J. Natn. Sci. Coun. Sri Lanka 1982 **10** (2) : 169—180

Sucrose Phosphorylase and Invertase Activities in Bacteria

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(*Date of receipt* : 31 *March* 1982)
(*Date of acceptance* : 13 *July* 1982)

Abstract: *Leuconostoc* species from coconut toddy and from cabbage, *Acetobacter xylinum* and a *Klebsiella* species were grown in a sucrose-phosphate medium. The *Klebsiella* sp. had the fastest rate of growth followed by *Leuconostoc* from cabbage, *Acetobacter xylinum* and *Leuconostoc* from toddy. The time taken by them to reach the late log phase was 9 - 10 h, 12 - 14 h, 24 - 30 h and 60 - 72 h respectively. The invertase from all four species was found in the 20,000g supernatant. The activity of invertase in *A. xylinum*, *Leuconostoc* (toddy), *Klebsiella* and *Leuconostoc* (cabbage) was 0.66, 8.06, 45.0 and 77.7 units per mg protein respectively. Invertase was inactivated when the crude enzyme preparation was stored at 0°C in half saturated neutral ammonium sulphate solution for 2 to 3 days. The invertase of *Klebsiella* and that of *Leuconostoc* sp from cabbage were inhibited by 5×10^{-2} M EDTA to the extent of 85% and 67% respectively suggesting that it may be a metallo-enzyme. The growth rate of the *Klebsiella* sp. was decreased by two-fold when 5×10^{-2} M EDTA was present in the culture medium. Sucrose phosphorylase activity was detected in the 20,000g supernatant of *A.xylinum*. This enzyme could not be detected in the other three bacteria even after the inhibition of invertase and phosphatase activities.

1. Introduction

The synthesis of sucrose in plants is catalysed by the enzyme sucrose synthetase. The energy required for this process is obtained by the plants from sunlight. The synthesis of sucrose in the laboratory without the investment of energy has been approached in two ways. The original approach utilized the reversal of the invertase reaction. The recent method called the "phosphate process" uses the reversal of the sucrose phosphorylase reaction.[3] The energized glucose-1-phosphate required for this process is obtained by the action of starch phosphorylase on starch in which the energy of the glucosidic bond in starch is conserved in glucose-1-phosphate. The overall reactions are shown in Figure 1.

Of the two sucrose hydrolyzing enzymes, invertases are widely distributed among the microorganisms. Much work has been done on yeast and mould invertases[9] but very little attention has been paid to bacterial invertase.

Unlike invertase, sucrose phosphorylase is not widely distributed among the microoganisms. Upto date it has been found only in *Pseudomonas saccharophila*,[5] *Pseudomonas putrefaciens* [6] and *Leuconostoc mesenteroides*.[8] The preparations of sucrose phosphorylase from *P. putrefaciens* are not stable. Therefore most of the research has been done with sucrose phosphorylase of *P. saccharophila* and *L. mesenteroides*.

