CHAPTER-III

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Favourable chemical changes brought about by micro-organisms during fermentation of legume based foods are extensively discussed earlier while reviewing fermentation. The most important biochemical changes during fermentation are breakdown of complex nutrients into simpler digestible forms, increase in some vitamins content along with the degradation of some anti-nutritional compounds and toxins. Experiments carried out with fermented foods of India viz. 'khaman' and 'idli' suggested their greater digestibility as compared to unfermented foods (Rajalakshmi and Vanaja, 1967). Earlier studies carried out in the laboratory showed that 'soyidli' was found to be well accepted and tolerated by severely malnourished children (Ramakrishnan, 1977). Bacteriological studies carried out showed that Leuconostoc mesenteroides, Lactobacillus fermenti, Lactobacillus delbrueckii and Bacillus Sp. play a dominant role in fermentation of some Indian fermented foods (Ramakrishnan et al, 1976). Presence of hemagglutinin degrading bacteria L. mesenteroides (HA) from fermented 'soyidli' batter was also reported by Ramakrishnan and Rao (1977).

As mentioned earlier, L. sativus is a hardy crop whose consumption is still not stopped by the poor people. Its nutritive value can be compared with other legumes, but it contains neurotoxin and other antinutritional compounds viz. trypsin inhibitor, phytate and flatulence producing oligosaccharides.

Preliminary studies carried out in this laboratory showed that fermentation of L. sativus dhal decreased raffinose family of oligosaccharides completely and neurotoxin to some extent. Therefore the present experiments were aimed to study the mode of breakdown of oligosaccharides and neurotoxin (ODAP) by bacteria isolated from either stored grains or fermented L. sativus dhal.

The results of the following experiments are reported and discussed in the thesis.

Section-I : Preparation of a fermented product from L. sativus dhal and the microbial profile during the fermentation.

Section-II : Isolation and identification of bacteria, capable of degrading flatulence producing oligosaccharides and neurotoxin, from stored grains.

Section-III : Studies on bacterial degradation of flatulence producing oligosaccharides with special reference to production of α-galactosidase and invertase.

Section-IV : Studies on bacterial degradation of neurotoxin (ODAP).

Section-V : Studies on the genetic basis of degradation of toxins and antinutritional factors in bacterial isolates with special reference to raffinose degradation.

Section - I

Preparation of a fermented product from L. sativus dhal and microbial profile of the same during fermentation

As described under 'Materials and Methods', a fermented product similar to 'khaman' (a traditional fermented product of Gujarat, India prepared from Bengal gram dhal) was prepared using L. sativus dhal for studies on breakdown of flatulence producing oligosaccharides and neurotoxin (ODAP) during fermentation. The characteristics of this fermented product are described in Table-70. It was found that during fermentation, the pH decreased and volume of the batter increased which is a characteristic of other Indian fermented foods (Mukherjee et al, 1965; Rajalakshmi and Vanaja, 1967; Steinkraus et al, 1967; Akolkar, 1977). Decrease in pH was also observed in other foods involving acid fermentation viz. 'sauerkraut' (Pederson and Albury, 1969), 'soyidli' (Ramakrishnan, 1979), 'sour-dough breads' (Wood et al, 1975; Wood, 1977), Nigerian 'ogi' (Akinrele, 1970), 'kenkey' fermented maize dough balls of Ghana (Christian, 1966; Nyako, 1977), Nigerian 'gari' (Akinrele et al, 1975) etc. Regarding the acceptability, taste and texture of the product from L. sativus dhal are quite acceptable as shown in Table-70. When fermented L. sativus batter was investigated for the microbial population using selective media it was found that it is only due to bacteria and not by yeast or any other fungi.

TABLE-70 : Characteristics of a fermented product from L. sativus dhal.

	Physical changes in the batter*			Sensory acceptability mean score**		
	% increase in volume of batter	pH of the unfermented batter	pH of the fermented batter	Taste	Texture	Colour
1) 'Khaman' - a traditional fermented food prepared from Bengalgram dhal.	123 ± 5.0	6.6 ± 0.07	4.60 ± 0.08	3.0	3.0	3.0
2) Fermented product from L. sativus dhal.	118 ± 7.0	6.5 ± 0.05	4.5 ± 0.14	3.0	2.5	2.5

* Average of four trials ± S.E.

** Ten subjects formed a panel of judges; scores are average of individual scores.
3 = very good, 2 = good, 1 = acceptable, 0 = not acceptable.

Yeast has been reported to play a role in other Indian food fermentations (Lewis and Johar, 1953; Desikachar et al, 1960; Batra and Milner, 1974). But this was not found to be the case in the present studies on fermentation of L. sativus dhal. Total counts of bacteria isolated from unfermented and fermented L. sativus batter are given in Table-71, and the details of identification of the major bacteria are given in Table-72.

The change in total counts of bacteria during the soaking and fermentation are reported in Table-71 which shows that the bacterial count starts increasing during just 4 h soaking of the dhal, and the total counts increased from 10^4 to 10^9 during fermentation. The morphological, cultural and biochemical characteristics of the dominant bacteria present in fermented L. sativus batter showed that they were namely Leuconostoc mesenteroides, Lactobacillus sp. I, Lactobacillus fermenti, Pediococcus sp., Bacillus sp. III and Streptococcus sp. II as described in Table-72. It is interesting to note that some of these organisms were initially present in the batter in very small amount, but the concentrations increased after fermentation (Table-73). L. mesenteroides and L. fermenti were present in higher concentration in the fermented batter, compared to other bacteria as shown in Table-73. These observations suggest that the fermentation of L. sativus dhal was brought about by bacteria like other Indian fermented foods. L. mesenteroides showed highest increase in its content during fermentation in the present investigation. This organism is

TABLE-71 : Changes in bacterial counts during soaking and fermentation of L. sativus dhal.

	Total bacterial counts/g (a)
Control dhal	$5.9 \pm 0.30 \times 10^4$
Soaked dhal	$8.9 \pm 0.54 \times 10^5$
L. sativus dhal batter fermented for 8 h.	$20.75 \pm 1.2 \times 10^7$
L. sativus dhal batter fermented for 16 h.	$1.7 \pm 0.10 \times 10^9$

(a) Values are mean of four different observations ± S.E.

TABLE-73 : Change in bacterial population of L. sativus dhal batter during fermentation.

Bacterial isolate	Counts per g of batter(a)		% of total bacterial counts in batter	
	Unfermented (b) (counts x 10^3)	Fermented (counts x 10^7)	Unfermented	Fermente
Lactobacillus fermenti	170 ± 4.6	52.57 ± 6.4	19	29
Lactobacillus sp. I	78.3 ± 6.0	16.35 ± 1.4	9	9
Leuconostoc mesenteroides	133 ± 10	73.7 ± 4.3	15	40
Bacillus sp. II	236 ± 12	12.65 ± 1.2	26	7
Pediococcus sp.	10 ± 0.6	22.10 ± 1.2	1	12
Streptococcus sp. II	275 ± 12.8	4.5 ± 0.40	31	3

(a) Values are mean of four different observations ± S.E.

(b) Ingredients soaked in water and ground to get a batter.

also found to be a dominant one during fermentation of soyidli (Akolkar, 1977), 'sauerkraut' (Pederson and Albury, 1954,1956), Phillipine vegetable blends (Orillo et al, 1969), 'kenkey' fermented maize dough of Ghana (Christan, 1970), Nigerian 'palm wine' (Okafor, 1975) and Nigerian 'gari' (Okafor,1977). Leuconostoc mesenteroides is the essential micro-organism for leavening of the batter and is also responsible, along with Streptococcus faecalis for acid production during 'idli' fermentation (Mukherjee et al, 1965). L. fermenti which is present in fermented L. sativus batter was also found to increase free sugar, decrease the pH and increase the volume of the batter by production of gas, and increase niacin in soyidli fermentation (Akolkar, 1977). Lactic acid bacteria are also known to be involved in the fermentation of several types of foods and to be responsible for acidity, flavour and desirable fermentative changes (Allen and Allen, 1933; Akinrele, 1964; Mukherjee et al, 1965; Orillo et al, 1969). Lactobacilli are found to be one of the essential micro-organisms during fermentation of soymilk, soy-sauce, Japanese-sake, Maxican puluque, sauerkraut, soyidli, sourdough bread, Nigerian ogi, 'Eragrostistef'- an Ethiopian fermented food (Sanchez and Morroquin, 1953; 1970; Pederson and Albury, 1969; Kline and Sugihara et al, 1971; Banigo and Muller,1972; Mital et al, 1974; Kodama and Yoshizawa, 1977; Ramakrishnan et al, 1976; Gashe, 1985). Bacillus sp. are reported to play important role in fermentation of African oil bean 'ugba' (Odunfa and Oyeyiola, 1985), 'natto' fermentation (Hesseltine and Wang, 1969), 'soyidli' fermentation (Akolkar, 1977), 'ogiri' fermentation (Odunfa, 1981a) and 'iru' fermentation

(Odunfa, 1981b). Pediococcus sp. are reported to play fermentative role in fermented products of Thailand (Tanasupawat and Daengsubha, 1983), 'sauerkraut' (Pederson and Albury, 1969), and a traditional Japanese 'miso' (Wang and Hesseltine,1979). Thus it can be concluded from this experiment that during fermentation of L. sativus dhal, L. mesenteroides and L. fermenti were present as dominant microflora resembling other Indian fermented foods involving acid production. This fermented product prepared from L. sativus dhal was analysed further for its oligosaccharides and neurotoxin content.

Section - II

Isolation and identification of bacteria, capable of degrading flatulence producing oligosaccharides and neurotoxin, from stored grains.

Earlier studies carried out in this laboratory (Ramakrishnan, 1979) showed that the bacteria involved in the fermentation may come from the grains used for fermentation and that the bacterial flora of different grains are different. Thus it appears that there may be microflora in stored grains which have the capacity to degrade toxins and antinutritional factors present in food grains. Since the major aim of the present investigations was to study the mode of breakdown of neurotoxin (ODAP) and flatulence producing oligosaccharides by bacteria, *in vivo* and *in vitro*, the experiment was carried out to isolate and characterize bacteria from stored grains which can hydrolyse these compounds.

The major grains like rice, bengal gram, black gram, soybean etc. used for preparation of legume based fermented foods were used for the isolation of bacteria as described under 'Materials and Methods'. Using a basal salt medium containing either raffinose as sole carbon source or neurotoxin (ODAP) as carbon and nitrogen source, it was possible to isolate the

bacteria capable of hydrolysing neurotoxin or flatulence producing oligosaccharides. The details regarding the method of isolation have already been described under 'Materials and Methods'. The morphological, cultural and biochemical characteristics of these bacterial isolates are given in Table-74. Using these tests they were characterized as Bacillus Sp. I (soybean grains), Bacillus sp. II (L. sativus seeds), Streptococcus sp. I (L. sativus seeds), Klebsiella aerogenes (rice and bajra grains), Spirillum sp. (Bengal gram grains) and Lactobacillus sp. II (black-gram grains). These microorganisms which are isolated from the stored grains used for preparation of fermented foods, have been reported during fermentation of several legume based fermented foods (Mukherjee et al, 1965. Pederson and Albury, 1969; Mital et al, 1974; Salinas and Herrera, 1974; Odunfa, 1981a,b).

The above bacterial isolates were screened further for their efficiency to degrade flatulence producing oligosaccharides and neurotoxin.

Section - III

Studies on bacterial degradation of flatulence producing oligosaccharides with special reference to production of α-galactosidase and invertase.

Results on standardization of methods for quantitative and qualitative analysis of raffinose family of oligosaccharides, effect of different food processing methods on oligosaccharide content of L. sativus seeds/dhal, capacity of bacterial isolates to degrade oligosaccharides, metabolism of raffinose in a bacterial isolate and studies on purification and characterization of enzymes for raffinose degradation from Bacillus sp. I are presented and discussed here.

Standardization of methods for quantitative and qualitative determination of raffinose family of oligosaccharides and their metabolites :

During the course of studies on bacterial degradation of oligosaccharides, it was necessary to standardize a simple method for the separation and estimation of flatus producing oligosaccharides from L. sativus samples as well as culture filtrates during the growth of bacteria.

A large number of methods have been reported for the separation and quantitation of raffinose family of oligosaccharides. Cerning and Filiatre (1976) have employed colorimetric quantitation of the oligosaccharides after separation by gel filtration. Other procedures principally employed for determination of these sugars are paper chromatography (French and Wild, 1953; Kawamura, 1967; Champagnol and Bourzeix, 1971), carboncolumn chromatography (Kawamura and Tada, 1967), centrifugal chromatography (Kawamura et al, 1967), thin layer chromatography (Tanaka et al, 1975), liquid chromatography (Hymowitz et al, 1972; Kim et al, 1973; Cegla and Bell, 1977; Robert Macrae and Ahmad Zand-Moghaddran, 1978; Conkerton et al, 1983) and gas chromatography (Sweely et al, 1963; Delente and Ladenburg, 1972; Wankhede and Tharanathan, 1976).

The short comings of the liquid chromatographic procedures are that either the two main components, raffinose and stachyose are separated only in the top of their peaks or the separation of monosaccharides and disaccharides from sucrose is not complete (Conkerton et al, 1983). Consequently the decomposition products of oligosaccharides cannot be measured using these methods. The most efficient method for this purpose is gas chromatography (GC) if optimum derivatization and separation conditions are ensured. These requirements were not fulfilled by earlier reported 'GC' methods except by the recently described method of Perl et al.(1984). Therefore a suitable, rapid and sensitive GC method for separation and estimation of

oligosaccharides and their metabolites was standardized in the present investigation.

In the beginning of the present studies, gas chromatograph was not available in the laboratory, hence a simple and sensitive thin layer chromatographic (TLC) method was also standardized. Results of standardization of the TLC and GC methods for the separation and estimation of mono-, di- and oligosaccharides are described further.

Analysis of sugars by TLC method :

Several investigators have reported TLC methods for the separation of oligosaccharides and simple sugars from many biological samples including food samples (Iato et al, 1968; Hansen, 1975; Tanaka et al, 1975; Gauchi et al, 1979; Ghebregzabher et al, 1976). Using these methods either the the sugars can be separated well only at lower concentration (5-20 µg of each) in a mixture or precoated plates are required for good separation of sugar.

When the mixture of standard sugars were spotted at high concentrations using silica gel-G plates, and when the TLC was run according to the standard methods, the distinct separation of sugars was not achieved. Therefore the method of Stefanis and Ponte (1968) was modified in present studies by changing the concentration of solvent system, type of binder

and spraying reagent (details of the method are described in 'Materials and Methods'). When different types of salts were tried for impregnating gel, viz., acetate, phosphate, borate and bisulphite, it was found that 0.1M sodium phosphate bring about separation of all the oligosaccharides and disaccharides studied, viz., raffinose, stachyose, verbascose, melibiose and sucrose. The important influence of phosphate salts on silica gel-G is that they cause it to be sufficiently insensitive to the negative interferences of inorganic salt impurities and non-saccharide components such as urea, amino acids, carboxylic acids etc.(Ovodov et al, 1967; Ghebregzabher et al, 1974). It was found that good separation of disaccharides and oligosaccharides from monosaccharides was achieved when the solvent system chloroform:acetic acid:water (6:10:1) was used to run the silica gel plates. Fig.9 shows the chromatographic separation of different sugars studied. Figure indicates that it was possible to separate stachyose, raffinose, melibiose and sucrose from each other, eventhough the three monosaccharides (glucose, galactose and fructose) were not separated from each other. It was found that when the plates were allowed to run twice or thrice, better separation of the sugars was obtained in the range of 20-200 µg of each sugar in a mixture. For the quantitative analysis of well separated disaccharides or oligosaccharides, Roe's method was used. On the other hand, monosaccharides viz. glucose, galactose and fructose were not separated using this method (Fig.9), hence they were quantitatively assayed using an indirect method (as described under

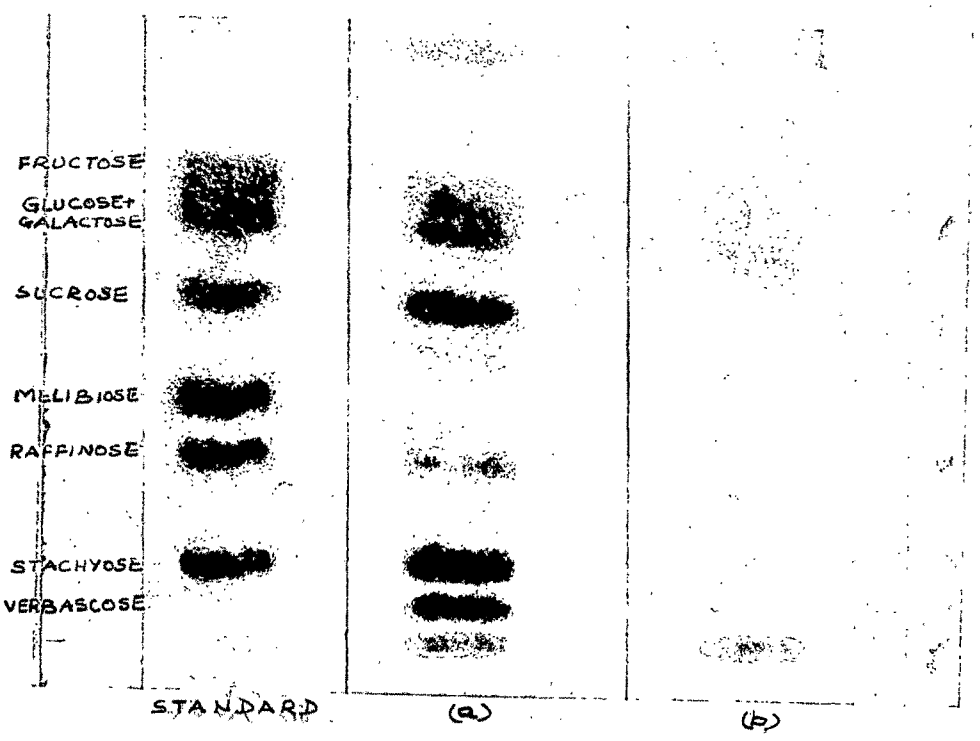


FIG.9 : Detection of oligosaccharides and their metabolites of (a) unfermented and (b) fermented L. sativus dhal samples, analysed by TLC method.

'Materials and Methods'). The estimated recovery of all the sugars were 70-90% when they were spotted (25-150 µg) on plates using different method (Tables-75-a, b, c). When the samples from raw L. sativus seeds were spotted they showed presence of verbascose, stachyose, raffinose, sucrose, glucose, galactose and fructose (Fig.9). Data given in Table-77 show quantitative distribution of oligosaccharides and their metabolites in unfermented and fermented L. sativus dhal. Later on these values were compared with those obtained using 'GC' method. Quantitative analysis of oligosaccharides present in soybean and bengal gram were carried out using this TLC method (as known standards) (Table-76), whose values were comparable with those reported by Kawamura and Tada (1954) and Cristofaro et al (1974). Thus these observations suggested that raw L. sativus pulse contain considerable amount of flatulence producing oligosaccharides viz. verbascose, stachyose and raffinose. Quantitative analysis of flatulence producing oligosaccharides is reported for the first time during present investigation.

In summary, the present TLC method in conjunction with the colorimetric methods, viz. glucose oxidase method, method of Dubois et al. and Roe's method can be used to separate and estimate oligosaccharides and their metabolites, where GC is not available. Verbascose, which is normally difficult to estimate by GC owing to its very high molecular weight, can be separated well from stachyose using the TLC method standardised in the present investigation.

