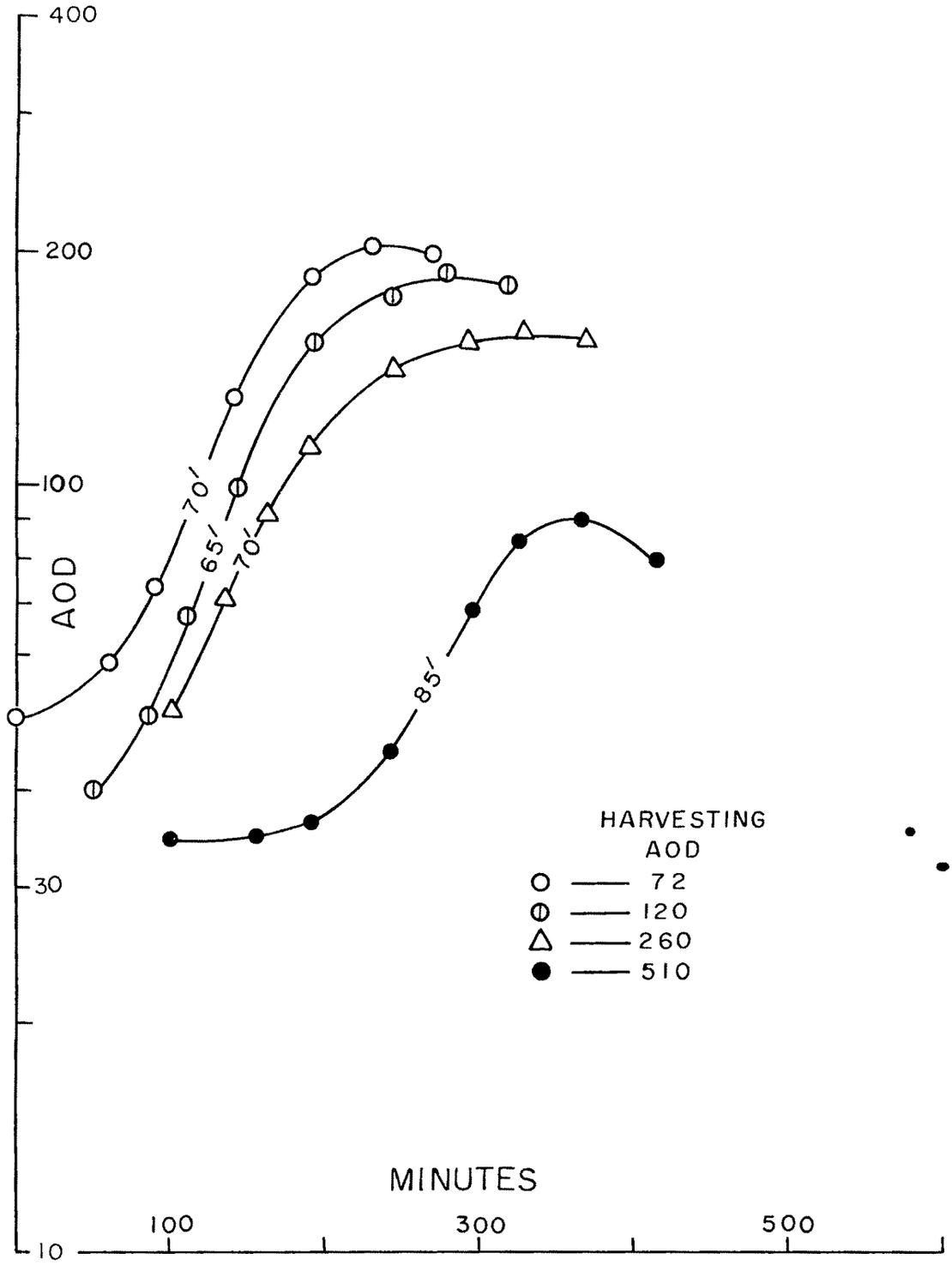
400
200
100
30
10
AOD
70'
65'
70'
85'
HARVESTING
AOD
72
120
260
510
MINUTES
100
300
500

FIG. 1-4

ndary cultures (obtained from primary cultures grown either in high- or low- pantothenate containing medium) was significantly slower (10-28 percent) than that obtained with the corresponding primary cultures (see foot notes under Table1.3 and Table1.4). This could be due to rapid depletion of some pantothenate containing factors like CoA, needed for the synthesis of cellular integumental structures. Intracellular conversion of CoA to ACP during growth in pantothenate-free medium in this strain as in S. faecalis 9790 (12,49) was quite likely.

1.3 SUMMARY

Studies were done in this chapter with Streptococcus faecalis R ATCC NO. 8043.

(i) S. faecalis R 8043 could not utilise β-alanine, DL-pantolactone or their mixture to replace its need for pantothenate. Pantothenate was essentially required for its growth.

(ii) Peak-growth was a function of the pantothenate concentration in the medium. Low concentration of pantothenate yielded normal exponential cells which were all prone to lysis after attainment of peak-growth.

(iii) Rate of growth was not affected by molarity of the phosphate buffer of the medium. Maximum peak-growth was attained between 0.15 and 0.30 M buffer. Lysis followed after peak-growth and its rate was maximum with 0.30 molar buffer. Lysis rates were slower both with higher and lower concentrations of the buffer.

(iv) (a) High- and low- pantothenate pregrown (primary culture) exponential cells of S. faecalis R 8043 gave significant growth response in a medium free of pantothenate but otherwise complete. (b) Secondary responses (peak-growths) in the pantothenate-free medium were dependent on the pantothenate levels in primary culture medium. In case of low-pantothenate grown primary culture secondary growth response depended on the harvesting AOD levels of the cultures. Secondary peak-growths of high-pantothenate pregrown cells were found to be higher than those obtained with low-pantothenate pregrown cells. (c) Generation time of high-pantothenate pregrown cells in the pantothenate free medium was about 10 percent higher (i.e., grew little slowly) than that obtained in the medium containing pantothenate. Generation time of low-pantothenate pregrown cultures in the pantothenate-free medium was about 28 percent higher (i.e., grew significantly slowly) than that obtained in medium containing low-pantothenate level (20 mμg/ml).