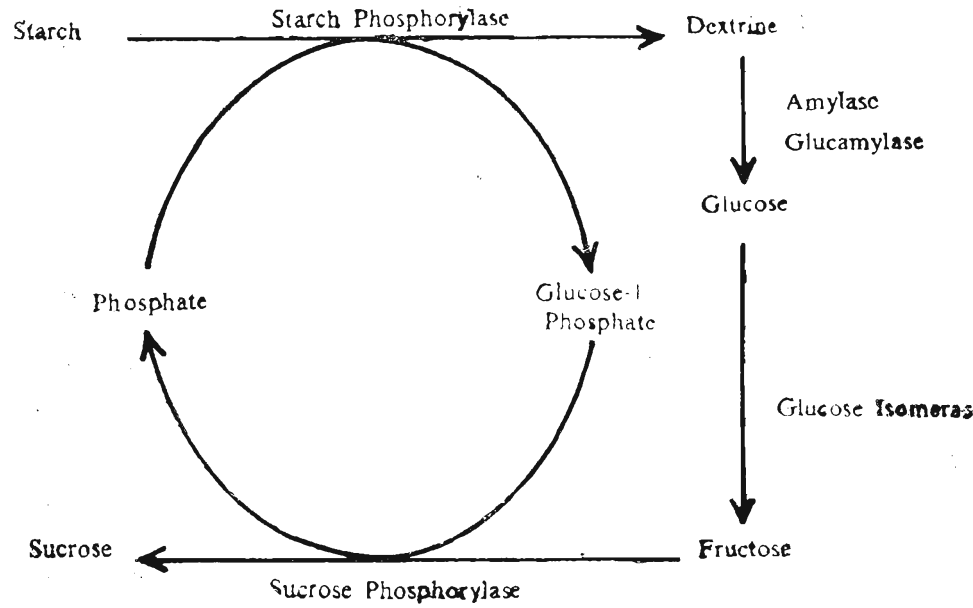

Figure 1. The synthesis of sucrose from starch by the enzyme catalysed phosphate process.

In this investigation a search for sucrose phosphorylase was made by studying the sucrose hydrolyzing enzymes of four locally found sucrose utilizing bacteria. The bacteria used were two species of *Leuconostoc*, *Klebsiella* sp. and *Acetobacter xylinum*.

2. Experimental

2.1 Identification and isolation of bacteria.

The species of *Klebsiella* was obtained from the department of Microbiology, Faculty of Medicine, University of Colombo and the *Acetobacter xylinum* from the Industrial Development Board, Moratuwa. The species of *Leuconostoc* were isolated from fresh toddy (pH 5.9) and from the outer leaves of cabbage. These two *Leuconostoc* species were identified by their physical appearance.[4]

2.2 Culture medium

The culture medium [12] contained 0.033 M KH_2PO_4 - Na_2HPO_4 buffer (pH 6.5),0.1% NH_4Cl, 0.05% $MgSO_4$, 0.005% Ferric ammonium citrate, 0.001% $CaCl_2$, 0.5% sucrose and distilled water. This medium was autoclaved at 15 pounds pressure for 10 min.

2.3 Growth of bacteria

All species were grown under aerobic conditions at room temperature (27° - 30° C). *Klebsiella* needed vigorous aeration. *A. xylinum* was grown at pH 5.5 while the other three species grew best at pH 6.5. In all cases, the inoculum constituted 10% (V/V) of the culture medium and cultures in log phase were used for inoculation.

2.4 Measurement of bacterial growth

Bacterial growth was measured by the "Dilution plating technique" or by measuring the turbidity in a Klett-Summerson colorimeter (filter No. 52).[12]

2.5 Harvesting of bacteria and the preparation of the crude enzyme extract.

Bacteria were harvested during the late log phase of growth. The crude extract was prepared at 4° C according to the flow chart in Figure 2.