TABLE-7a : Recovery of sugars in TLC method*

Sugar	Concentration spotted (µg)	OD at 420 nm		Recovery (%)
		Before spotting (direct)	After elution from TLC	
Fructose	25	0.14	0.13	93
	50	0.29	0.24	83
	75	0.46	0.39	85
	100	0.58	0.52	90
	125	0.72	0.67	93
	150	0.88	0.80	91
Raffinose	25	0.05	0.04	80
	50	0.08	0.08	100
	75	0.14	0.12	86
	100	0.18	0.17	94
	125	0.21	0.20	95
	150	0.25	0.24	96
Sucrose	25	0.09	0.08	89
	50	0.16	0.14	88
	75	0.23	0.21	91
	100	0.30	0.25	83
	125	0.37	0.34	92
	150	0.50	0.38	76
Stachyose	25	0.03	0.03	100
	50	0.07	0.06	86
	75	0.10	0.08	80
	100	0.13	0.11	85
	125	0.16	0.13	81
	150	0.19	0.17	89
Fructose (in a mixture of fructose + glucose + galactose).	25	0.15	0.14	93
	50	0.30	0.30	100
	75	0.44	0.43	98
	100	0.61	0.59	97
	125	0.76	0.74	97
	150	0.92	0.90	98

* Sugars were estimated by Roe's method

TABLE-75b : Recovery of sugars in TLC method*

Sugar	Concentration spotted (µg)	OD at 490 nm		Recovery (%)
		Before spotting (direct)	After elution from TLC	
Fructose	25	0.18	0.14	78
	50	0.35	0.26	74
	75	0.53	0.42	79
	100	0.71	0.55	77
	125	0.86	0.64	74
	150	1.09	0.85	78
Glucose	25	0.25	0.20	80
	50	0.49	0.35	71
	75	0.76	0.55	72
	100	0.97	0.79	81
	125	1.29	0.92	71
	150	1.52	1.33	87
Galactose	25	0.23	0.19	83
	50	0.51	0.40	78
	75	0.76	0.64	84
	100	1.03	0.75	73
	125	1.30	0.98	75
	150	1.55	1.25	81
Melibiose	25	0.13	0.12	92
	50	0.29	0.28	96
	75	0.43	0.42	98
	100	0.59	0.59	100
	125	0.71	0.70	99
	150	0.85	0.82	96
Mixture of glucose + galactose + fructose	25**	0.64	0.62	97
	50**	1.32	1.28	97
	75**	1.96	1.85	94

* Sugars were estimated by Dubois's method.

** Amount of each sugar in the mixture.

TABLE-75c : Recovery of sugars in TLC method*

Sugar	Concentration spotted (µg)	O.D at 540 nm.		Recovery (%)
		Before spotting (direct)	After elution from TLC	
Glucose	25	0.15	0.12	80
	50	0.32	0.28	88
	75	0.45	0.37	82
	100	0.61	0.49	80
	125	0.76	0.68	89
	150	0.89	0.73	82
Glucose in a mixture of glucose + galactose + fructose	25	0.15	0.12	80
	50	0.30	0.27	90
	75	0.47	0.36	77
	100	0.60	0.49	82
	125	0.77	0.69	90
	150	0.92	0.75	82

* Sugars were estimated by glucose oxidase method.

TABLE-76 : Distribution of oligosaccharides and simple sugars in seeds of L. sativus, Glycine max and Cicer arietinum analysed by TLC method. Components, expressed as mg/gm of the total dry material[a].

	Fructose	Galactose	Glucose	Melibiose	Sucrose	Raffinose	Stachyose	Verbascose
1) L. sativus (Kesari dhal)	5.50 ± 0.35	0.80 ± 0.05	3.23 ± 0.24	0	19.93 ± 0.85	6.33 ± 0.24	18.69 ± 0.73	11.77 ± 0.88
2) Glycine max (Soybean)	4.98 ± 0.10	0	1.02 ± 0.06	0	54.06 ± 2.0	12.63 ± 0.49	47.73 ± 2.5	0
3) Cicer arietinum (Bengal gram)	3.66 ± 0.14	2.03 ± 0.09	4.04 ± 0.08	0	27.8 ± 0.75	9.78 ± 0.5	22.33 ± 0.71	39.13 ± 0.63

(a) Values are expressed as mean of four observations ± S.E.

TABLE-77 : Distribution of oligosaccharides and simple sugars in unfermented and fermented *L. sativus* dhal analysed by TLC method, components, expressed as mg/gm of the total dry material [a].

Sample	Fructose	Galactose	Glucose	Sucrose	Raffinose	Stachyose	Verbascose
Unfermented	5.70 ± 0.25	0.80 ± 0.04	3.53 ± 0.20	20.43 ± 0.82	6.33 ± 0.20	19.90 ± 0.95	12.85 ± 0.66
Fermented	2.07 ± 0.01	0.0	4.34 ± 0.29	1.05 ± 0.0	0.25 ± 0.02	0.0	0.0
	(36)[b]	(0)	(123)	(5.0)	(4.0)	(0)	(0)

(a) Values are mean of four observations ± S.E.

(b) Values in parentheses are percent of control values.

Gas chromatographic analysis of sugars :

During the present investigation, using UCW-98 liquid stationary phase, oligosaccharides and their metabolites in L. sativus samples were separated and quantitatively estimated using GC. (The details of the methods are described under "Materials and Methods").

Fig.10 shows the chromatographic separation of standard sugars, from which it can be seen that stachyose, raffinose, sucrose and melibiose can be separated very well using this method. Glucose and galactose, which could not be separated by Perl et al. (1984) using SP-2250 column at higher concentration were separated in the present method without altering the elution pattern of other sugars. Using this method glucose and galactose were eluted with a difference of 44 seconds in the retention time (Table-78). The sensitivity of estimation using this method was 100 times higher than that reported by Perl et al. (1984). Data given in Table-78 and Fig.11 show the detector response of various sugars at different concentrations, which indicate that though the detector response of stachyose was less than that for other sugars, it could still be separated and estimated. Moreover, sensitivity for each sugar was not affected by the presence of other sugars in a mixture. As to derivatization, it was found that preparation of TMS derivatives takes less time than preparation of TMS oximes, and further no precipitates were formed during

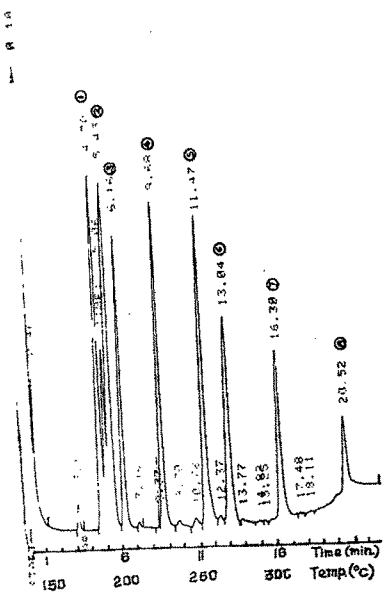
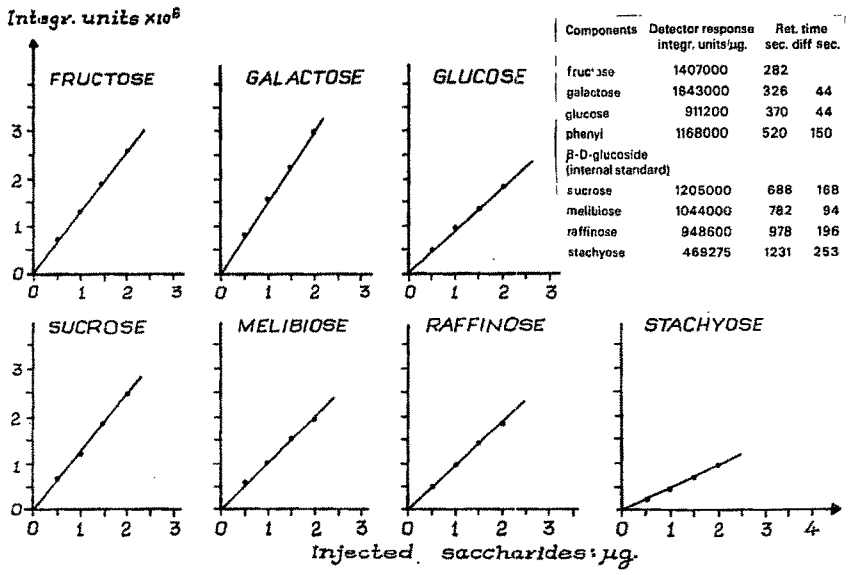


FIG. 10

Fig.10 Gas chromatogram of different oligosaccharides and their metabolites on a 50 cm. long ucw-98 column.
Peaks: 1=fructose; 2=galactose; 3=glucose ; 4=phenyl β-D-glucoside (internal-standard); 5=sucrose, 6=melibiose, 7=raffinose; 8=stachyose.



Components	Detector response integr. units/µg.	Ret. time sec.	diff sec.
fructose	1407000	282	
galactose	1843000	326	44
glucose	911200	370	44
phenyl β-D-glucoside (internal standard)	1168000	520	150
sucrose	1205000	688	168
melibiose	1044000	782	94
raffinose	948600	978	196
stachyose	469275	1231	253

FIG. 11

Fig. 11 Detector response of TMS derivatives on a 50 cm. UCW-98 column.

TABLE-78 : Detector response of TMS derivatives of sugars on a 50 cm long UCW-98 column in GC.

Component	Detector response (integr. units/µg of sugar)	Retention time	
		Seconds	Difference in seconds
Fructose	14,07,000	282	0
Galactose	16,43,000	326	44
Glucoside	9,11,200	370	44
Phenyl β -D-glucose (Internal Standard)	11,68,000	520	150
Sucrose	12,05,000	688	168
Melibiose	10,44,000	782	94
Raffinose	9,48,600	978	196
Stachyose	4,69,275	1231	253

this preparation. In the present experiment, the derivatization of sugars with TMS was carried out at 70-75°, because oligosaccharides are sparingly soluble in pyridine at room temperature. During derivatization, addition of trifluoroacetic acid (TFA) was preferred, instead of chloromethylsilane, because it can tolerate proportion of water (moisture) during sample preparation. TFA also catalyses the rate of trimethylsilylation of sugars. Thus the present method is superior to other reported methods (Sweeley et al, 1963; Delente and Ladenburg, 1972; Li and Schahmann, 1980; Perl and Szakacs, 1981; Perl et al, 1984) because it separates and quantitatively estimates all the oligosaccharides and their metabolites present in biological samples, simultaneously with very high sensitivity.

The present method of GC determination is a modification of method of Sweeley et al. (1963) who showed that trimethylsilyl ethers of sugars upto tetrasaccharides are sufficiently volatile for use in GC, since then the trimethylsilyl derivative method has been applied in the determination of oligo- and disaccharides in food samples.

The other derivatization methods reported for sugars are derivatives such as trifluoroacetates (Nakamura and Tamura, 1970), isopropylidene acetyl derivatives (Anderson and Boggs, 1966), trimethylsilylated oximes (Mason and Slover, 1971; Perl et al, 1984) and methyl ether derivatives (Ovodov and Evtushenko, 1967). Nakamura and Tamura (1970) separated

oligosaccharides as their trifluoroacetates with less retention time (7 min) using OV-1 column, but increased volatility of these derivatives of monosaccharides (compared to TMS derivatives) are in fact disadvantageous because there is a little difference between their retention times. It is, however, likely that trifluoroacetates will be increasingly used for oligosaccharides, where volatility is of prime importance for oligomers of high molecular weight. The acetates of monosaccharides are less readily formed compared to TMS derivatives and can form anomeric/isomeric derivatives as an added disadvantage (Shapira, 1969) although it is the best derivatization method for alditols and other polyhydric compounds. The other isopropylidene derivatives are useful only for separation of monosaccharides but longer time is required for their preparation (, Dutton, 1973).

Since the GC method standardized during present investigation, could give very sensitive and rapid results it was used for quantitative and qualitative analysis of processed L. sativus seeds or dhal samples. The method was also used for analysis of the products of stachyose, raffinose and melibiose metabolism in Bacillus sp. I.

When quantitative analysis of mono-, di- and oligosaccharides were carried out using TLC and GC (Fig.12 and Table-79) method, it was found that L. sativus dhal contains considerably good amount of flatulence producing oligosaccharides, viz. verbascose (1.2%), stachyose (1.9%) and raffinose (0.6%)

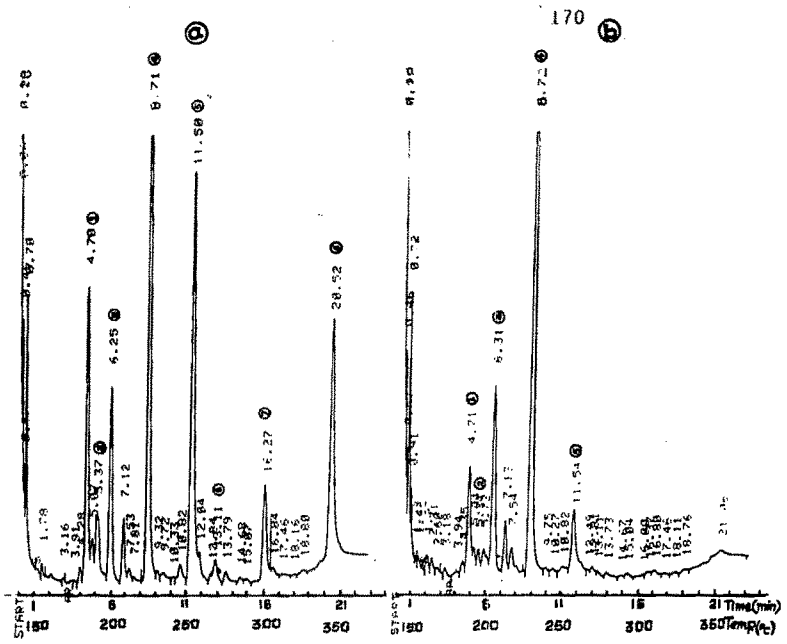


Fig: 12

Fig.12 *Gas chromatograms of the oligosaccharides and their metabolites of (a) unfermented and (b) fermented L.sativus dhal samples on a 50 cm. long ucw-98 column. Details of the types of peaks are shown in fig.1. Detailed data in Table-I.*

TABLE-79 : Distribution of oligosaccharides and simple sugars in processed food samples of L. sativus dhal. Components, expressed as mg/gm of the dry material(a)

Treatments	Fructose	Galactose	Glucose	Sucrose	Melibiose	Raffinose	Stachyose	Verbascose
Control (Unprocessedpraw flour)	6.11 ± 0.11	0.99 ± 0.04	3.94 ± 0.16	22.6 ± 0.58	0.562 ± 0.02	6.52 ± 0.49	20.29 ± 0.90	12.85 ± 0
Fermentation (8 h)	10.63 ± 0.62 (174) (b)	2.11 ± 0.12 (215)	15.52 ± 1.2 (395)	21.45 ± 0.82 (187)	0.99 ± 0.05 (177)	8.04 ± 0.31 (123)	10.22 ± 0.50 (50)	5.17 ± 0.10 (40)
Fermentation (14 h)	3.07 ± 0.10 (50)	0.32 ± 0.03 (32)	6.25 ± 0.10 (159)	1.02 ± 0.06 (09)	0.05 ± 0.0 (09)	0.25 ± 0.05 (04)	0.0 (0)	0.0 (0)
Soaking and boiling	1.24 ± 0.09 (20)	0.28 ± 0.01 (28)	1.28 ± 0.10 (33)	4.28 ± 0.15 (37)	0.213 ± 0.01 (38)	2.44 ± 0.23 (37)	6.55 ± 0.37 (32)	4.88 ± 0.32 (38)
Soaking and auto-claving	0.98 ± 0.09 (16)	0.28 ± 0.02 (28)	0.73 ± 0.06 (19)	2.64 ± 0.17 (23)	0.15 ± 0.02 (27)	0.60 ± 0.05 (09)	2.03 ± 0.09 (10)	1.42 ± 0.10 (11)
Roasting	3.01 ± 0.08 (49)	0.49 ± 0.05 (50)	2.27 ± 0.10 (58)	8.29 ± 0.14 (72)	0.380 ± 0.0 (68)	5.75 ± 0.11 (88)	17.17 ± 0.42 (85)	11.51 ± 0.60 (97)

(a) Values are mean of four observations ± S.E.

(b) Values in parentheses are percent of control values.

as mentioned in Table-77 & 79 like other legumes. The quantitative composition of the oligosaccharides in the unfermented Lathyrus sativus seed is comparable to that of other edible legumes. Kawamura (1954) determined the oligosaccharide content of soybean and reported that it contained sucrose 3.7 percent, raffinose 1.0 percent and stachyose. Vishalakshi et al.(1980) also found sucrose, raffinose and stachyose in three varieties of Phaseolus vulgaris. Stachyose was the major oligosaccharide found in all the varieties. Verbascose was found in trace amounts in raw kidney beans (Vishalakshi et al, 1980) and soybean (Kawamura and Tada, 1967). Recently quantitative analysis of raffinose family of oligosaccharides have been carried out from dry seeds of various plants using high performance liquid chromatography by Kuo et al. (1988). Among seeds of 29 different crops examined by these group of workers, cotton, garden pea, soybean, cowpea and alfalfa contained more than 50 mg of raffinose saccharides per g of defatted meal and all leguminosae species contained more raffinose than stachyose. Thus the present studies on the analysis of raffinose family of oligosaccharides in L. sativus are reported for the first time which also contains toxins as described earlier. Therefore, to utilize this legume as a more acceptable source of inexpensive proteins, it is desirable to reduce the flatus factors also.

Common food processing methods are reported to breakdown flatulence producing oligosaccharides in different legumes

(Ku et al, 1976; Iyengar and Kulkarni, 1977; Rao and Belavady, 1978; Reddy and Salunkhe, 1980b; Olson et al, 1981; Jood et al, 1985), before they are consumed. Therefore, it was of interest to study effect of commonly used processing methods of food, viz. soaking and boiling/autoclaving, parching, roasting, germination and fermentation on the oligosaccharide content of L. sativus dhal or seeds.

Effect of processing on flatulence producing oligosaccharides of L. sativus seed/dhal :

The results of GC analysis (except for verbascose) and TLC analysis (only for verbascose) of raw and processed L. sativus samples are given in Tables-79 & 80. Data given in Table-81 indicates the percentage increase or decrease in content of oligosaccharides and simple sugars during various processing of L. sativus dhal or seeds. Data given in Table-79 show that soaking and boiling of the dhal decreased raffinose, stachyose and verbascose significantly. Sucrose and water soluble monosaccharides also decreased during this process. Present findings are similar to those reported by other investigators for some other legumes (Ku et al, 1976; Olson et al, 1982a; Silva and Braga, 1982; Bianchi et al, 1985; Jood et al, 1985), but differ from Rao and Belavady (1978) who found significant increase in oligosaccharides on cooking of whole seeds of Italian pulses. Reddy et al. (1980) also have reported increase in stachyose and sucrose and slight

TABLE-30 : Distribution of oligosaccharides and simple sugars in processed food samples of L. sativus seeds. Components, expressed as mg/gm of dry meterial [a].

Treatment	Fructose	Galactose	Glucose	Sucrose	Melibiose	Raffinose	Stachyose	Verbascose
Control (unprocessed/ raw seeds)	5.95 ± 0.91	0.96 ± 0.05	4.00 ± 0.20	22.16 ± 0.50	0.544 ± 0.06	5.95 ± 0.46	19.78 ± 1.37	11.77 ± 0.88
Parching	2.67 ±0.28 (45)[b]	0.399 ± 0.10 (42)	1.36 ± 0.11 (34)	10.64 ± 0.58 (48)	0.346 ± 0.02 (64)	3.97 ± 0.11 (67)	10.96 ± 0.80 (55)	7.07 ± 0.17 (60)
Germination (12 h)	8.66 ± 0.60 (146)	1.28 ± 0.03 (133)	11.12 ± 0.97 (278)	40.50 ± 1.54 (183)	1.172 ± 0.09 (215)	9.96 ± 0.71 (167)	11.02 ± 0.54 (56)	4.27 ± 0.26 (36)
Germination (24 h)	13.80 ± 0.72 (232)	1.96 ± 0.09 (204)	16.03 ± 0.80 (400)	58.50 ± 2.3 (264)	1.428 ± 0.43 (262)	4.98 ± 0.4 (84)	5.58 ± 0.49 (28)	1.074 ± 0.07 (9)
Germination (48 h)	16.72 ± 0.72 (281)	2.86 ± 0.11 (298)	23.79 ± 0.87 (594)	69.53 ± 2.07 (314)	0.158 ± 0.02 (29)	1.817 ± 0.06 (31)	0	0
Germination (72 h)	17.03 ± 0.87 (286)	2.99 ± 0.10 (311)	30.62 ± 0.91 (766)	94.78 ± 3.4 (428)	0.0 (0)	0.18 ± 0.0 (3)	0	0

(a) Values are mean of four observations ± S.E.