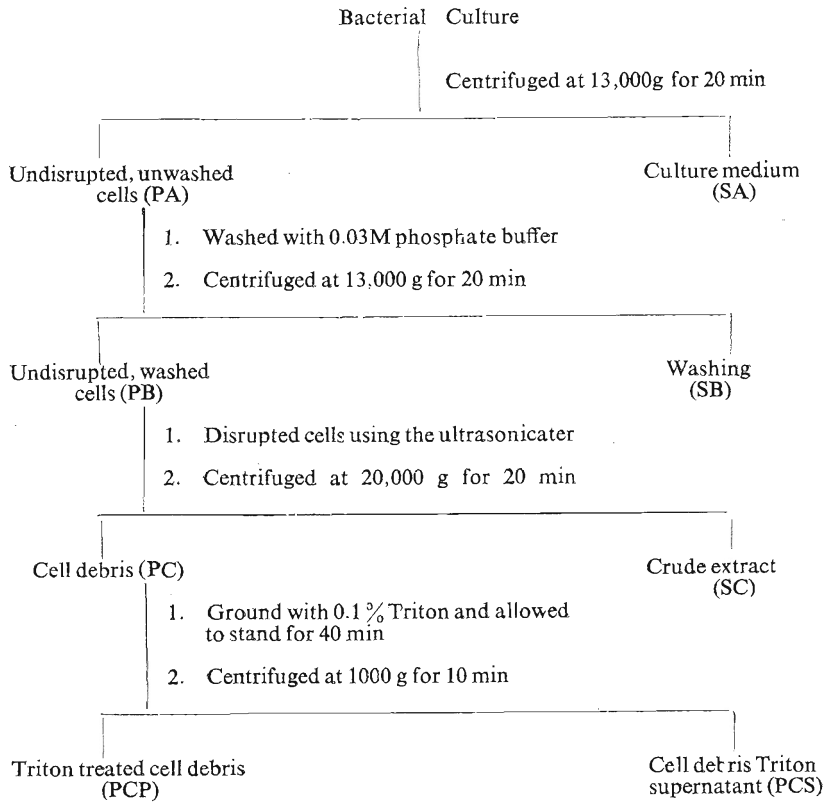

Figure 2. The preparation of crude enzyme extract from bacterial cells

2.6 Assay for enzymes

2.6.1 *Invertase*

Invertase activity was measured by incubating appropriate amounts of enzyme in 0.033M phosphate buffer (pH 6.7) containing 0.2M sucrose at 37°C for 1h. The total volume of the incubation mixture was 2.0 ml. The reducing sugars formed were measured using the DNS (dinitrosalicylic acid) reagent at 540nm.[7] A unit of invertase activity is defined as the amount of enzyme which releases 1 μ mole of reducing sugar per hour at 37°C under the given experimental conditions.

2.6.2 *Sucrose Phosphorylase*

Sucrose phosphorylase was assayed by measuring the rate of reduction of NADP in a coupled system consisting of sucrose phosphorylase, phosphoglucomutase and glucose-6-phosphate dehydrogenase.[12] The assay mixture contained potassium phosphate buffer pH 7.0 (0.033M), sucrose (0.08M), $MgSO_4$ (6×10^{-4}M), NADP (5×10^{-4}M), phosphoglucomutase (90 μl containing 50 μg), glucose - 6-phosphate dehydrogenase (90 μl containing 5 μg) and the test solution (enzyme extract). The total volume of the reaction mixture was 3ml. The increase in optical density was measured at 340 nm using a Pye-Unicam, SP 800 recording Spectrophotometer.

2.7 Estimation of protein

Protein was estimated by the method of Lowry et al.[10]

3. Results and Discussion

3.1 Identification of bacteria

Both the toddy bacterium and the cabbage bacterium formed a film of polysaccharide on the surface of the culture medium after two days of incubation. This biochemical criterion was used to identify them as *Leuconostoc*. They also had the physical appearances assigned to *Leuconostoc*. The colonies of toddy and cabbage species were light brown and white in colour respectively, but they were both gummy in nature.

3.2 Measurement of bacterial growth

The growth curves for the four bacteria are shown in Figure 3. *Klebsiella* reached the late log phase of growth in 9-10h of incubation. The species of *Leuconostoc* (cabbage) and *A. xylinum* reached the late log phase in 10-12 h and 24-30 h respectively. *Lecconostoc* sp. (toddy) had the slowest growth rate and reached the late log phase in 60-72 h.