(b) Values in parentheses are percent of control values.

TABLE-31 : Percentage loss or gain in the content of oligosaccharides and simple sugars during processing of *L. sativus* dhal/seeds.

Treatment	Fru-cose	Gala-ctose	Glu-cose	Sucro-se	Meli-biose	Raffi-nose	Stach-yose	Verbas-cose
Fermentation of dhal (8h)	+ 74	+115	+ 295	+ 87	+ 77	+25	- 50	- 60
Fermentation of dhal (14h)	- 50	- 68	+ 59	- 91	- 91	- 96	-100	-100
Soaking and boiling of dhal	- 80	- 72	- 67	- 63	- 62	- 63	- 68	- 62
Soaking and autoclaving of dhal	- 84	- 72	- 81	- 76	-73	- 91	- 90	- 89
Roasting of dhal	- 51	- 50	- 42	- 28	- 32	- 12	- 15	- 03
Parching of seeds	- 55	- 58	- 66	- 52	- 36	- 33	- 46	- 40
Germination for 12 h	+ 46	+ 33	+ 178	+ 82	+115	+ 68	- 44	- 64
Germination for 24 h	+132	+104	+ 300	+164	+162	- 16	- 72	- 91
Germination for 48 h	+181	+198	+ 494	+213	- 71	- 69	-100	-100
Germination for 72 h	+186	+211	+ 666	+328	-100	- 97	-100	-100

'+' Indicates % increase and '-' indicates % degradation or decrease

- Values of unprocessed seeds/dhal are considered as controls or 100%.

decrease in verbascose during cooking of blackgram in 1:10 water ratio for 3 h at 90°C. The present experiment shows that soaking and autoclaving is more effective in removal of flatulence producing oligosaccharides (80% removal) than boiling. Soaking and cooking was considered as a good method of removal of flatulence factors by Garg et al. (1980) because in their experiments, cooking of chickpeas and peas caused decrease in gas production in relation to that of uncooked seeds. The solubility, molecular weight, location and natural binding form of the sugars within the cell play an important role in the extent to which saccharides are extracted from soybean seeds (Ku et al, 1976). A 50% reduction in flatulence potential measured by hydrogen production was observed when rats were fed extracted California beans by Olson et al. (1982). A significant reduction in subjective gastrointestinal distress was reported by human subjects interviewed after eating cooked extracted beans compared to cooked nonextracted beans.

Data given in Table-79 show that roasting of L. sativus dhal does not breakdown stachyose, verbascose and raffinose to a significant extent, whereas monosaccharides were sensitive to heat. Similar observations were made by Faki et al. (1983) who found that roasting was ineffective in removal of flatus effect of chickpeas, cowpeas and horse grams and their isolated carbohydrate fractions.

When L. sativus dhal was soaked and fermented for 8 h, there was highly significant increase in free sugars, viz.,

fructose, galactose and glucose which can be due to breakdown of oligosaccharides and higher polysaccharides. Excess of glucose was detected due to breakdown of starch during fermentation. During 8 h fermentation, there was 50-60% degradation of verbascose and stachyose, whereas slight increase in raffinose was observed. During fermentation of dhal for 14 h complete reduction in oligosaccharide content was observed(Fig.9&12) Fructose and galactose also decreased after complete fermentation. Odunfa (1982b)also observed the increase in reducing sugars during first 12 h of fermentation in 'iru' fermentation, which decreased during later stages of fermentation. It is possible that sugars have been used up during the growth of the bacteria involved in the fermentation, because sugars are the preferred carbon source for microbial growth and metabolism. Complete reduction of oligosaccharides can be explained due to microbial α -galactosidase and invertase (sucrose hydrolase) activities during fermentation. Similar results are reported during fermentation of 'tempeh' (Shallenberger et al, 1967), 'soymilk' (Mital and Steinkraus, 1975), 'oncom' (Fardiaz and Markakis, 1981), chick peas and cow peas (Zamora and Fields, 1979a)and 'iru' (Odunfa, 1982b)due to the production of microbial α -galactosidase. In the present studies on fermentation of Kesari dhal, Leuconostoc mesenteroides, L. fermenti, Lactobacillus sp., Pediococcus sp. and Bacillus sp. were found to be the major bacteria present as mentioned in Section-I. The efficiency of these bacteria to degrade raffinose family of oligosaccharides will be discussed later. Role of Lactobacilli,

and Bacillus sp. in removal of flatus factors were reported in 'soymilk' and 'ugba' (fermented oil bean product) respectively (Mital and Steinkraus, 1975; Odunfa and Oycyiola, 1985). Pediococcus sp.are also known to have ∝-galactosidase activity (Gonzalez and Kunka, 1986).

In the case of seeds when the effect of parching was observed, there was approximately 40% decrease in verbascose, stachyose and raffinose perhaps due to chemical breakdown of all these sugars at high temperature in presence of moisture. The effect of parching was studied because it has been proved to increase the nutritive value in other legumes (Rajalakshmi et al, 1964; Carvalho et al, 1977).

During germination of L. sativus seeds there was highly significant increase in all the monosaccharides amongst which increase in glucose was highest (Table-80). This increase can be accounted due to breakdown of polysaccharides and oligosaccharides, during germination. Jaya and Venkataraman (1981) also reported three times increase in fructose and 10 times increase in glucose + galactose during germination of chickpeas and green grams. Data given in Table-80 also show that sucrose content increased during 48 h germination, which may be due to the action of ∝-galactosidase which cleaves selectively galactose from raffinose, stachyose and verbascose and leaves behind sucrose (Gupta and Wagle, 1980). At 12 h germination, there was highly significant decrease in verbascose compared to stachyose, along with increase in raffinose due to breakdown of

verbascose and stachyose. In subsequent stages of germination, all the three oligosaccharides almost completely degraded. These results are in agreement with those of Jood et al. (1986), Reddy and Salunkhe (1980b) and Sathe et al. (1983). In the present experiment, early reduction in verbascose content compared to stachyose was observed, which may be due to the reason that during germination, α-galactosidase first attacks verbascose followed by stachyose and raffinose. Increase in melibiose during early stages of germination could be due to the partial hydrolysis of stachyose and raffinose. Melibiose is also reported as an intermediate product of hydrolysis of soybean oligosaccharides, when soymilk was treated with enzymes of Aspergillus saito (Sugimoto and Van Buren, 1970).

It can be concluded from this experiment that soaking plus boiling/autoclaving, germination and fermentation decrease all the flatulence producing oligosaccharides present in L. sativus. Germination and fermentation can be considered as better methods of processing L. sativus because they also increase the digestibility as well as increase the nutritive value by breakdown of other toxic compounds and increase in vitamins (Banerjee and Banerjee, 1950; Zamora and Fields, 1979ab; Reddy et al, 1980; Lay and Fields, 1981; Sathe et al, 1983). Significant increase in vitamin B_1, B_2, B_6, B_{12} and niacin were found during fermentation of several legumebased foods as described in Section-I. Reddy et al. (1980) while evaluating different processing methods using rat bioassay method found that germinated blackgram seeds and fermented steamed product (idli)

significantly produced lower flatulence than the cooked blackgram products. Becker et al. (1974) also reported that the disappearance of raffinose and stachyose during autolysis in California, small white beans reduced flatulence as measured by hydrogen production in rats. Increase in the content of soluble amino acids occurred during germination of great northern beans (Sathe et al, 1983). Decrease in phytate and trypsin inhibitory activity were observed by different investigators during germination of legumes (Kakade and Evans, 1966; Chen and Pan, 1977; Gupta and Wagle, 1980; Reddy et al, 1982). Soaking and boiling cannot be preferred to fermentation and germination because there is a loss of water soluble vitamins and minerals during this processes (Raghunath and Belavady, 1979; Swaminathan, 1974; Olsen et al, 1982).

Degradation of flatulence producing oligosaccharides by bacterial isolates :

Since the basic aim of the present investigation was to study the mode of breakdown of flatulence producing oligosaccharides by bacteria, the different bacterial isolates from stored grains and fermented foods (isolation and identification of the isolates are described in the previous sections) were screened for their capacity to breakdown raffinose. Other flatulence producing oligosaccharides viz. stachyose and verbascose are not readily available therefore raffinose which is easily available was used for all the experiments on bacterial degradation unless and until specified. Data given in Table-82

show the ability of different bacterial isolates to hydrolyse raffinose when inoculated to the basal medium containing raffinose as sole carbon source. Bacillus sp. I isolated from stored soybean grains was found to be the most efficient bacterial strain for utilization of raffinose. Pediococcus sp. and Bacillus sp. III isolated from L. sativus batter as well as L. mesenteroides and L. fermenti isolated from fermented batters could hydrolyse raffinose to some extent. Thus Bacillus sp. I (having maximum capacity to hydrolyse raffinose) isolated from stored soybean grains was used for the further studies.
Data given in Table-82 show that raffinose in the medium is broken down by Bacillus sp. I and not concentrated in the cells within 24 h suggesting that there is a complete utilization of raffinose during growth. The strain was studied for its capacity to utilize other oligosaccharides and their metabolites. For this, bacteria were grown in basal salt medium containing 0.5% of each sugar (stachyose, raffinose, melibiose or sucrose) as sole source of carbon as described under 'Materials and Methods'. It can be seen from Tables-83-86 and Figs.13-16 that the culture shows a similar growth pattern with all the different sugars studied. The growth curve exhibited an initial lag period of 3 hours, followed by an exponential period till 6 hours and then stationary phase after that. Decrease in turbidity reading was observed after 48 h growth with all the sugars due to the lysis of bacteria. Subsequently, pH as well as the sugar content in the culture filtrate decreased during the growth, suggesting the utilization of the respective sugar by Bacillus sp. I. It can be seen from the Figs.13-16 that as growth increases, there is a

FIG. 13 : Growth curve of Bacillus sp. I in raffinose broth and utilization of raffinose.

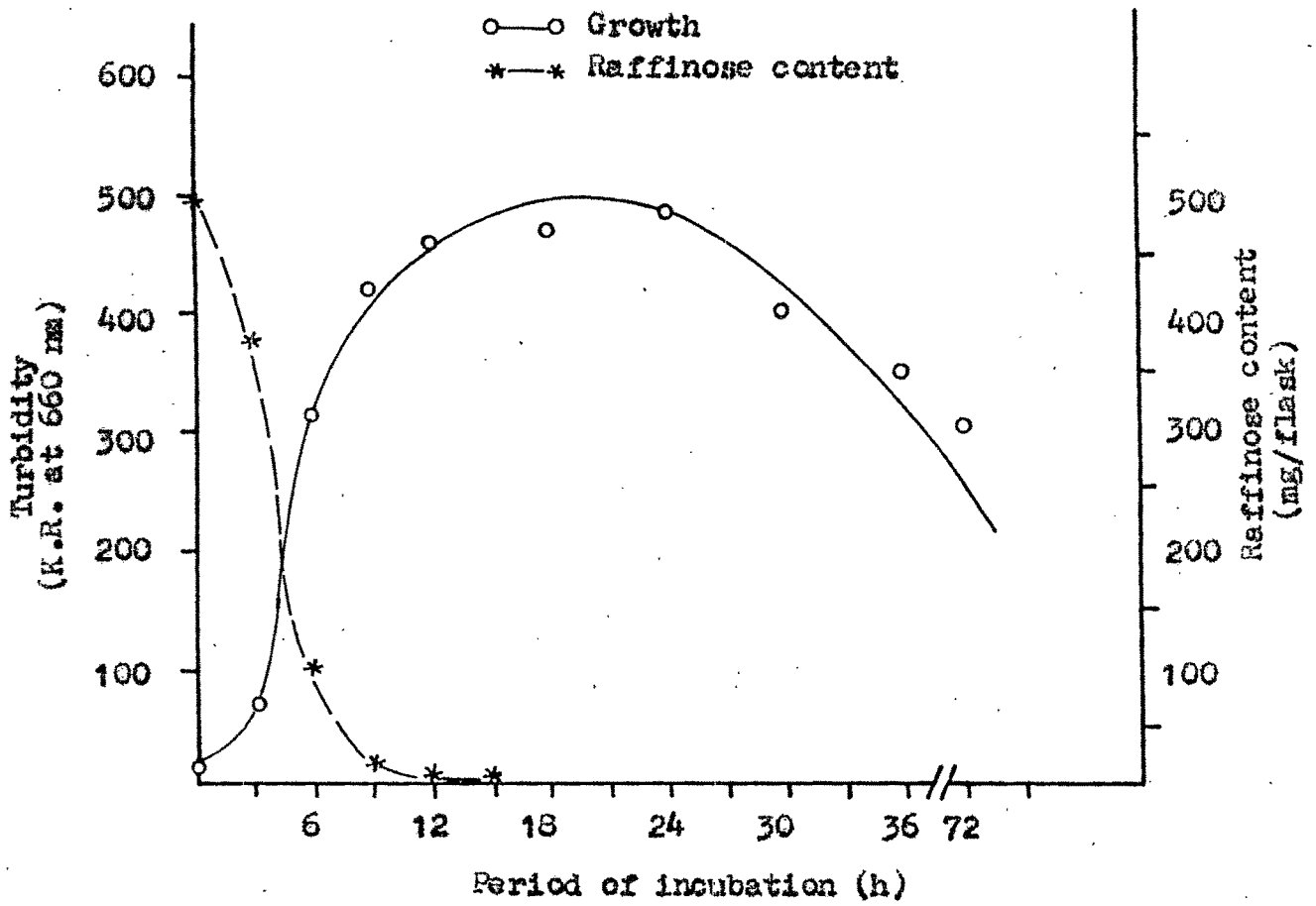


FIG.14 : Growth curve of Bacillus sp. I in stachyose broth and utilization of stachyose.

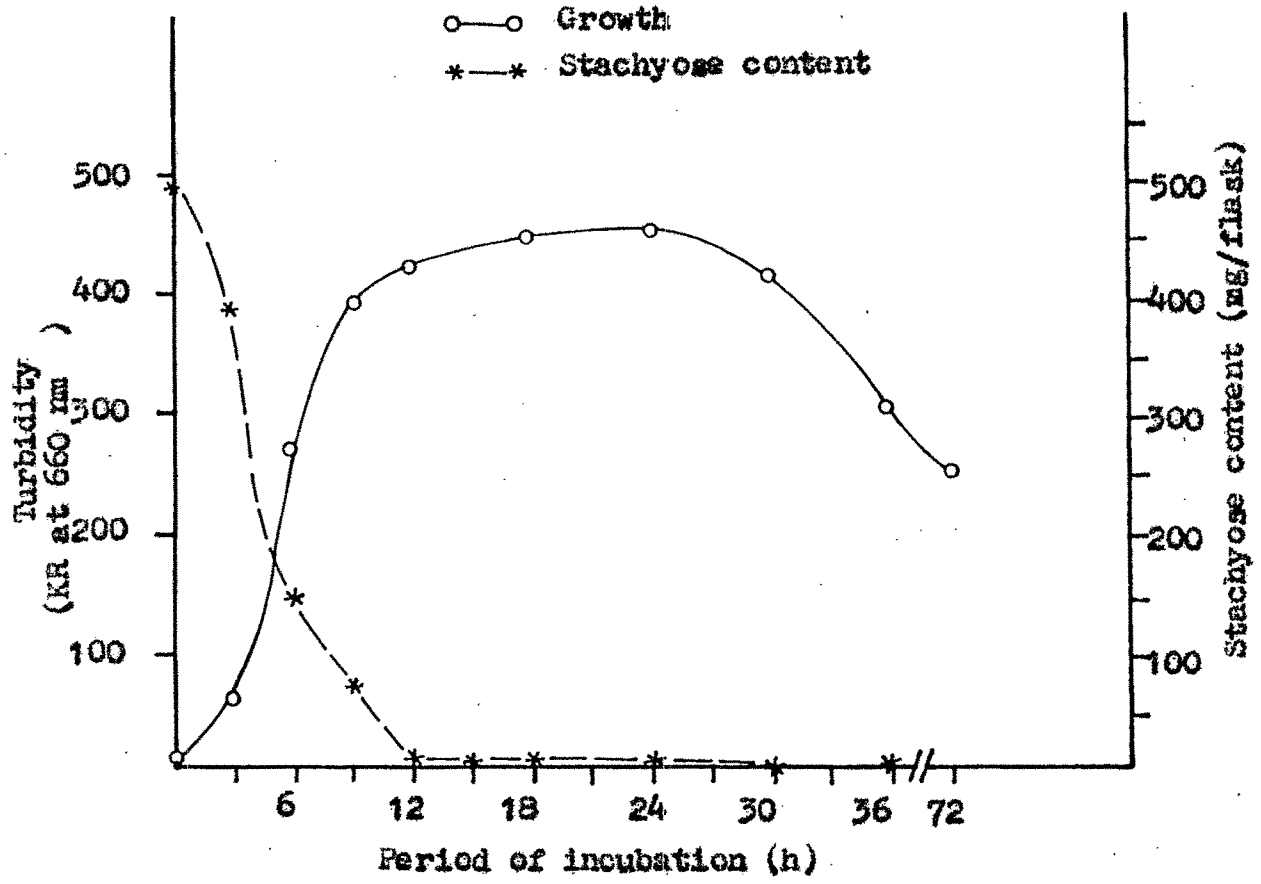


FIG.15 : Growth curve of Bacillus sp. I in melibiose broth and utilization of melibiose.

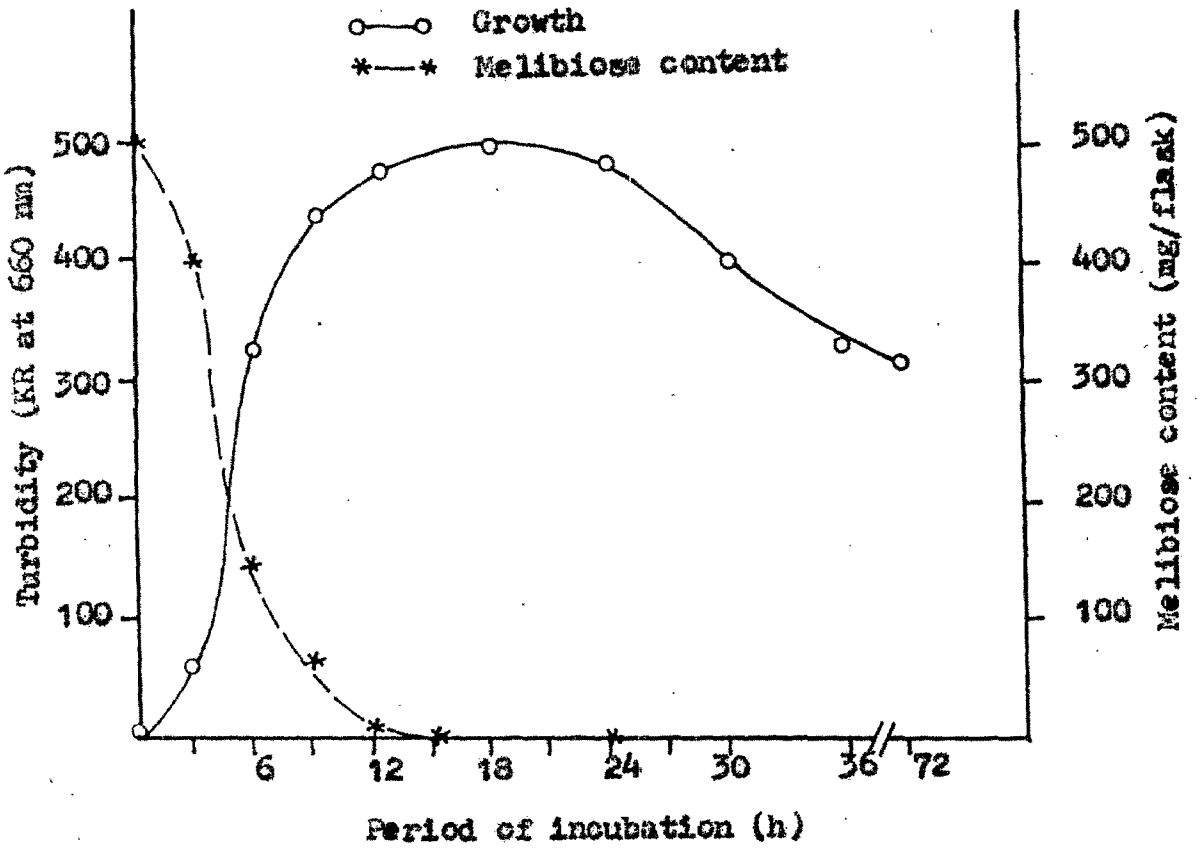


FIG.16 : Growth curve of Bacillus sp. I in sucrose broth and utilization of sucrose.