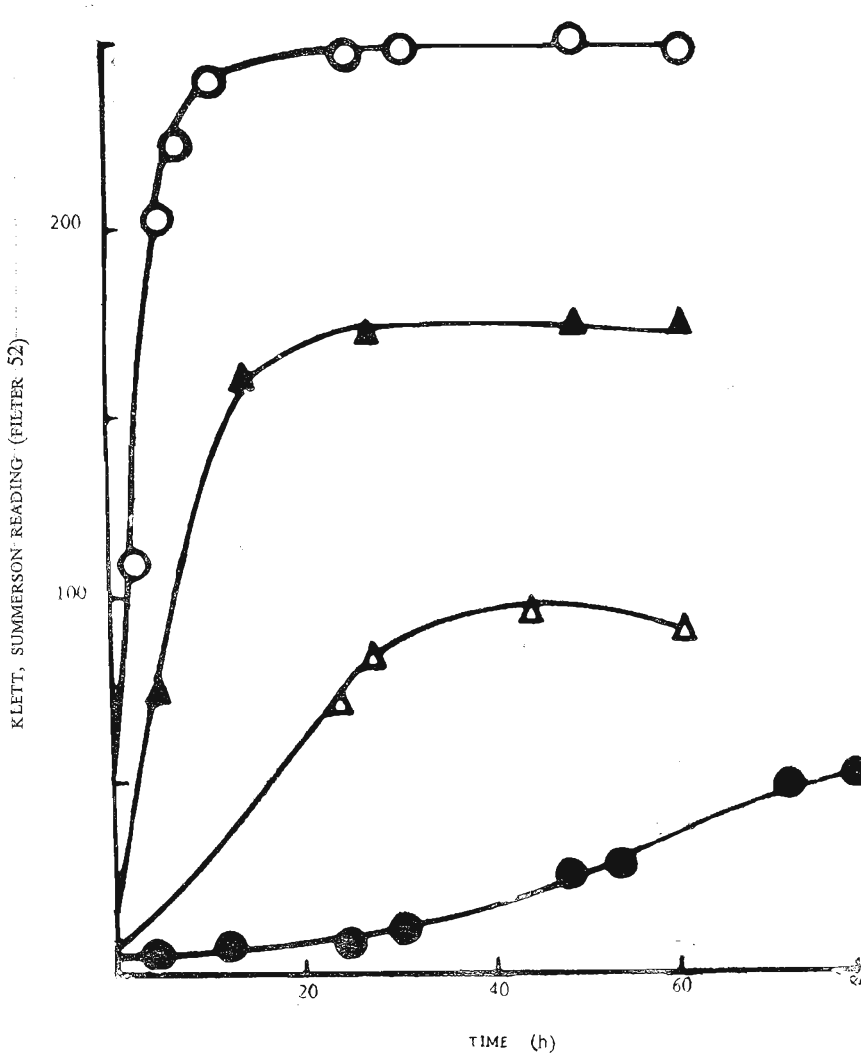

Figure 3. Growth curves of bacteria. ○—○, *Klebsiella sp.*; ▲—▲, *Leuconostoc sp.* (Cabbage): Δ—Δ, *Acetobacter xylinum*: ● — ●, *Leuconostoc sp.* (toddy)

3.3 Enzyme activity

The culture medium (SA), crude extract (SC), and the cell debris triton supernatant (PCS) were assayed for invertase and sucrose phosphorylase as described in 2.6.1 and 2.6.2 respectivly. Invertase activity was found in the crude extract of all four bacteria (Table 1)and not in the culture medium and in the cell debris triton supernatant. This shows that the invertase in all four bacteria is not membrane bound and it is found in the soluble fraction after cell disruption. Furthermore, as in the case of yeast[9], the invertase in these bacteria is not released into the culture medium.

TABLE 1. Measurement of Invertase Activity

Type of bacteria	Sample	Total volume (ml)	Protein (mg/ml)	Enzyme activity units (μ moles/hr)	Specific activity (units/mg Protein)	Total activity (units)
Klebsiella	SA	500	0	0	0	0
	SC	25	0.16	7.2	45.0	180.0
	PCS	10	0.26	0	0	0
Leuconostoc	SA	500	0	0	0	0
(Cabbage)	SC	80	0.09	7.0	77.7	560
	PCS	20	0.53	0	0	0
Leuconostoc	SA	500	0	0	0	0
(Toddy)	SC	20	0.61	4.92	8.06	98.4
	PCS	20	0.49	0	0	0
Acetobacter	SA	500	0	0	0	0
xylinum	SC	25	0.30	0.20	0.66	5.0
	PCS	20	0.25	0	0	0

SA, culture medium; SC, crude extract; PCS, cell debris triton supernatant.