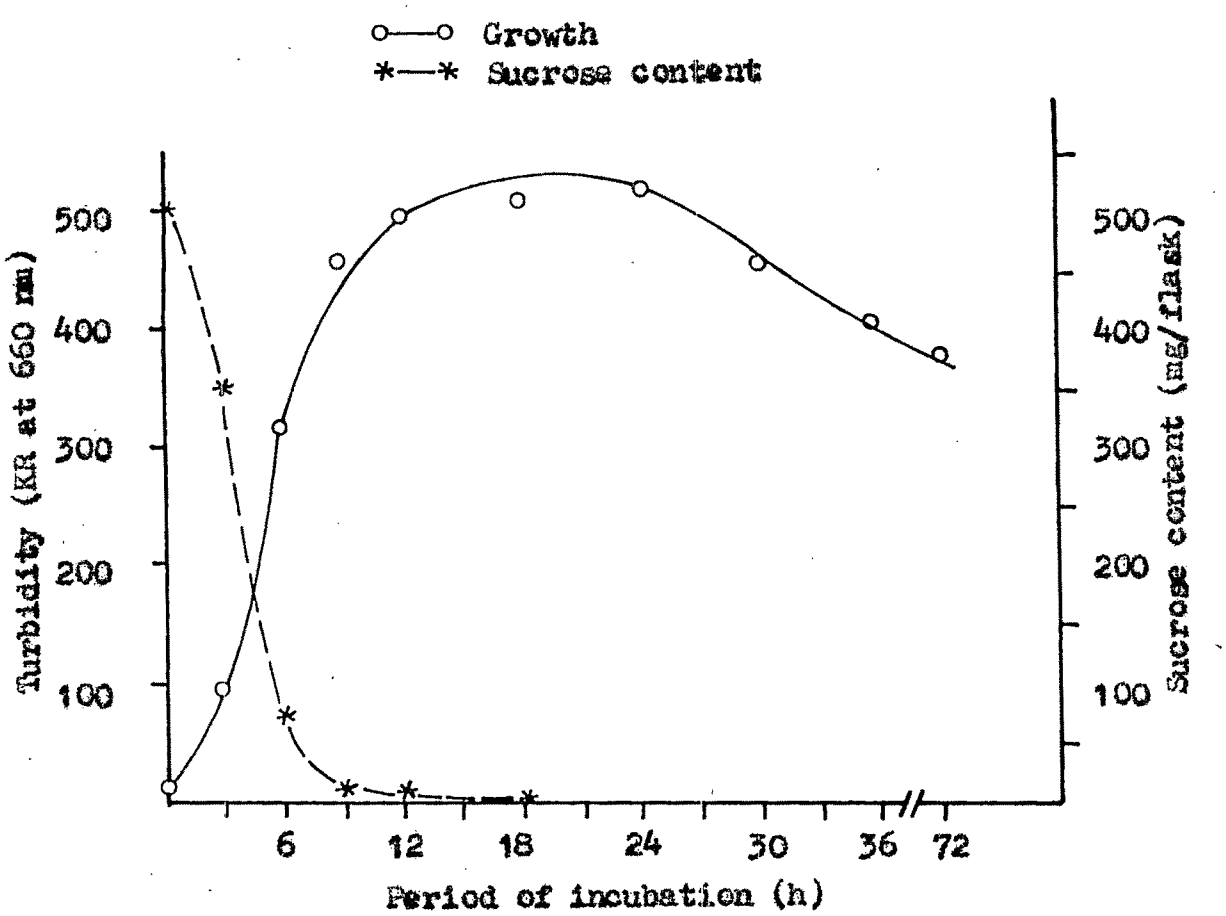


TABLE-83 : Growth of Bacillus sp. I in raffinose broth and degradation of raffinose.

Period of incubation (h)	Final pH of the medium	Growth in terms of turbidity (KR at 660 nm)	Raffinose content in the culture filtrate (mg/flask)		Degradation of raffinose (%)
			Control	Experimental	
0	7.0	15	500	500	0
3	6.8	64	500	378	24
6	6.0	315	500	102	80
9	5.8	420	500	25	95
12	5.7	455	500	5	99
18	5.5	470	500	0	100
24	5.2	480	500	0	100

100 ml of basal salt medium (pH 7.0) containing 0.5% raffinose were sterilized in 500 ml side-armed conical flask, inoculated with 20 x 10^6 counts of bacteria, and was then incubated at 30° in shaker (140 rpm). Growth was measured in terms of turbidity at the above indicated intervals of incubation period using Klett-Summerson colorimeter and also samples were withdrawn at those intervals to estimate raffinose in the culture filtrate (Raffinose estimation described under 'Materials and Methods). The control flask was kept at 2°. The experiment was carried out in triplicates and the data given above are the mean of three observations.

TABLE-84 : Growth of Bacillus sp. I in stachyose broth and degradation of stachyose.

Period of incubation (h)	Final pH of the medium	Growth in terms of turbidity (KR at 660 nm)	Stachyose content in the culture filtrate		Degradation of Stachyose (%)
			Control	Experimental	
0	7.0	5	500	500	0
3	6.9	65	500	390	22
6	6.4	270	500	144	71
9	5.8	395	500	76	85
12	5.6	425	500	4	99
18	5.4	450	500	0	100
24	5.5	455	500	0	100

Details of the experiment are same as in Table-83 except 'Stachyose' was used as a 'C' source instead of raffinose in the medium during the growth of bacteria.

TABLE-85 : Growth of *Bacillus* sp. I in melibiose broth and degradation of melibiose.

Period of incubation (h)	Final pH of the medium	Growth in terms of turbidity (KR at 660 nm)	Melibiose content in the culture filtrate (mg/flask)		Degradation of melibiose (%)
			Control	Experimental	
0	7.0	5	500	500	0
3	6.9	60	500	405	19
6	6.2	322	500	152	70
9	5.8	440	500	60	88
12	5.5	475	500	0	100
18	5.3	500	500	0	100
24	5.2	485	500	0	100

Details of the experiment are same as in Table-83 except melibiose was used as a 'C' source instead of raffinose in the basal salt medium during the growth of bacteria.

TABLE-86 : Growth of *Bacillus* sp. I in sucrose broth and degradation of sucrose.

Period of incubation (h)	Final pH of the medium	Growth in terms of turbidity (KR at 660 nm)	Sucrose content in the culture filtrate (mg/flask)		Degradation of sucrose (%)
			Control	Experimental	
0	7.0	8	500	500	0
3	6.8	98	500	345	31
6	6.3	320	500	75	85
9	5.6	455	500	8	98
12	5.5	495	500	0	100
18	5.5	510	500	0	100
24	5.4	525	500	0	100

Details of the experiment are same as in Table-83 except sucrose was used as 'C' source instead of raffinose during the growth.

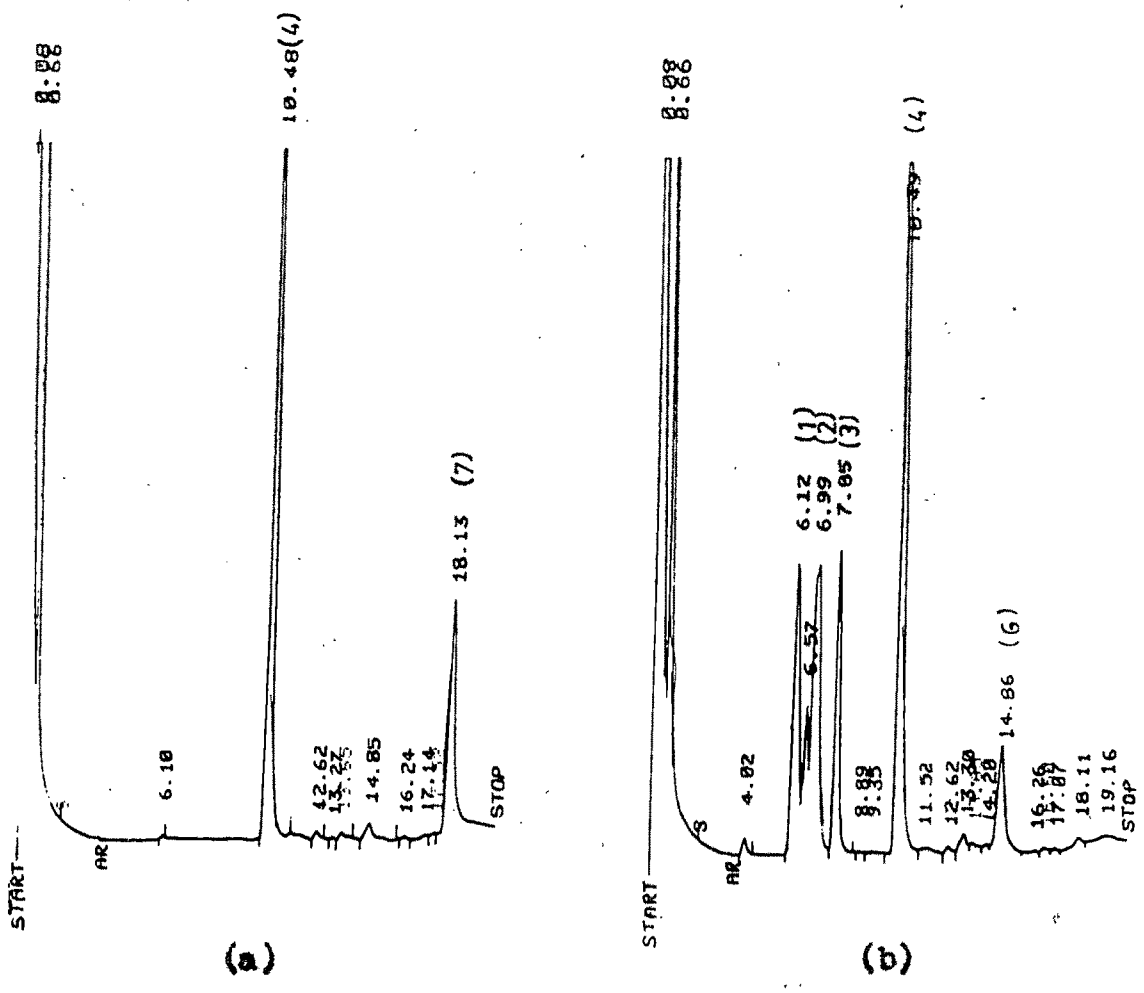
rapid decrease in the sugar content. Thus it can be concluded from this experiment that Bacillus sp. I can utilize oligosaccharide viz. stachyose and raffinose and their metabolites viz. sucrose and melibiose as sole carbon source.

Analysis of the products of oligosaccharide degradation during growth of Bacillus sp. I :

During the growth of Bacillus sp. I in raffinose, melibiose and sucrose broth the subsequent degradative products were analysed by TLC and GC as described under 'Materials and Methods'.

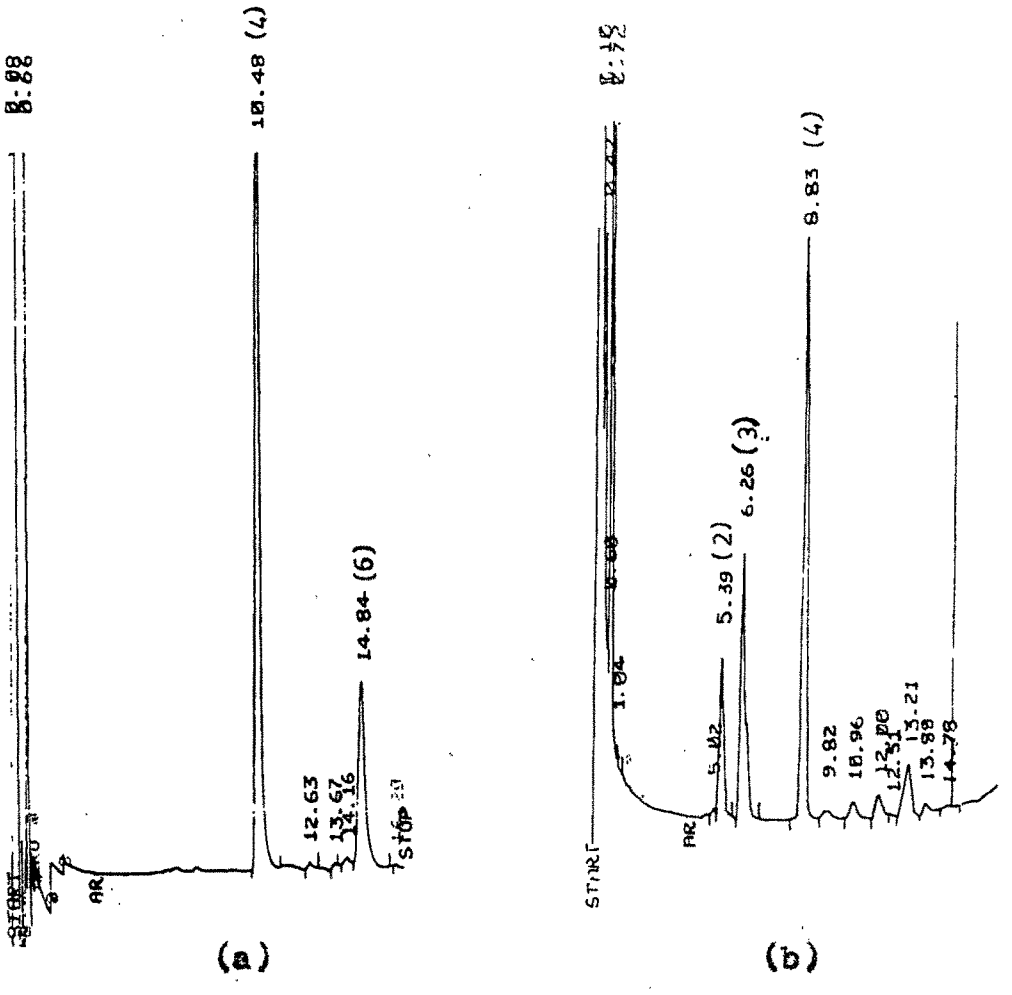
Fig.17 shows that when the cells were grown on basal medium containing raffinose as sole carbon source, melibiose, fructose, glucose and galactose were detected in the culture filtrate after 6 h of incubation. Analysis of the culture filtrate by TLC (Fig.20) or GC (Fig.19) did not show the presence of sucrose as a degradative product of raffinose utilization, eventhough fructose, glucose and galactose were detected. This could be ascribed to the higher levels of β-D-fructofuranosidase activity in bacteria compared to α-galactosidase activity during utilization of raffinose in this culture. The degradative products of melibiose and sucrose utilization are shown in Figs.18 & 19. When the culture filtrates were analysed after 12 hours of growth, the complete disappearance of tri- and disaccharides was observed as shown in Figs.17-19-20, indicating a complete utilisation of sugars.

FIG.17 : Gas chromatograms of products of raffinose utilization by Bacillus sp. I, at (a) 0 h. growth and (b) 6 h growth.



Peaks : 1 = fructose; 2 = galactose;
3 = glucose;
4 = phenyl β-D-glucoside (internal standard);
5 = sucrose; 6 = melibiose
7 = raffinose.

FIG.18 : Gas chromatograms of products of melibiose utilization by Bacillus sp. I, at (a) 0 hr growth and (b) 6 h growth.



Details of the peaks are given in Fig.17.

FIG.19 : Detection of products of melibiose and sucrose utilization by Bacillus sp. I, analysed by TLC method.

(1) Cells were grown in melibiose broth for '0' h (control); (2) Cells were grown in melibiose broth for 6 h; (3) Cells were grown in melibiose broth for 12 h; (4) Standard sugars; (5) Cells were grown in sucrose broth for '0' h. (control); (6) Cells were grown in sucrose broth for 6 h; (7) Cells were grown in sucrose broth for 12 h.

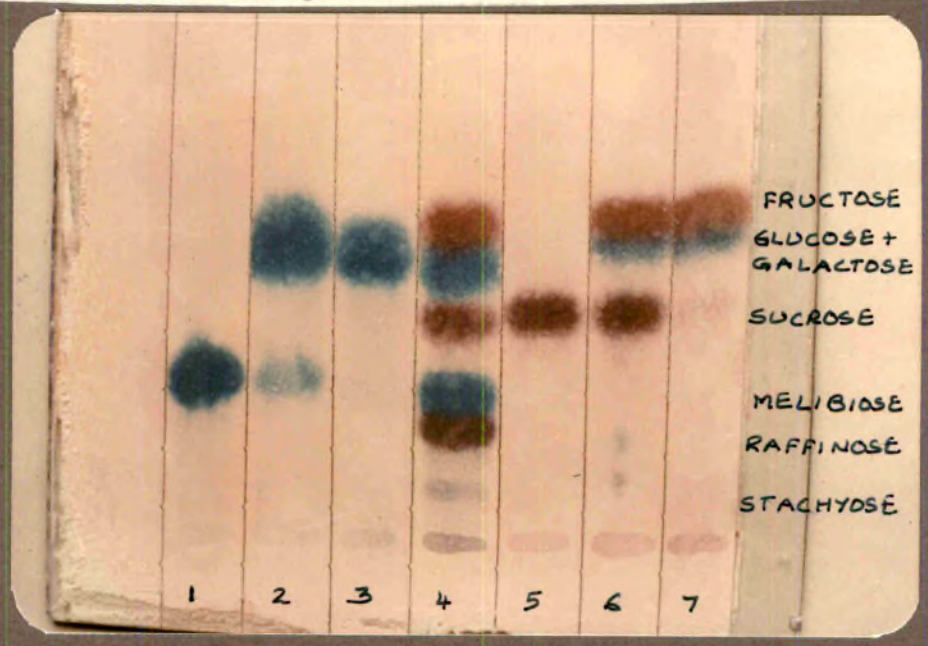
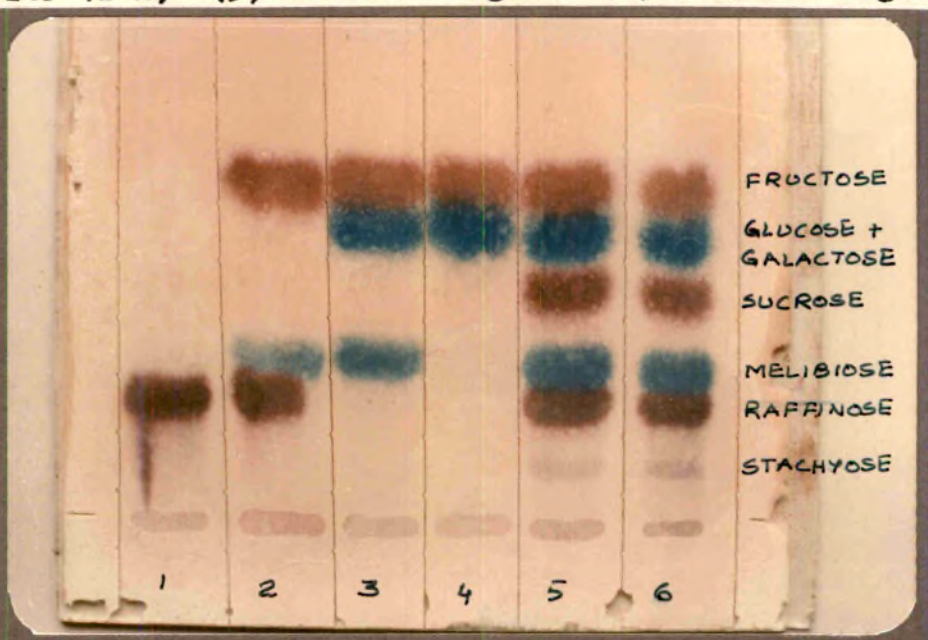


FIG.20 : Detection of products of raffinose utilization by Bacillus sp. I analysed by TLC method:

(1) Cells were grown in raffinose broth for '0' h (control); (2) Cells were grown in raffinose broth for 3 h; (3) Cells were grown in raffinose broth for 6 h; (4) Cells were grown in raffinose broth for 12 h; (5) Standard sugars (6) standard sugars.



Detection of raffinose hydrolysing enzyme system in Bacillus sp.I:

Data given in Table-83 and Fig.13 show that raffinose in the medium was utilized completely during the growth of Bacillus sp. I. This suggests that bacteria possess enzymes which hydrolyse raffinose family of oligosaccharides. Studies were therefore carried out to investigate the capacity of the culture filtrate and bacterial cell free extract to hydrolyse raffinose in vitro.

As discussed in the 'Introduction' (Chapter-I), α-galactosidase and invertase are the major enzymes responsible for the breakdown of raffinose into glucose, fructose and galactose in bacteria. Therefore, these two enzymes were investigated during the present studies on Bacillus sp. I.

For this experiment, bacteria were grown in raffinose broth for 6, 12 and 24 hours. The culture was then centrifuged at 8,000 xg for 10 min at 4°C. The supernatant (SN_1) was collected separately and the cells were suspended in buffer and disrupted (details of the method are described earlier) and cell homogenate (SN_2) was prepared. This homogenate was then centrifuged at 20,000 xg for 45 min and the supernatant (SN_3) was collected. All the samples (SN_1, SN_2 and SN_3) were then assayed for the presence of raffinose degrading enzymes.

Data given in Table-87 indicate that the culture filtrate (SN_1) did not possess α-galactosidase or invertase activity

whereas cell-free extracts (SN_2 and SN_3) exhibited activities of both the enzymes. Since both the enzymes were absent in culture filtrate but present in cell free extract (SN_2 and SN_3) it was concluded that in Bacillus sp. I, α-galactosidase and invertase are intracellular and cytoplasmic. Raffinose hydrolysing micro-organisms have been reported from several other sources also as described in Table-20. The intracellular localization of α-galactosidase has been reported in E. Coli (Schmitt and Rotmann, 1966; Schmid and Schmitt, 1976), B. steaothermophilus (Pederson and Goodman, 1980), Pediococcus sp. (Gonzalaez and Kunka, 1986), Micrococcus sp., Bacillus sp. (Akiba and Horikoshi, 1976), Lactobacillus sp. (Mital et al, 1973) and Mongscus pilosus (Wong et al, 1986). In contrast to this, extracellular α-galactosidase has been reported in S. carlsbergensis (Lazo et al, 1978), Pycnoporous cinnabarinus (Ohtakara et al, 1984), Bacteroides fragilis (Berg et al, 1980) etc. Invertase is found to be extracellular or secreted into the medium in yeasts (Wickerham, 1958; Iizuka and Yamamoto, 1972) and molds (Mandels, 1956; Maruyama and Onodera, 1979). α-Galactosidase in most of the eukaryotic organisms is present in soluble fraction (Dey and Pridham, 1972). However, the same enzyme has been also reported in particulate fraction of renals of pig (Debris et al, 1962) in mitochondrial fraction of rat brain (Subba Rao and Pieringer, 1970), in membrane fraction of bovine liver (Fleischer and Fleischer, 1969), in chloroplast, mitochondrial and microsomal fraction of spinach leaves (Gatt and Baker, 1970) and in cell wall of several plant tissues (Thomas and Webb, 1979; Asamizu et al, 1981).

TABLE-87 : Detection of raffinose hydrolysing enzymes in the cell-free extract and the culture filtrate of Bacillus Sp. I.

Period of growth (hr)	Raffinose hydrolysing enzymes* (units/flask)					
	Cultrate filtrate (SN_1)		Cell homogenate (SN_2)		Cell free extract (SN_3)	
	α-galacto-sidase	Invertase	α-galacto-sidase	Invertase	α-galacto-sidase	Invertase
6	0	0	8.80	4.24	9.00	4.64
12	0	0	17.60	9.88	18.28	10.64
24	0	0	12.80	8.52	14.24	9.48

20×10^6 counts were inoculated into 100 ml raffinose broth in 250 ml conical flasks, and incubated at 30° on shaker (140 rpm). After the incubation, culture was centrifuged at 8000 xg for 10 min. at 0-4°. The culture filtrate (SN_1) was removed and the cells were sonicated in appropriate amount of 50 mM sodium phosphate buffer, pH 7.0. The cell homogenate (SN_2) was centrifuged at 20,000 xg for 30 min. at 0-4° and the supernatant viz. cell free extract (SN_3) was removed. The samples SN_1, SN_2 & SN_3 were then used for enzyme assays as described under "Materials and Methods".

* - α-galactosidase units are defined as the amount of enzyme required to libera te one micromole of paranitrophenol under assay conditions.

\- Invertase units are defined as the amount of enzyme required to liberate one micromole of glucose under assay conditions.

Further studies on α-galactosidase and invertase were carried out using cell-free extract of Bacillus sp. I. Table-88 and Figs. 21-25 give the optimum conditions required for the maximum activities of both the enzymes during the assay. The optimum pH for α-galactosidase and invertase was found to be 7.0 (Fig.21). The optimum substrate concentration for α-galactosidase was 1.0 µmoles of α-PNPG whereas for invertase, it was 10.0 µmoles of sucrose (Fig.22). The enzyme activity was linear upto 10 minute of incubation in the case of α-galactosidase and 30 minutes in the case of invertase (Fig.23). The enzyme activity was proportional upto 50 µg. of the crude protein in the case of α-galactosidase and 300 µg of protein in the case of invertase (Fig.24). The optimum temperature for both the enzymes was found to be 37°C (Fig.25).

Results on the stability of raffinose hydrolysing enzymes of Bacillus sp. II in crude extract are presented in Table-89. When the crude cell-free extract of bacteria was stored at 7-8°, both α-galactosidase and invertase activity were reduced after 6 h storage. Addition of 2-mercaptoethanol (1 mM) to the crude enzyme extract increased the stability of both the enzymes. Hence for further experiments 2-mercapto-ethanol was added to increase the stability of enzymes.

TABLE-88 : Optimum conditions for the activities of α-galactosidase and invertase of Bacillus sp.I.

	α-galactosidase	Invertase
Optimum pH	7.0	7.0
Optimum temperature (°C)	37	37
Period of incubation for assay (min.)	10	30
Optimum enzyme concentration (µg protein)	50	300
Optimum substrate concentration (µmoles)	1.0	10.0

FIG.21 : Effect of pH on the activity of crude ∝-galactosidase and inverta of Bacillus sp. I.

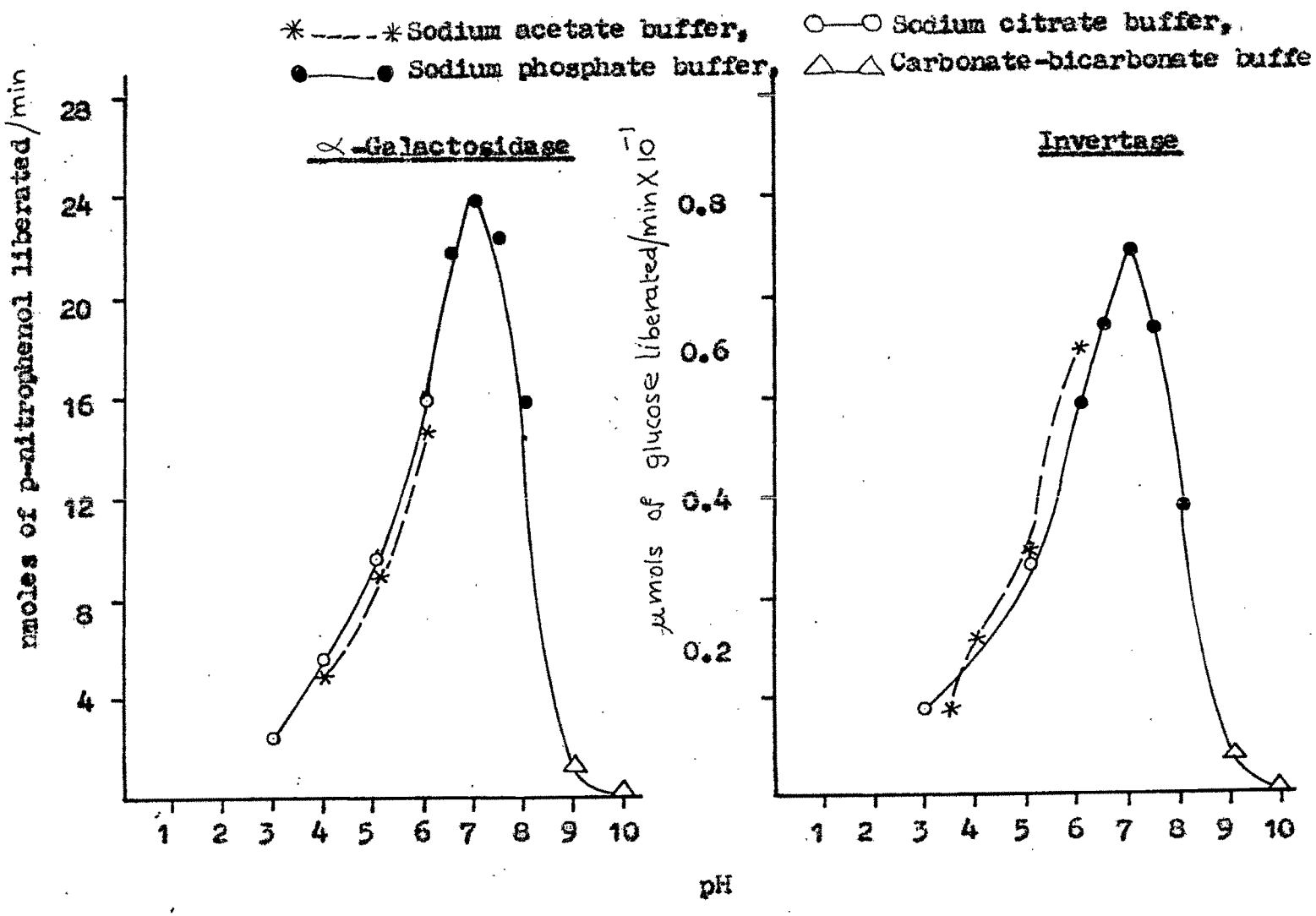


FIG.22 : Effect of substrate concentration on the activity of crude α-galactosidase and invertase activity of Bacillus sp. I.

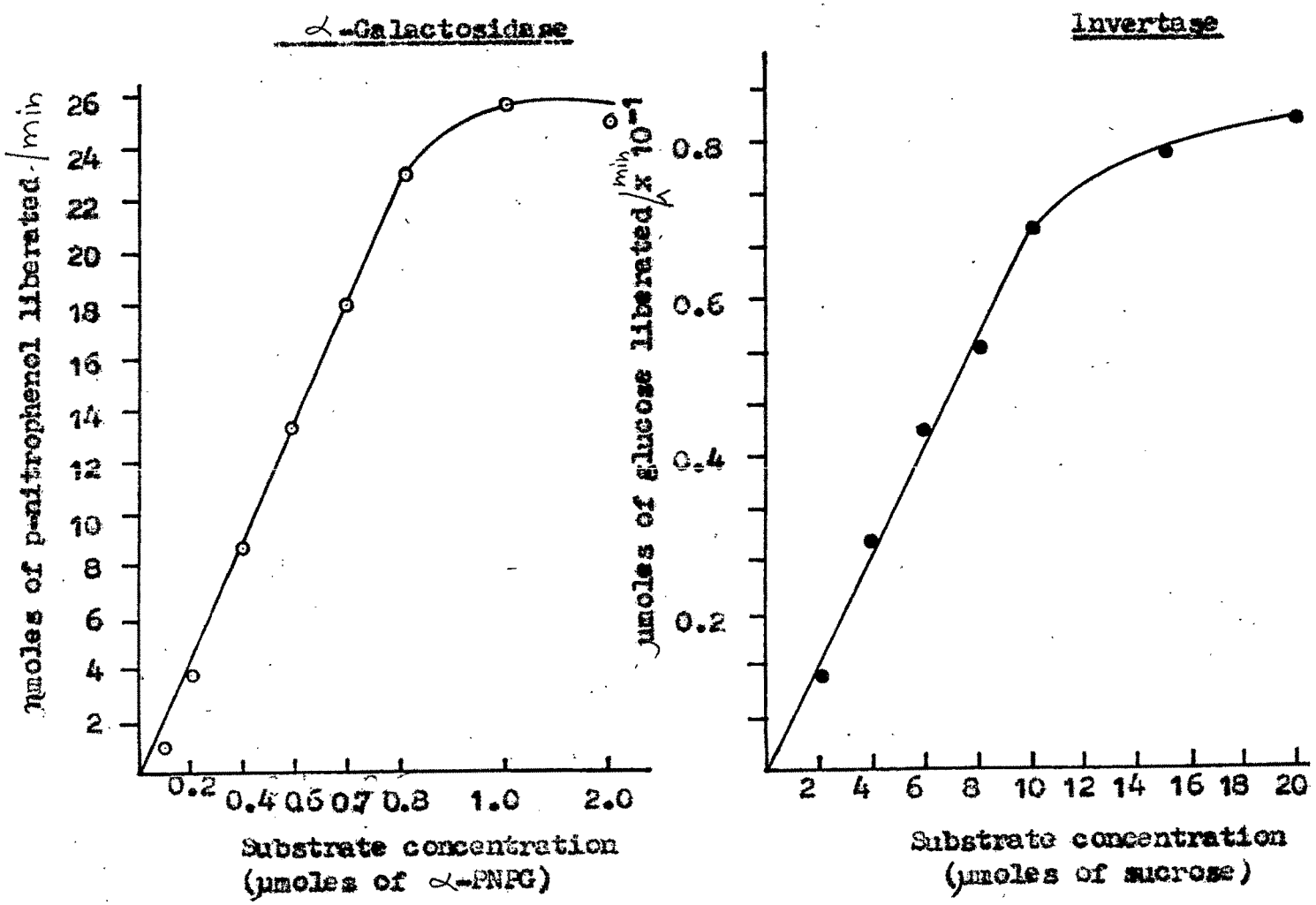


FIG. 23 : Effect of period of incubation on the activity of crude α-galactosidase and invertase of Bacillus sp. I.

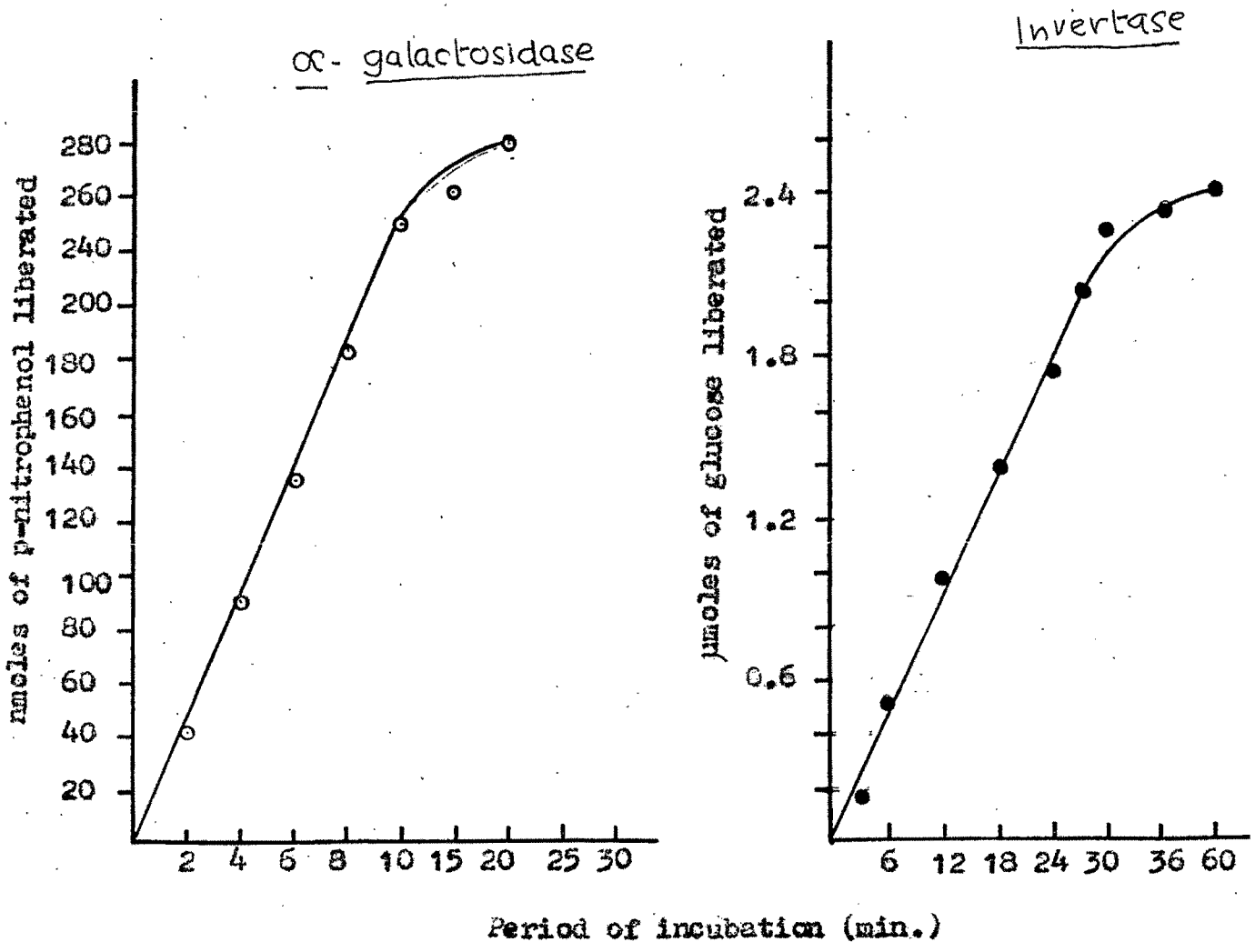


FIG.24 : Effect of crude enzyme concentration on α-galactosidase and invertase activity of Bacillus sp. I.