The specific activities of invertase in *Acetobacter xylinum*, in the *Leuconostoc* from toddy, in the *Klebsiella* and in the *Leuconostoc* from cabbage are 0.66, 8.06, 45.0 and 77.7 units /mg protein respectively. Specific activity of invertase appears not to be related to the growth rate of the bacteria.

Sucrose phosphorylase activity was seen only in the soluble fraction of *Acetobacter xylinum*. The little increase in absorbance at 340 nm observed in the control (Figure 4) is probably due to the presence of small amounts of endogenous substrates.

Phosphorolysis of sucrose by sucrose phosphorylase conserves energy. Although having this enzyme appears to be advantageous for an organism, evolution appears to have selected the non-energy conserving pathway for sucrose utilization using invertase. This is evident from our results in that all the four bacteria have invertase while only one of them (*A. xylinum*) has sucrose phosphorylase.

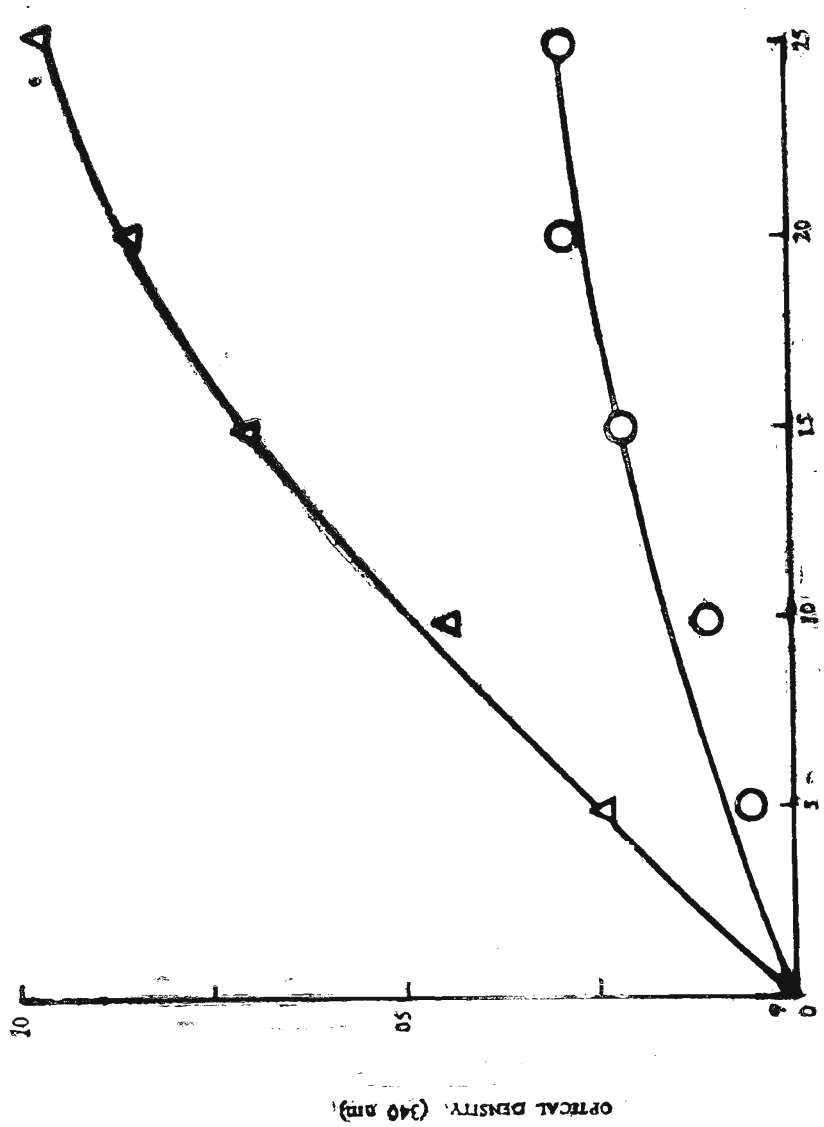

Figure 4. Sucrose phosphorylase activity of the crude extract of *A. xylinum*, Δ—Δ, Test; ○—○, Control.

3.4. Inhibition of invertase activity

3.4.1 *Effect of EDTA on invertase activity*

Figure 5 shows the effect of EDTA on invertase activity of the *Klebsiella* sp. and of the *Leuconostoc* sp. from cabbage. 5x10 $^{-2}$M EDTA inhibited the invertase activity of *Klebsiella* and *Leuconostoc* to the extent of 85% and 67% respectively.

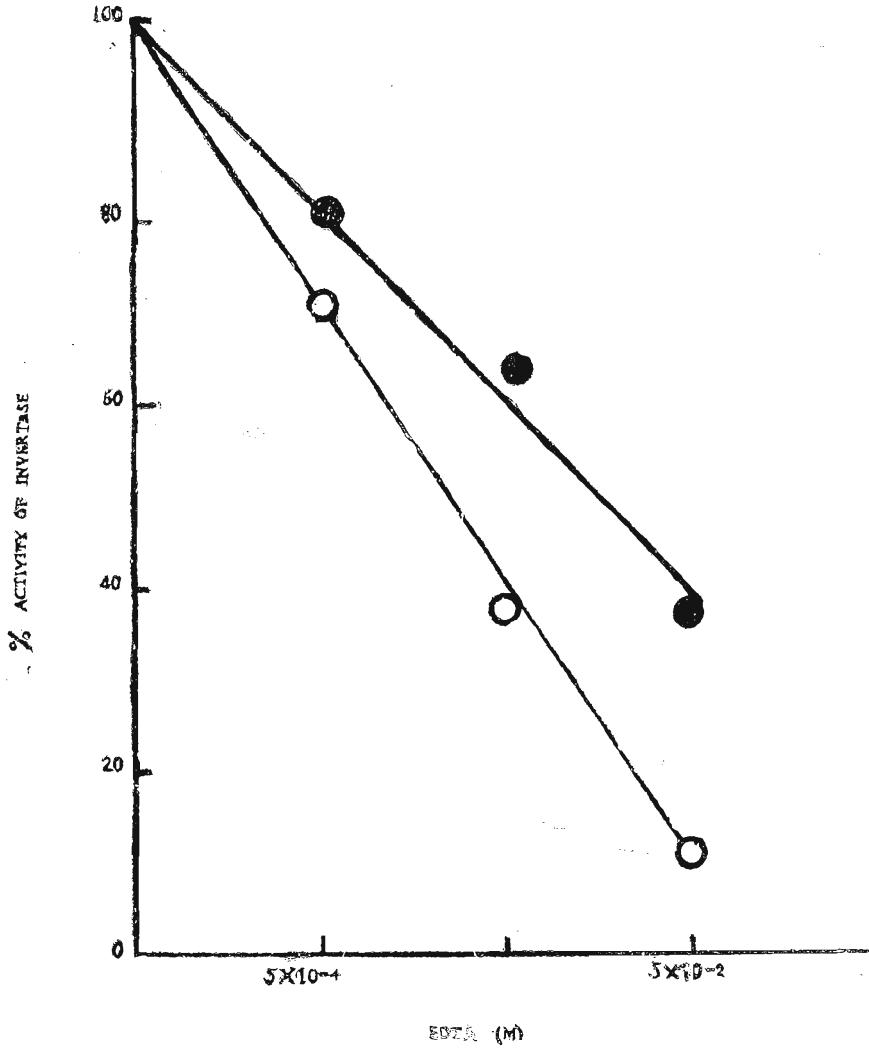

Figure 5. Effect of EDTA on invertase activity.
●—●, *Leuconostoc sp.*; ○—○, *Klebsiella sp.*