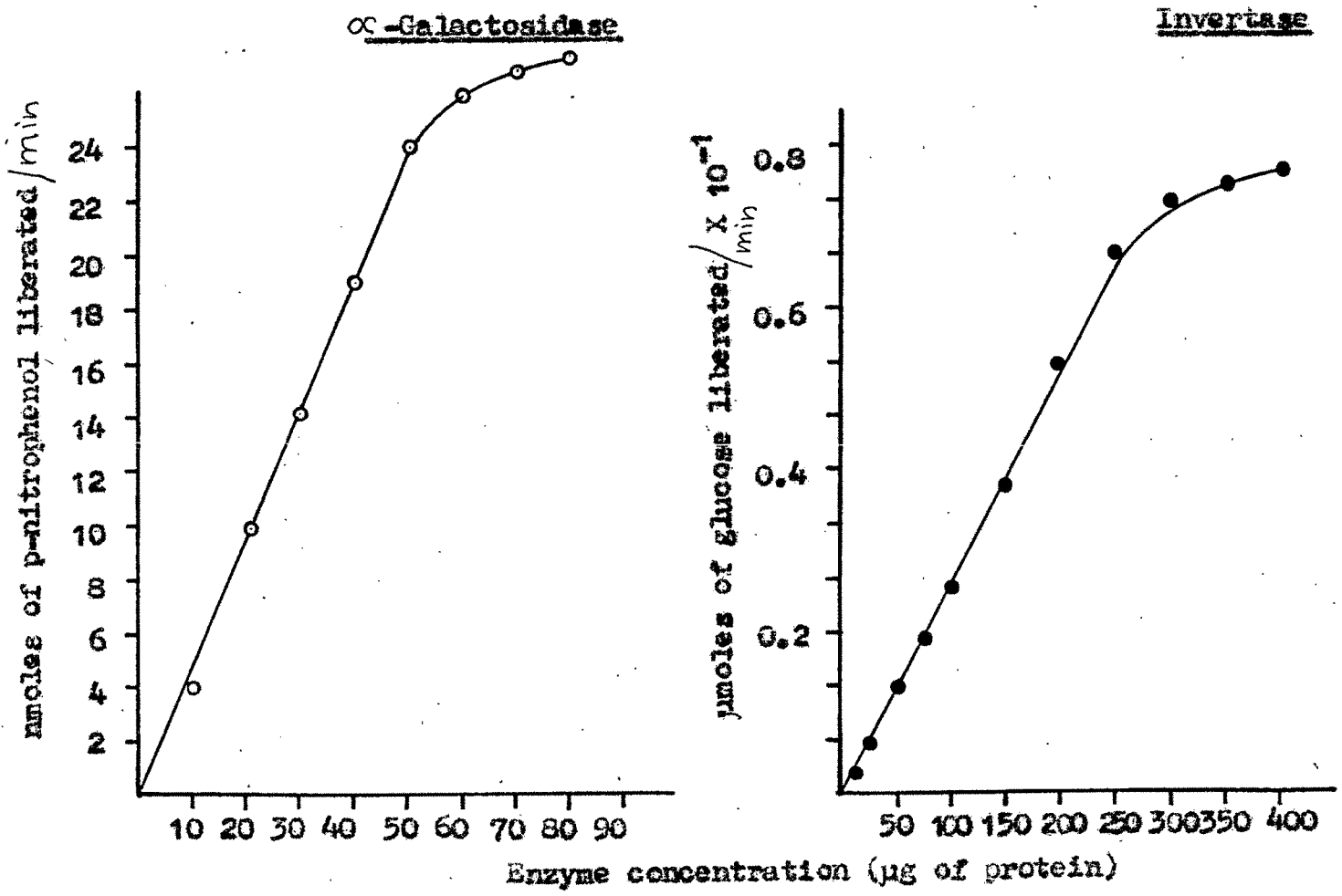


FIG. 25 : Effect of temperature on the activity of crude ∝-galactosidase and invertase of Bacillus sp. I.

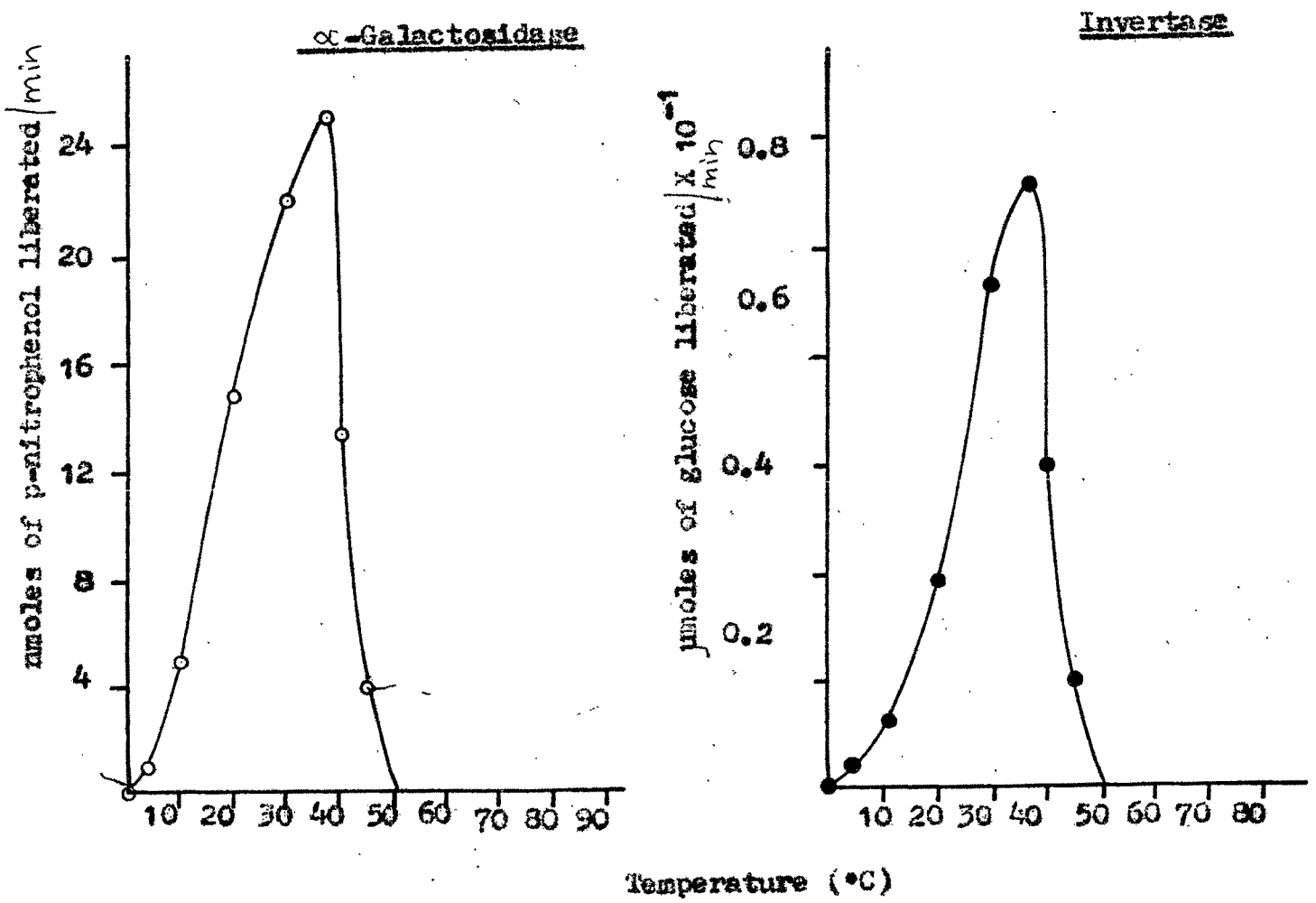


TABLE-89 : Effect of mercaptoethanol on the stability of raffinose hydrolysing enzymes of Bacillus sp. I.

Period of storage (hr)	Specific activity of enzymes (units of enzyme/mg protein)							
	Without mercaptoethanol				With mercaptoethanol			
	∝-galactosidase		Invertase		∝-galactosidase		Invertase	
0	0.328	(100)*	0.202	(100)	0.330	(100)	0.225	(100)
6	0.306	(93)	0.181	(90)	0.316	(96)	0.220	(99)
12	0.153	(47)	0.128	(63)	0.300	(91)	0.216	(96)
18	0.155	(35)	0.072	(36)	0.286	(87)	0.196	(86)
24	0.086	(26)	0.036	(18)	0.265	(80)	0.182	(81)
36	0.052	(16)	0.017	(8)	0.246	(75)	0.160	(71)
48	0.032	(10)	0.009	(4)	0.229	(69)	0.135	(60)

Crude enzyme preparations were stored at 7-8° in 50 mM sodium phosphate buffer, pH 7.0. Aliquotes were withdrawn at different time intervals and assayed for the residual enzyme activity.

* Values in parentheses are percentage residual activity.

Cultural conditions and nutritional requirement for maximum production of raffinose hydrolysing enzymes by Bacillus sp.I :

Before carrying out purification of enzymes, studies were carried out to find out the optimum conditions under which Bacillus sp. I will synthesize maximum amount of α-galactosidase and invertase in the cells. The results of these studies are presented in Tables-90 to 99.

It can be seen from the data presented in Table-90 that organism showed the maximum amount of growth and production of both the enzymes when the initial pH of the medium was 6.5 to 7.0. As far as the effect of amount of inoculum added is concerned, it showed linear increase in growth of bacteria and both the enzyme production, upto inoculum size of 4×10^6 cells/flask (Table-91). Data on the effect of period of incubation (Table-92) show that production of both the enzymes were optimum at 12 hr of incubation time. Data given in Table-93 show the effect of raffinose concentration in the medium on growth and production of enzymes. A linear increase in growth and production of enzymes was observed till 0.4% of raffinose in basal medium. Raffinose concentration higher than 1.0% inhibited the production of both the enzymes, but with a very high increase in turbidity. Data given in Table-94 show that aeration of the culture during incubation significantly increased the growth as well as production of both the enzymes.

TABLE-90 : Effect of initial pH of the medium on growth and production of raffinose hydrolysing enzymes of *Bacillus* sp. I.

pH		Growth in terms of turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
Initial	Final	Initial	Final	α-Galactosidase	Invertase
2.0	2.0	0	0	0	0
3.0	3.0	0	0	0	0
4.0	3.8	0	65	0.40	0.26
5.0	4.0	0	210	1.62	1.00
6.0	4.5	0	410	3.54	2.03
6.5	5.0	0	462	4.25	2.53
7.0	5.7	0	480	4.26	2.45
7.5	6.0	0	405	3.20	1.95
8.0	7.2	0	255	2.05	1.16
9.0	9.0	0	22	0.02	0.05
10.0	10.0	0	0	0.0	0.0

25 ml of basal medium were taken in 100 ml conical flask and the pH of the medium was adjusted desired values using either 1N NaOH or 1N HCl. The medium was then sterilized by autoclaving a 121° for 15 min. Raffinose solution (20%) was sterilized separately and then added to each fla to final concentration 0.5%. The medium was then inoculated with (5×10^6 cells/flask) and inc at 30° for 18 h. Control flasks were kept at 2°. Enzyme activity was measured as described und 'Materials and Methods'.

TABLE-91 : Effect of inoculum size on growth and production of raffinose hydrolysing enzymes by *Bacillus sp.* I

Inoculum size (number of cells added per flask)	pH of the medium		Growth in terms of turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	∝-galactosidase	Invertas
0.5×10^6	7.0	7.0	0	48	0.32	0.25
1.0×10^6	7.0	6.5	0	160	1.25	0.72
2.0×10^6	7.0	6.0	0	285	2.46	1.35
3.0×10^6	7.0	6.0	0	410	3.18	2.02
4.0×10^6	7.0	5.6	0	485	4.15	2.45
5.0×10^6	7.0	5.5	0	500	4.28	2.56
7.5×10^6	7.0	5.5	0	550	4.64	2.72
10.0×10^6	7.0	5.5	0	600	4.8	2.84

Details of the experiment are same as in Table-90, except for the variation in the inoculum size.

TABLE-92 : Effect of period of incubation on growth and production of raffinose hydrolysing enzymes of *Bacillus sp.* I.

Period of incubation (h)	pH of the medium		Growth in terms of turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-galactosidase	Invertase
0	7.0	7.0	0	10	0	0
3	7.0	6.8	0	65	0.43	0.31
6	7.0	6.2	0	275	2.26	1.35
9	7.0	6.0	0	395	3.28	2.34
12	7.0	5.5	0	445	4.05	2.48
18	7.0	5.5	0	470	4.26	2.51
24	7.0	5.2	0	485	3.95	2.25
36	7.0	5.2	0	405	3.56	2.00
72	7.0	5.0	0	355	3.05	1.83

Details of the experiment are same as in Table-90, except for the variation in the time of incubation for growth of the culture.

TABLE-93 : Effect of raffinose concentration in the medium on growth and production of raffinose degrading enzymes of *Bacillus* sp. I.

Concentration of raffinose in basal medium (%)	pH of the medium		Growth in turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-galactosidase	Invertase
0	7.0	7.0	0	0	0	0
0.1	7.0	6.0	0	105	0.82	0.52
0.2	7.0	6.2	0	230	1.85	1.15
0.3	7.0	5.8	0	345	2.62	1.67
0.4	7.0	5.4	0	495	3.96	2.33
0.5	7.0	5.2	0	520	4.28	2.50
0.75	7.0	5.0	0	560	4.55	2.82
1.00	7.0	5.0	0	600	2.56	1.77
1.5	7.0	4.0	0	600	0.65	0.33
2.0	7.0	3.5	0	600	0	0

Details of the experiment are same as in Table-90, except for the variation in the raffinose concentration in the medium.

TABLE-94 : Effect of aeration on growth and production of raffinose hydrolysing enzymes of Bacillus Sp. I.

	pH of medium		Growth in turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-galacto-sidase	Invertase
Stationary culture	7.0	6.6	0	150	1.20	0.73
Shake culture	7.0	5.6	0	525	4.30	2.43

Details of the experiment are same as in Table-90, except for the variation in conditions of incubation.

The effect of glucose was studied by adding different concentrations of glucose in raffinose broth during the growth of bacteria. Data presented in Table-95 indicate that at higher concentrations (more than 0.4%), glucose inhibited the production of both the enzymes with concomitant increase in turbidity.

After determining the optimum cultural conditions for the growth of Bacillus sp. I and production of α-galactosidase and invertase an attempt was made to study the nutritional requirements of bacteria and effect of other components present in L. sativus seeds.

Different sugars were compared for the growth and enzyme production. As shown in Table-96, melibiose, raffinose, stachyose, and galactose could induce production of α-galactosidase, whereas invertase was produced during growth in almost all the sugars tried except in case of cellulose, cellobiose and lactose which were not readily utilizable by the bacteria for their growth. Studies were made on the effect of substituting ammonium sulfate by various other nitrogen sources (Table-97). All the ammonium salts tried gave the same amount of growth as well as enzyme production whereas urea inhibited growth and production of enzyme. Organic nitrogen sources viz. peptone, yeast-extract and casein hydrolysate increased the growth of bacteria, but did not increase the production of both enzymes significantly.

TABLE-95 : Effect of different concentration of glucose on growth and production of raffinose hydrolysing enzymes of *Bacillus* sp. I.

Concentration of glucose (%)	pH of the medium		Growth in terms of turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-Galactosidase	Invertase
Control*	7.0	5.5	0	480	4.25	2.52
0.025	7.0	5.5	0	500	4.20	2.55
0.05	7.0	5.5	0	515	4.40	2.57
0.10	7.0	5.3	0	525	4.60	2.68
0.20	7.0	5.2	0	550	4.65	2.70
0.30	7.0	5.2	0	575	3.69	2.40
0.40	7.0	4.8	0	600	1.80	0.85
0.50	7.0	4.2	0	600	0	0
0.75	7.0	4.0	0	>600	0	0
1.0	7.0	3.8	0	>600	0	0
2.0	7.0	3.5	0	>600	0	0
3.0	7.0	3.5	0	>600	0	0

Details of the experiment are same as in Table-90, except the different concentration of glucose ✱ was added in raffinose broth.

* Control did not contain any glucose in the medium.

TABLE-96 : Effect of different sugars on growth and production of raffinose hydrolysing enzymes of *Bacillus sp.* I.

Sugar	pH of the medium		Growth in turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	∝-galactosidase	Invertas
Glucose	7.0	4.5	0	450	0	1.85
Galactose	7.0	4.8	0	380	4.36	1.75
Fructose	7.0	4.8	0	410	0	1.67
Sucrose	7.0	5.1	0	500	0	3.18
Lactose	7.0	7.0	0	15	0	0
Cellobiose	7.0	6.9	0	45	0	0
Maltose	7.0	5.2	0	500	0	1.83
Melibiose	7.0	5.5	0	425	4.28	2.05
Raffinose	7.0	5.4	0	395	4.30	2.55
Cellulose	7.0	7.0	0	20	0	0
Stachyose	7.0	5.5	0	400	3.89	2.61

Details of the experiment are same as in Table-90, except the medium contained 0.5% of different sugars, instead of raffinose.

TABLE-97 : Effect of different nitrogen sources on growth and production of raffinose hydrolysing enzymes of Bacillus sp. I.

Nitrogen source	Final pH of the medium	Growth in terms of turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
		Initial	Final	∝-galactosidase	Invertase
Ammonium nitrate	5.8	0	450	4.0	2.35
Ammonium chloride	5.5	0	520	4.45	2.52
Ammonium sulphate	5.4	0	525	4.56	2.66
Casein	5.8	0	550	3.26	1.50
Casein hydrolysate	5.6	0	525	4.05	2.52
Peptone	5.5	0	600	4.58	2.62
Urea	8.2	0	280	1.80	1.21

Details of the experiment are same as in Table-90, except the medium contained 0.2% of different nitrogen sources.

When the effect of different vitamins was studies, it was found that none of them significantly affected the growth of bacteria as well as production of raffinose hydrolysing enzymes (Table-98). Table-99 gives the data on effect of addition of different amino acids (which are present in legumes) to the medium. It was found that addition of amino acids did not affect the production of raffinose hydrolysing enzymes in Bacillus sp. I.

In summary, Bacillus sp. I grow well and produce raffinose hydrolysing enzymes in a basal salt medium containing 0.5% raffinose, when initial pH of the medium adjusted to 7.0 and incubated at 30 for 12 h. The vitamins, and amino acids commonly present in legumes do not affect the production of raffinose hydrolysing enzymes.

TABLE-98 : Effect of different vitamins on growth and production of raffinose hydrolysing enzymes of *Bacillus* sp. I.

Type of vitamin	pH of the medium		Growth in turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-Galacto-sidase	Invertase
Control	7.0	5.5	0	510	4.32	2.54
Thiamine	7.0	5.5	0	490	4.65	2.50
Riboflavin	7.0	5.6	0	450	4.30	2.52
Niacin	7.0	5.5	0	550	4.55	2.85
Biotin	7.0	5.2	0	600	5.08	3.08
Pyridoxine	7.0	5.5	0	550	5.00	2.98
Pantothenic acid	7.0	5.8	0	400	3.60	2.02
Inositol	7.0	5.6	0	495	4.20	2.50
Folic acid	7.0	5.6	0	500	4.30	2.50
Carotene	7.0	5.5	0	510	4.30	2.50

Details of the experiment are same as in Table-90, except raffinose broth contained different vitamins at concentrations of 0.01%.

TABLE-99 : Effect of different amino acids on growth and production of raffinose hydrolysing enzymes of Bacillus sp. I.

Type of amino acid	pH of the medium		Growth in turbidity (KR at 660 nm)		Enzyme activity (units/flask)	
	Initial	Final	Initial	Final	α-Galacto-sidase	Inverta
Control[a]	7.0	5.5	0	510	4.32	2.54
Lysine	7.0	5.7	0	450	4.28	2.50
Leucine	7.0	5.7	0	475	4.28	2.46
Isoleucine	7.0	5.8	0	475	4.35	2.50
Glutamic acid	7.0	5.9	0	450	4.30	2.50
Aspartic acid	7.0	5.9	0	450	4.30	2.36
Phenylalanine	7.0	5.6	0	500	4.40	2.64
Proline	7.0	5.5	0	500	4.38	2.68
Alanine	7.0	5.7	0	500	4.36	2.45
Methionine	7.0	5.6	0	525	4.42	2.72
Threonine	7.0	5.6	0	500	4.30	2.45
Tyrosine	7.0	6.0	0	425	3.65	2.02
Tryptophan	7.0	5.8	0	490	3.89	2.12
Histidine	7.0	5.5	0	550	4.45	2.60
Glycine	7.0	5.5	0	550	4.58	2.75
Cysteine	7.0	5.5	0	550	4.62	2.82

Details of the experiment are same as in Table-90, except raffinose broth contained 0.1% of different amino acids (stock, concentrated solution of each amino acid was prepared and autoclaved separately at 15 psi for 15 min).
'a' - Control did not contain any amino acid.