This inhibition by EDTA indicates that invertase of these bacteria might require a metal ion for its catalytic activity. Thus it is possible that bacterial invertase is a metallo enzyme. Confirmation of this observation will be of considerable interest. The other invertase that has been shown to require metal ion for its catalytic activity has been the coconut invertase.[1]

3.4.2 *Effect of* $(NH_4)_2 SO_4$ *on invertase activity*

The crude extract of *Klebsiella* sp. of specific activity 7.3 units/mg protein was stored in half saturated $(NH_4)_2 SO_4$ [12] overnight at 4°C and then centrifuged at 20,000 g for 20 mins.

Invertase activity was not observed either in the 20,000 g supernatant or in its pellet fraction indicating that half saturated ammonium sulphate inhibits invertase activity under these storage conditions (Table 2).

TABLE 2. Effect of Ammonium Sulphate on Invertase Activity.

Ammonium sulphate (Concentration)	Optical density (540 nm)	Invertase activity (μ moles/h)
0.0	615	7.3
50%	0	0

3.5. Measurement of phosphorylase activity in the presence of inhibitors for invertase and phosphatase.

As sucrose phosphorylase and invertase compete for the same substrate, attempts were made to detect the sucrose phosphorylase that may be present in very small amounts by inhibiting the invertase using EDTA and half saturated ammonium sulphate.

Experiments were also designed to inhibit the phosphatase that may hydrolyse the glucose-1-phosphate formed, which forms the substrate for phosphoglucomutase in the assay system of sucrose phosphorylase.

The phosphatase inhibitors used were 0.01M NaF, 0.025M tartrate and 0.5% formaldehyde.[2,11,13]

The assay mixture for sucrose phosphorylase was as described in section 2.6.2, but it contained in addition EDTA (0.05M), NaF (0.01M), tartrate (0.025M) and formaldehyde (0.5%).

No sucrose phosphorylase activity could be detected in the two species of *Leuconostoc* from cabbage and toddy and the *Klebsiella sp.*

3.6 Effect of EDTA on bacterial growth.

Since sucrose phosphorylase is an 'adaptive' enzyme, an experiment was performed to induce sucrose phosphorylase in the *Klebsiella* sp. by growing them in a sucrose-phosphate medium containing EDTA, the inhibitor of invertase.

Figure 6 shows the effect of EDTA on the growth of *Klebsiella.* The growth rate of bacteria decreased by twofold when $5x10^{-2}$ M EDTA was present in the culture medium. The extract failed to show any sucrose phosphorylase activity indicating that the sucrose, even in the presence of EDTA, did not induce the synthesis of sucrose phosphorylase in this *Klebsiella* species.

4. Conclusion

These studies show that the invertase activity is not directly related to their growth rates in the three species of bacteria namely the *Klebsiella* sp. and the two species of *Leuconostoc.* However *Acetobacter xylinum* showed a good growth in the sucrose-phosphate medium at pH 5.5 but had very low invertase activity. This suggests the involvement of an enzyme other than invertase in the breakdown of sucrose in this bacterium. Studies have shown this additional enzyme to be sucrose phosphorylase.

Studies on sucrose phosphorylase from *Pseudomonas sacchrophila*[12] have shown the enzyme to be found in the soluble fraction of the cell. The molecular weight as determined by Sephadex gel filtration was 80,000 to 100,000 while that determined by SDS-gel electrophoresis was 50,000.[12] This suggests that the enzyme is composed of two identical subunits. The optimum pH for the phosphorylysis of sucrose has been shown to be 7.0.[14]

The sucrose phosphorylase from *Acetobacter xylinum* is found in the soluble fraction of the cell as it is in *Pseudomonas saccharophila.*[14] The optimum pH for the growth of *A. xylinum* is 5.5.

Work on the purification and kinetic studies of sucrose phosphorylase is in progress. It appears that invertase is the most common enzyme for sucrose hydrolysis in bacteria and sucrose phosphorylase is present only in certain bacteria in which the invertase is absent or present in very low levels.

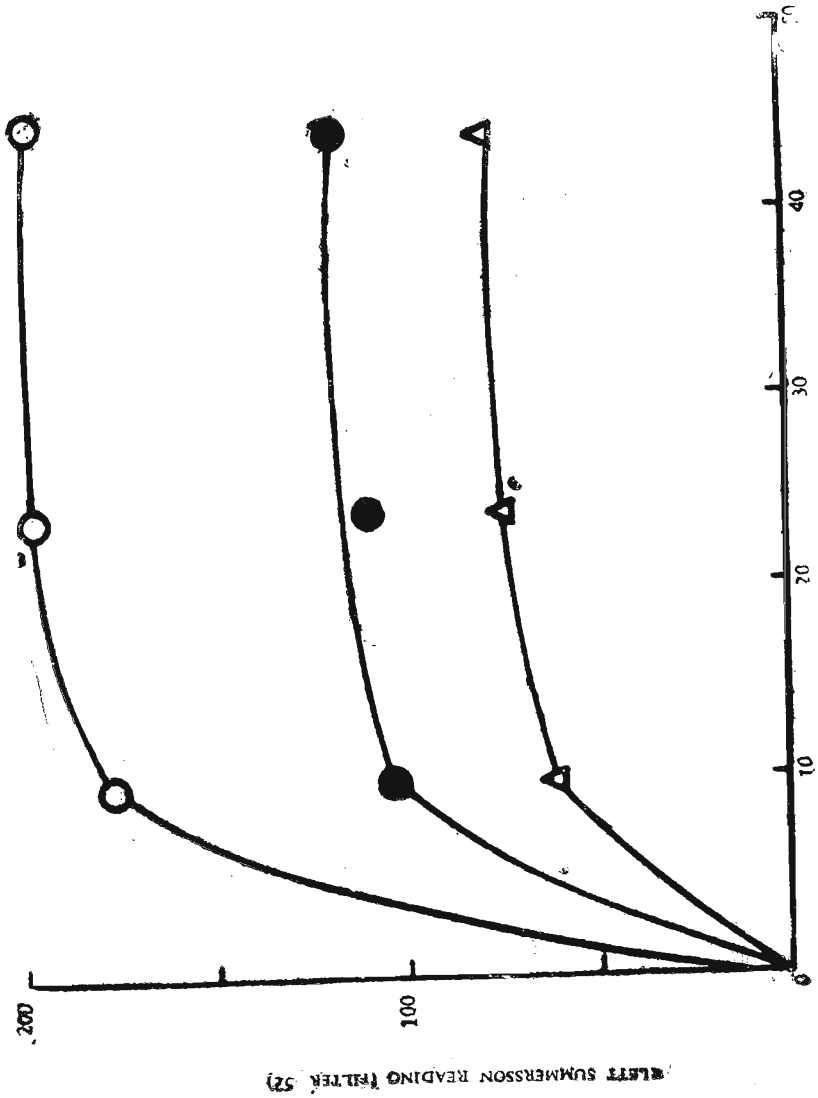

Figure 6. Effect of EDTA on growth of *Klebsiella sp.* ○—○, no EDTA; ●—●, 5×10^{-4} M EDTA; Δ—Δ, 5×10^{-2} M EDTA.

Acknowledgements

We thank the Natural Resources Energy and Science Authority of Sri Lanka for providing the grant for this study and the University of Colombo, Sri Lanka for providing the facilities. Our thanks to Dr. (Mrs.) M. C. P. Canagaratna for the helpful discussion and the Department of Microbiology and Industrial Development Board, for providing us with a sample of *Klebsiella* sp. and *Acetobacter xylinum* respectively. The technical assistance of Messrs O. D. H. R. Weerasena and R. D. Sothary is gratefully acknowledged.

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