Studies on regulation of raffinose hydrolysing enzymes production in Bacillus sp. I :

All known regulatory mechanisms in micro-organisms are mediated through allosteric proteins, the activity of which is altered by binding of small molecules. Hence they serve as sensitive detectors of the intracellular concentration of key metabolites and modulate the overall metabolic activity of the cell in such a manner as to maximize the rate of growth, by ensuring the conversion of nutrients into cell material with maximal efficiency. The key regulatory mechanisms in bacteria are end product inhibition (e.g., feed back control of pyrimidine synthesis), enzyme induction : negative control (induction of β-galactosidase), enzyme induction : positive control (induction of enzymes that metabolise arabinose), catabolite repression (glucose repressing of β-galactosidase synthesis) and end product repression. (cf. Stanier et al, 1984).

The growth rate of a bacteria is strongly regulated by its nutritional, chemical and physical environment. In larger perspective, the control of growth rate involves a number of factors; the transport of nutrients into the cell and their distribution through the pathways of intermediary metabolism, synthesis of specific proteins, regulation of synthesis of the components of protein forming systems and control over the processes providing for the growth and division of cell wall and replication of genome (Donald Nierlich, 1978).

Enzymatic adaptation has been known for a long time in bacteria, yeasts and molds (Stanier, 1951). Since the pioneer studies on β-galactosidase from E. Coli (Monod, 1947; Monod et al, 1951) extensive knowledge has been gained on the induction and catabolite repression of the synthesis of this enzyme and the effect of glucose ("catabolite repression") (Magasanik, 1961; Schwartz and Beckwith, 1970). Now it is believed that catabolite repression in bacteria is mediated by cyclic AMP and intracellular receptor CAP protein (de Crombrugghe et al, 1971). However, the role of cAMP as the unique regulator has been challenged (Dessein et al, 1978a,b). Catabolite repression has also been found in yeast invertase production (Gascon and Ottolenghi, 1972), but here again the role of cAMP is the subject of controversy (Sy and Ritcher, 1972; Vander Plat, 1974). Regulation of α-galactosidase and invertase production in micro-organisms at transcriptional and translational level has been studied by use of inhibitors viz. 8-hydroxyquinoline (Frazer and Geanor, 1974), 5-fluorouracil (Kepes, 1963), chloramphenicol (Elorza et al, 1977), 5-fluoro-deoxyuridine, 5-azoguanine, actinomycin-D, cyclohexamide (Maheshwari et al, 1983) and temperature sensitive mutants (Martinez et al, 1982).

In the present experiment, an attempt was made to study the regulation of production of α-galactosidase from raffinose degrading, Bacillus sp. I. The experiment presents an evidence of induction of α-galactosidase at transcriptional

level using rifampicin as an inhibitor of transcription. Rifampicin, the active semisynthetic derivative of rifamycin-B inhibits the synthesis of all cellular RNA in susceptible bacteria while DNA synthesis is unaffected (Calvori, et al, 1965). Since it forms a tight complex with bacterial DNA dependent RNA polymerase and inhibits transcription, it can be used to understand the regulatory mechanisms in bacteria.

Figs.26, 27 & 28 show the growth and levels of raffinose hydrolysing enzymes when Bacillus sp. I was grown in basal salt media containing different sugars as 'C' source. When the growth of bacteria in different sugars was compared, it was found that it was more in case of glucose and sucrose (Fig.26). Among the sugars studied, raffinose, stachyose, melibiose and galactose induced the production of α-galactosidase. Glucose and sucrose did not induce the production of α-galactosidase, eventhough the growth was highest in presence of both the sugars, (Fig.27). The induction of α-galactosidase was increased when the growth of cells was increased in the case of galactose, raffinose, melibiose and stachyose. The kinetics of invertase production by Bacillus sp. I grown on various sugars is shown in Fig.29. Cells incubated in the presence of all the sugars showed production of invertase indicating that the synthesis of this enzyme is constitutive in Bacillus sp. I. Cells grown with sucrose shows an early synthesis of invertase during the growth of bacteria compared to the other sugars. Synthesis of α-galactosidase took place in cells grown in galactose or α-D-galactose containing sugars (Fig.27). Cells incubated

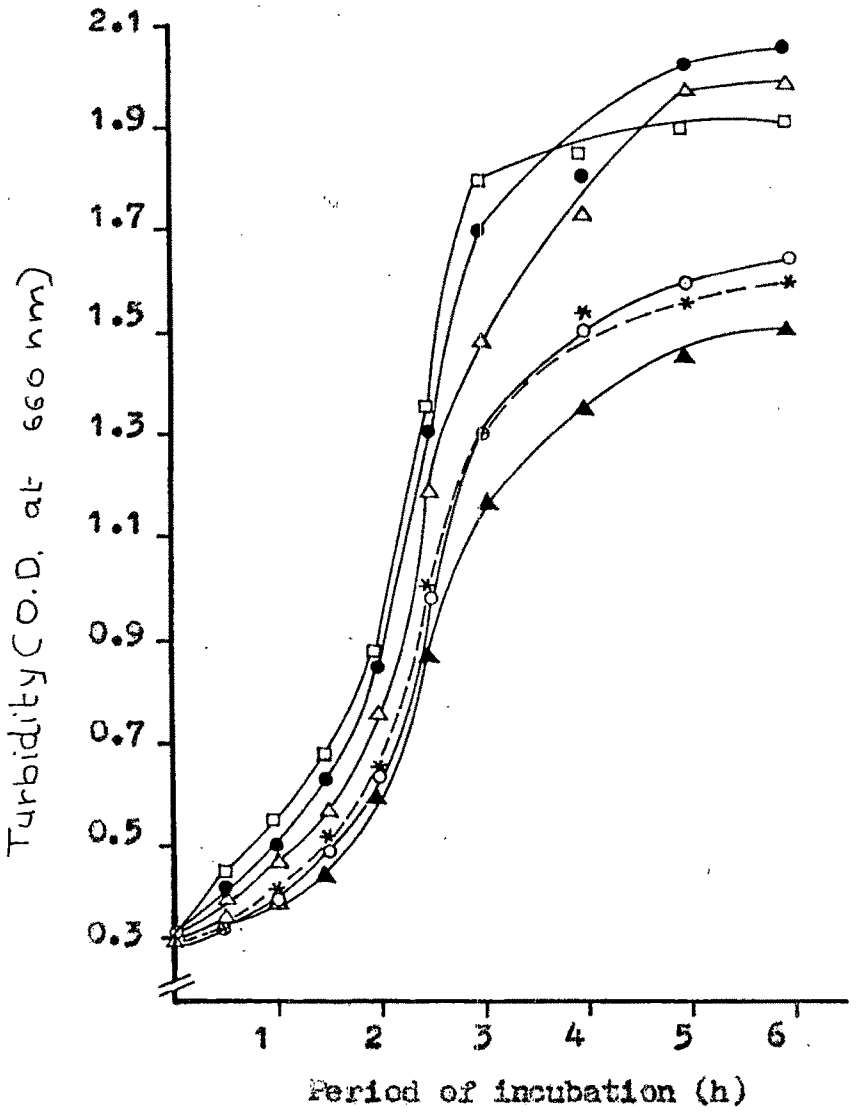


FIG.26 : Growth of Bacillus sp. I in presence of various sugars.
Cells were grown in a basal salt medium containing following sugars as carbon source : raffinose (○—○), melibiose (*---*), stachyose (▲—▲), galactose (△—△), glucose (□—□), sucrose (●—●).

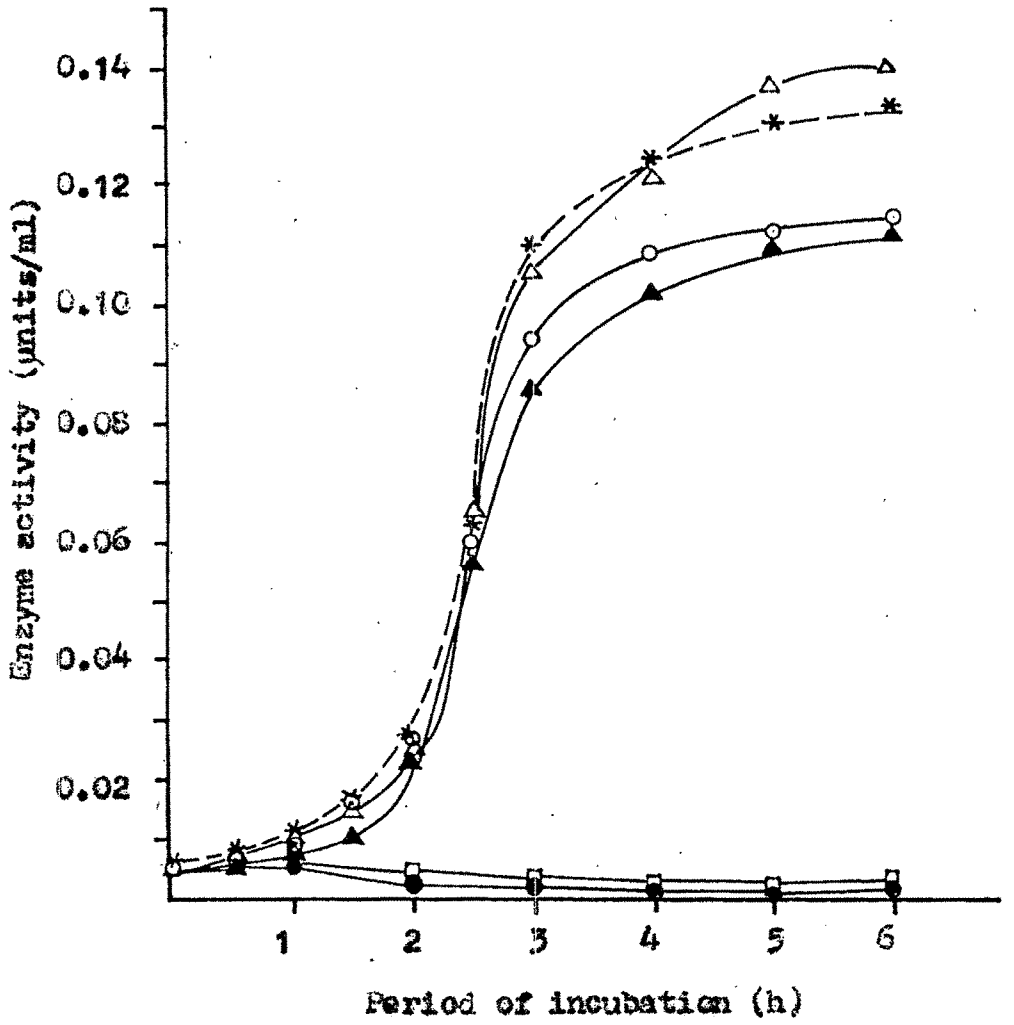


FIG.27 : Effect of carbon sources on ∝-galactosidase production during growth of Bacillus sp. I. Cells were grown in basal medium containing following sugars as carbon source : raffinose (o—o), melibiose (*---*), stachyose (▲—▲), galactose (△—△), glucose (□—□), sucrose (●—●). Enzymatic activity expressed as units per ml of culture.

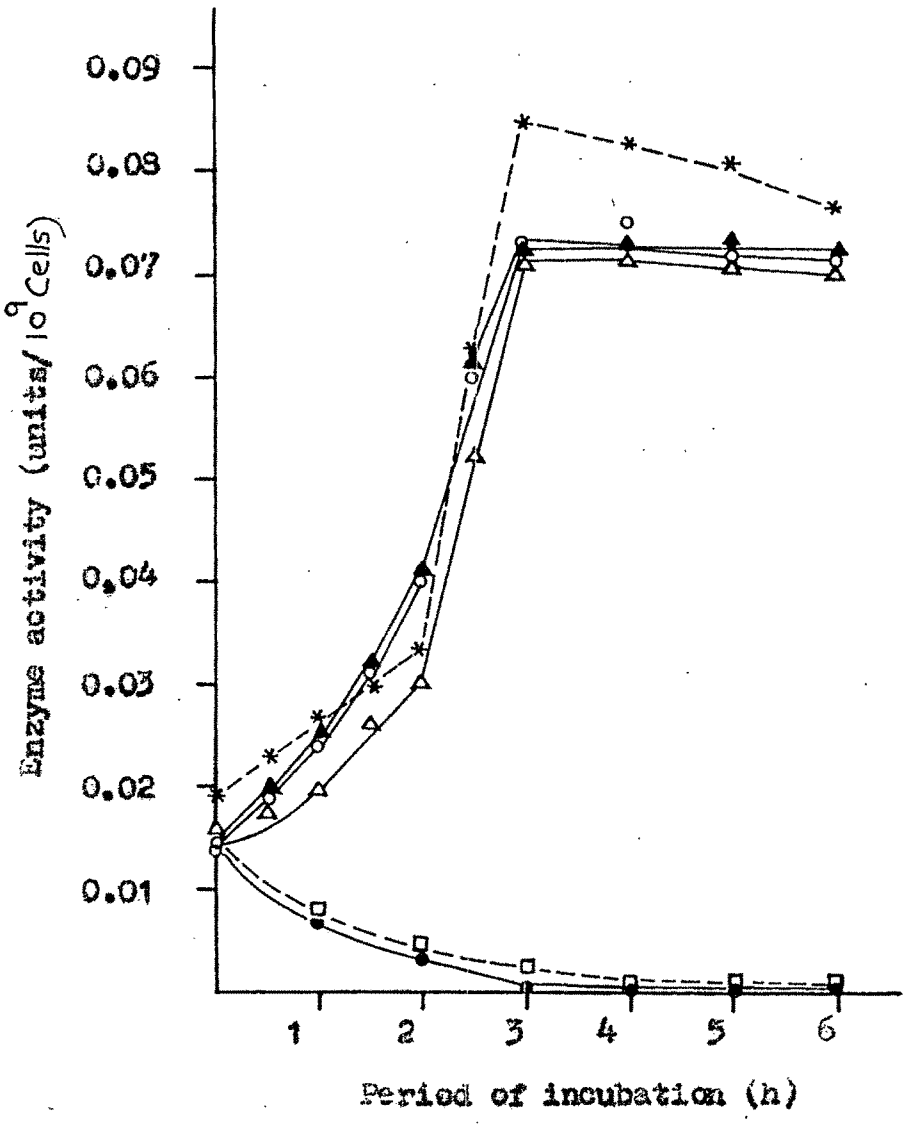


FIG.28 : Effect of carbon sources on ∝-galactosidase production during growth of Bacillus sp. I. Cells were grown in basal medium containing following sugars as carbon source : raffinose (o—o), melibiose (*--*), stachyose (▲—▲), galactose (△—△), glucose (□—□), sucrose (●—●). Enzymatic activity is expressed as enzyme units per of cells.

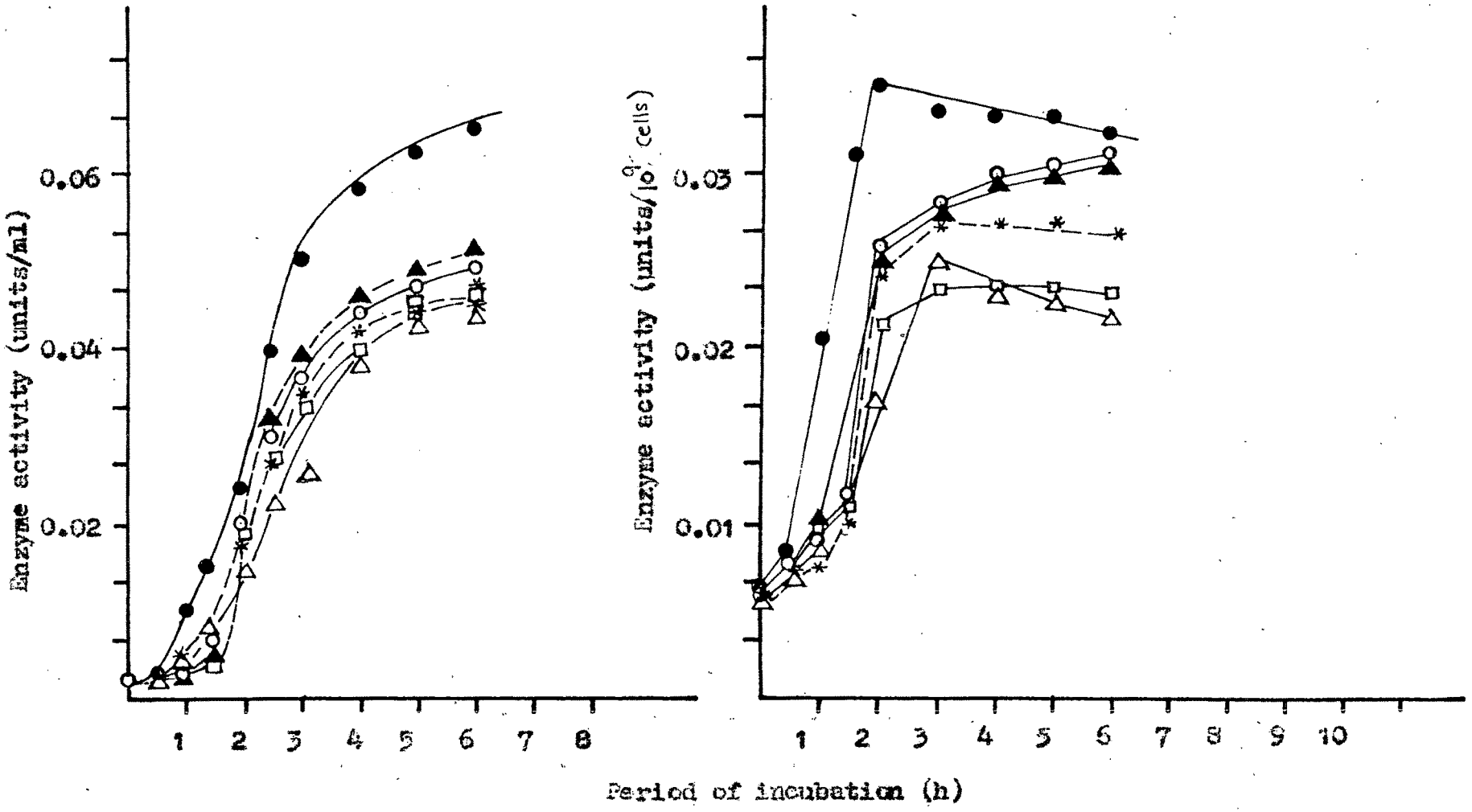


FIG.29 : Effect of carbon sources on invertase production during growth of Bacillus sp. I. Cells were grown in basal medium containing following sugars as carbon source : raffinose (○—○), melibiose (✳---✳), galactose (△—△), stachyose (▲—▲), glucose (□—□), sucrose (●—●).

with melibiose showed highest activity when the values were expressed as enzyme units/cell, whereas stachyose, raffinose and galactose showed the same levels of induction of ∝-galactosidase but lower than that of melibiose (Fig. 28). Thus it can be concluded from these data that ∝-galactosidase is an inducible enzyme whereas invertase is a constitutive enzyme in Bacillus sp. I.

The effect of glucose was studied on the growth and production of ∝-galactosidase and invertase, to see whether the regulation of production of these enzymes occur by catabolite repression. When the cells were grown in presence of 0.1% or 0.5% glucose in raffinose broth the diauxic growth pattern was not seen (Fig.30). When ∝-galactosidase activity was plotted against period of incubation, it was seen that induction of enzyme was not affected by glucose, when the cells were grown in raffinose broth (Fig.31). Similar results were obtained with invertase also (Fig.32). These experiments suggested that glucose did not bring about any catabolic repression on the synthesis of ∝-galactosidase or invertase.

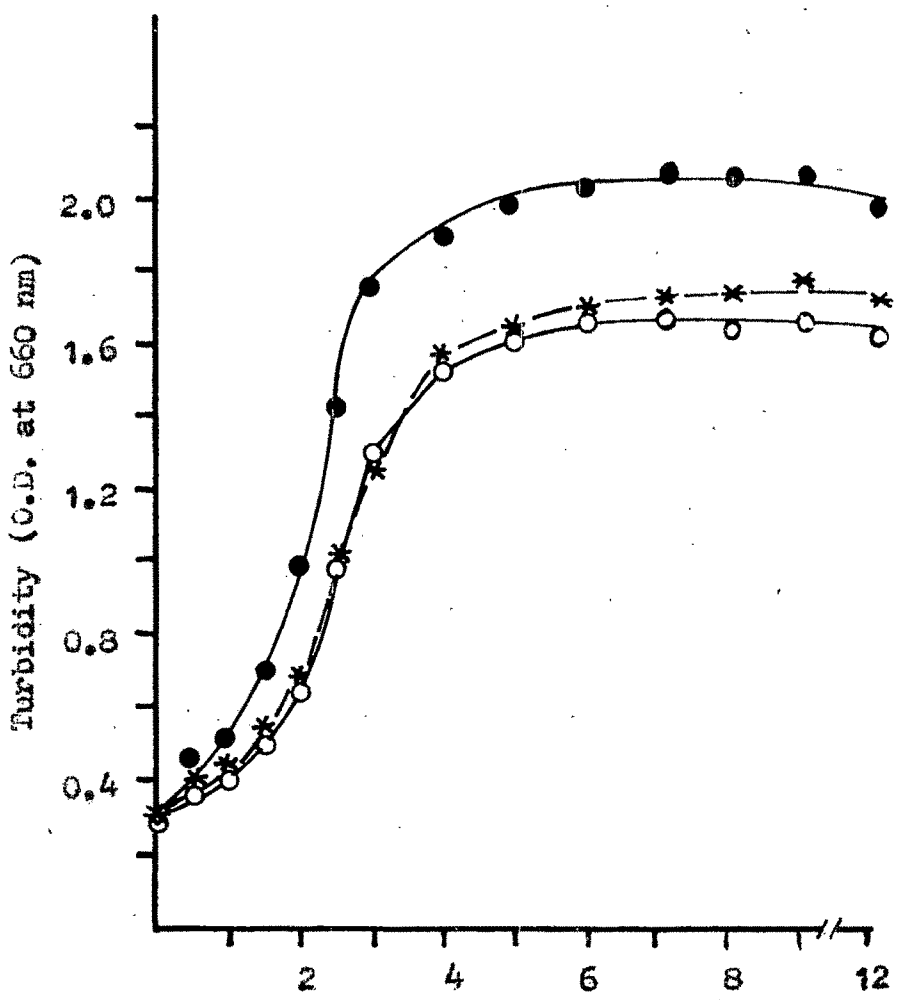


FIG.30 : Effect of glucose on growth of Bacillus sp. I when grown in raffinose broth.

Controls without addition of glucose are represented as (o—o). Experimental with addition of 0.1% glucose are represented as (*---*) and with 0.5% glucose are represented as (•—•).

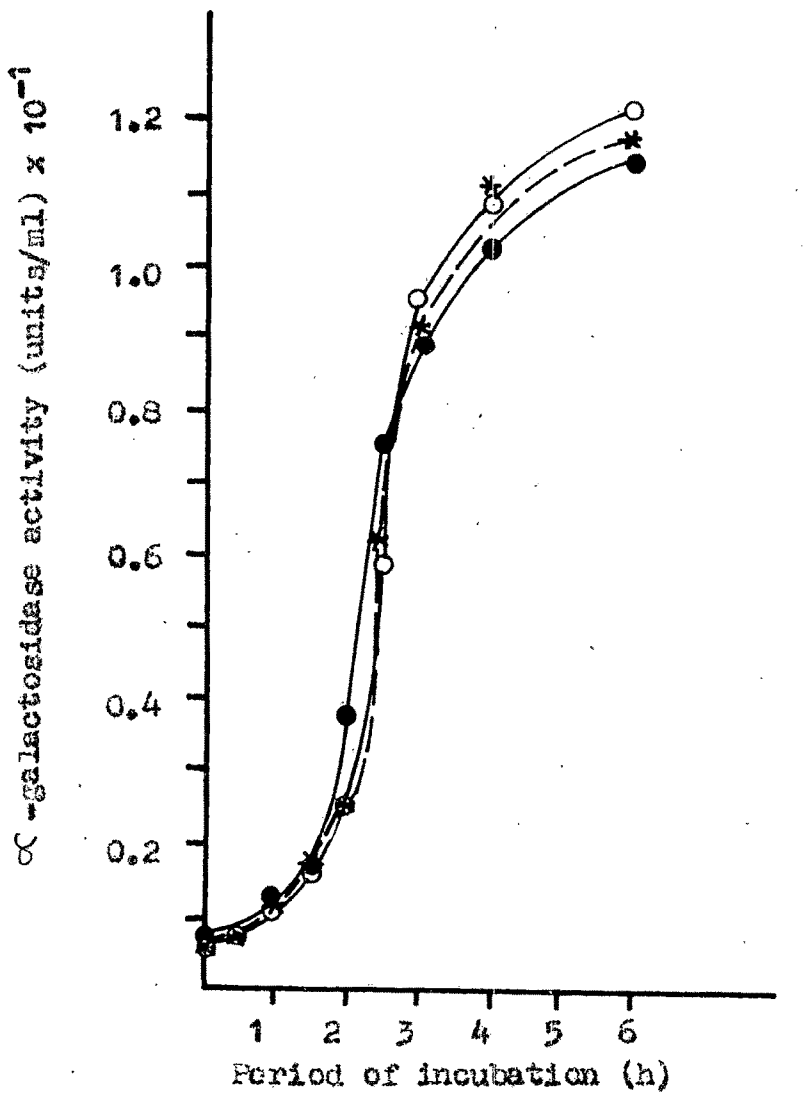


FIG.31 : Effect of glucose on production of ∝-galactosidase when Bacillus sp. I was grown in raffinose broth.

Controls without addition of glucose are represented as (o—o). Experimental with addition of 0.1% glucose are represented as (*---*) and with 0.5% glucose are represented as (●—●).

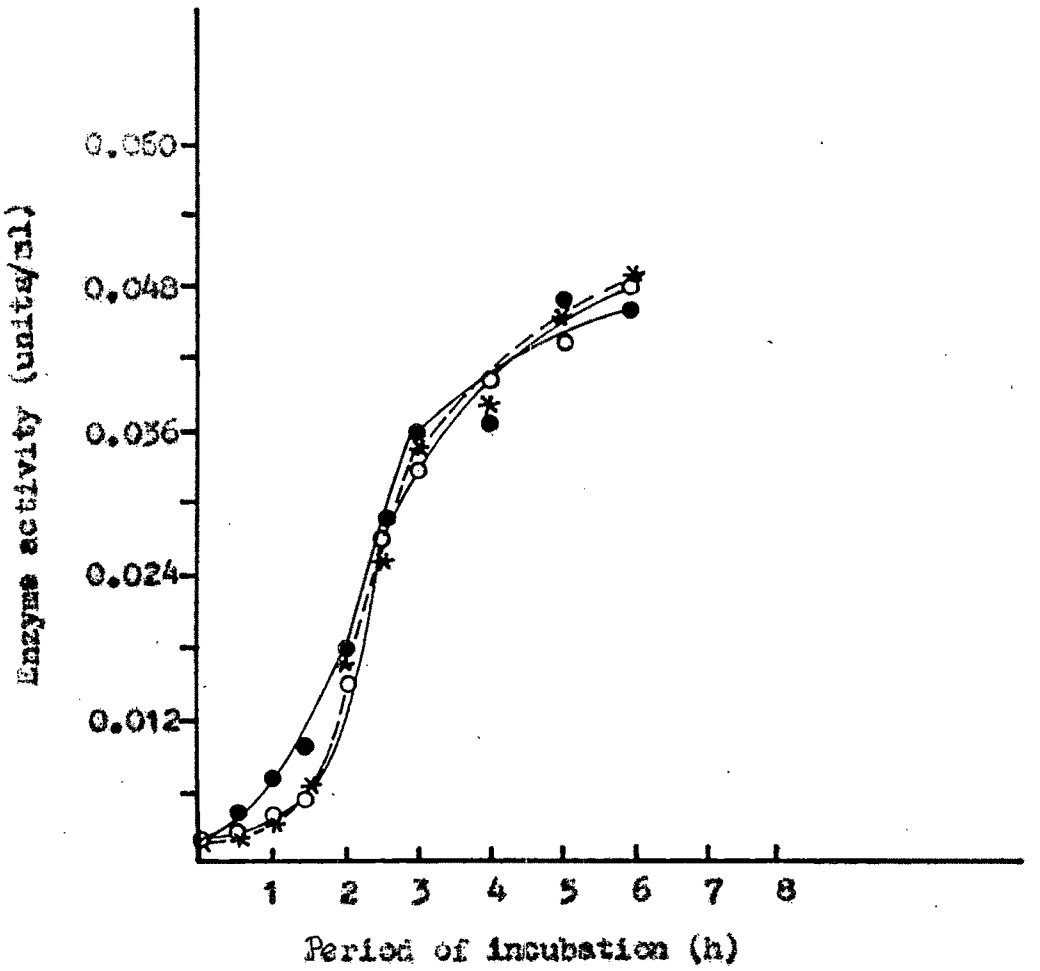


FIG.32 : Effect of glucose on production of invertase when Bacillus sp. I was grown in raffinose broth. Controls without addition of glucose are represented as (○—○). Experimental with 0.1% glucose are represented as (*---*) and with 0.5% glucose are represented as (●—●).

Effect of rifampicin on ∝-galactosidase induction :

In order to study the regulation of production of ∝-galactosidase in Bacillus sp. I effect of rifampicin on induction was studied assuming the molecular mechanisms of ∝-galactosidase production can take place at several levels, viz. transcription of DNA into ∝-galactosidase mRNA; mRNA translation into enzymic protein. The effect of

The effect of rifampicin on the induction of this enzyme was studied with a view to understand whether the induction of this enzyme was regulated at the level of transcription. For this experiment it was necessary to standardise the concentration of rifampicin required to inhibit RNA synthesis and not protein synthesis. First, the effect of rifampicin in protein synthesis was studied by incorporation of radioactive C^{14}-leucine into protein by cells during their growth. It was found that rifampicin at 50 µg/ml concentration did not inhibit synthesis of proteins, but at higher concentrations, it affected the incorporation of ^{14}C-leucine into proteins in Bacillus sp. I (Fig.33). On the other hand, RNA synthesis, as determined by incorporation of (H^3)-uridine, was severely affected by rifampicin at 50 µg/ml concentration as indicated in Fig. 34. (The details of this experiment are described under 'Materials and Methods). Thus it was evident from this experiment that rifampicin at concentration of 50 µg/ml affected transcription of mRNA but not translation of proteins of Bacillus sp. I.

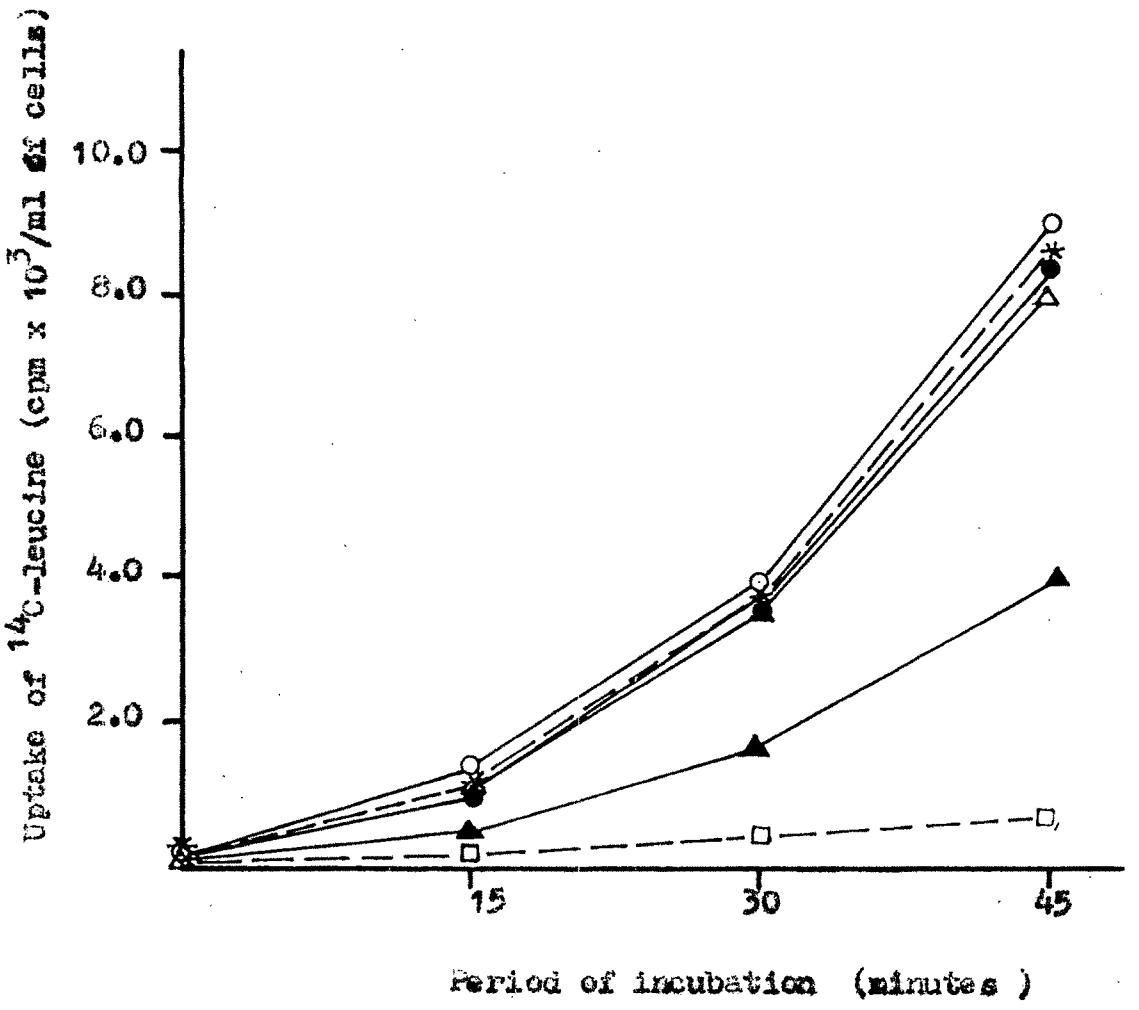


FIG.33 : Effect of rifampicin concentration on incorporation of ^{14}C-labelled leucine into proteins during growth of Bacillus sp. I in raffinose broth.

Controls without addition of rifampicin are represented as (○—○). Experimental contained following concentration of rifampicin : 10 µg/ml (✳---✳), 20 µg/ml (●—●), 50 µg/ml (△—△), 100 µg/ml (▲—▲) and 200 µg/ml (□---□).

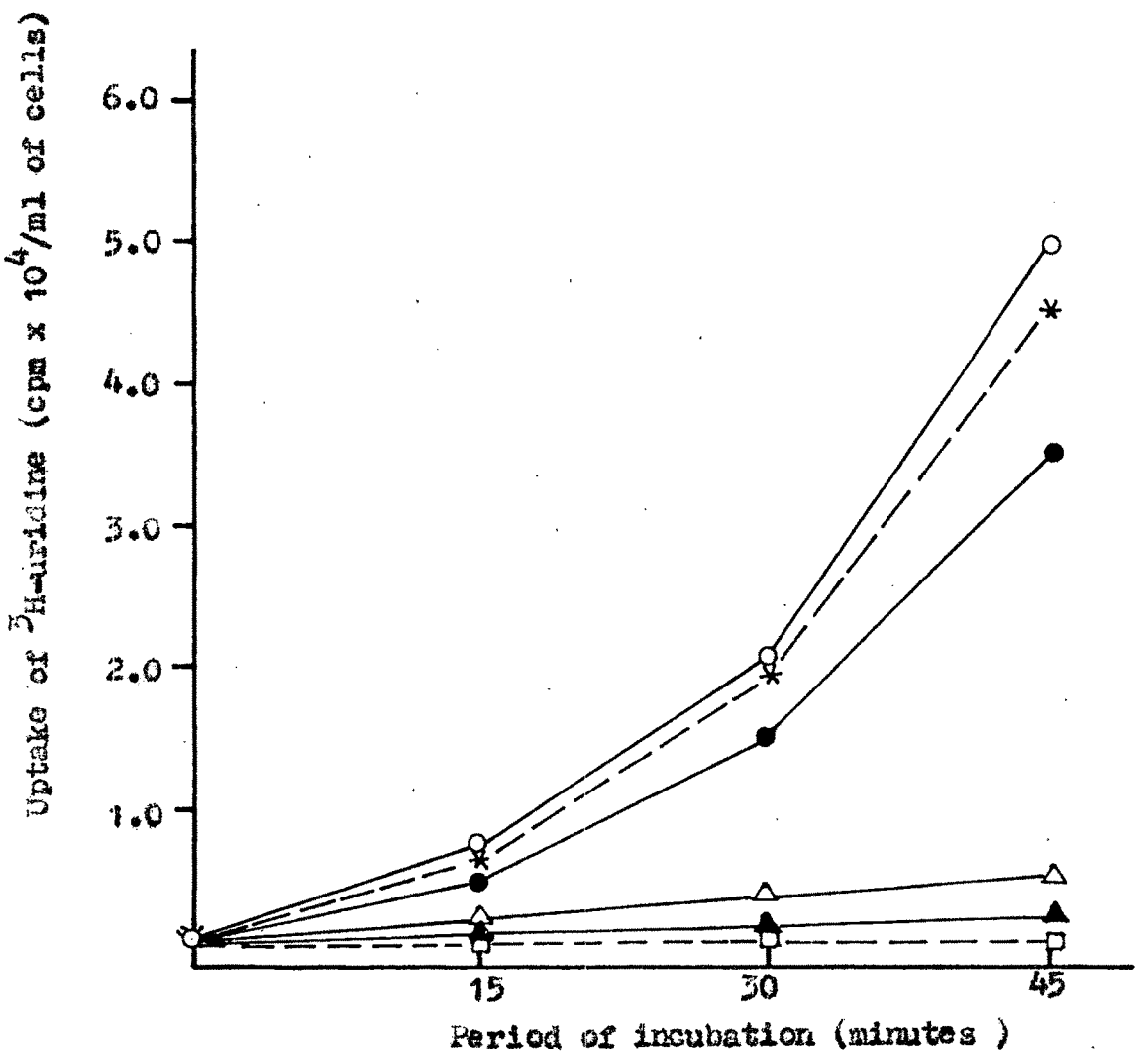


FIG.34 : Effect of rifampicin concentration on incorporation of ^{3}H uridine into RNA during growth of Bacillus sp.I in raffinose broth.
Controls without addition of rifampicin are represented as (○—○). Experimental contained following concentration of rifampicin: 10 µg/ml (*--*), 20 µg/ml (●—●), 50 µg/ml (△—△), 100 µg/ml (▲—▲) and 200 µg/ml (□--□).

For further studies this concentration of rifampicin was used. Figs. 35 & 36 show the effect of rifampicin (50 µg/ml) on growth and expression of ∝-galactosidase. When rifampicin was added at different time intervals during the induction of growth and ∝-galactosidase, it was found that the further induction was inhibited. Thus it can be concluded that addition of rifampicin which will inhibit only transcription and not translation, during the growth, inhibited the production of ∝-galactosidase suggesting that the production of this enzyme is at DNA level or ∝-galactosidase synthesis is dependent on DNA synthesis. This interpretation is based on the fact that rifampicin inhibits transcription of DNA function directly inhibiting RNA polymerase by covalent binding with 'β' subunit of the enzyme (Burgess, 1969). Thus the repressive effect of transcriptional inhibitor on the inducible appearance of ∝-galactosidase activity in cells is indicative of "de novo" synthesis of the enzyme.

Thus the present experiments suggested that in Bacillus sp. I ∝-galactosidase is an inducible enzyme, synthesis of which is not regulated by catabolite repression. In contrast to this invertase production seems to be constitutive in Bacillus sp. I which is also not under the control of catabolite repression.

Alpha galactosidase have been found to be inducible in various other bacteria also viz. E. Coli (Schmitt, 1968; Kawamura et al, 1976; Koppel et al, 1953), A.aerogenes

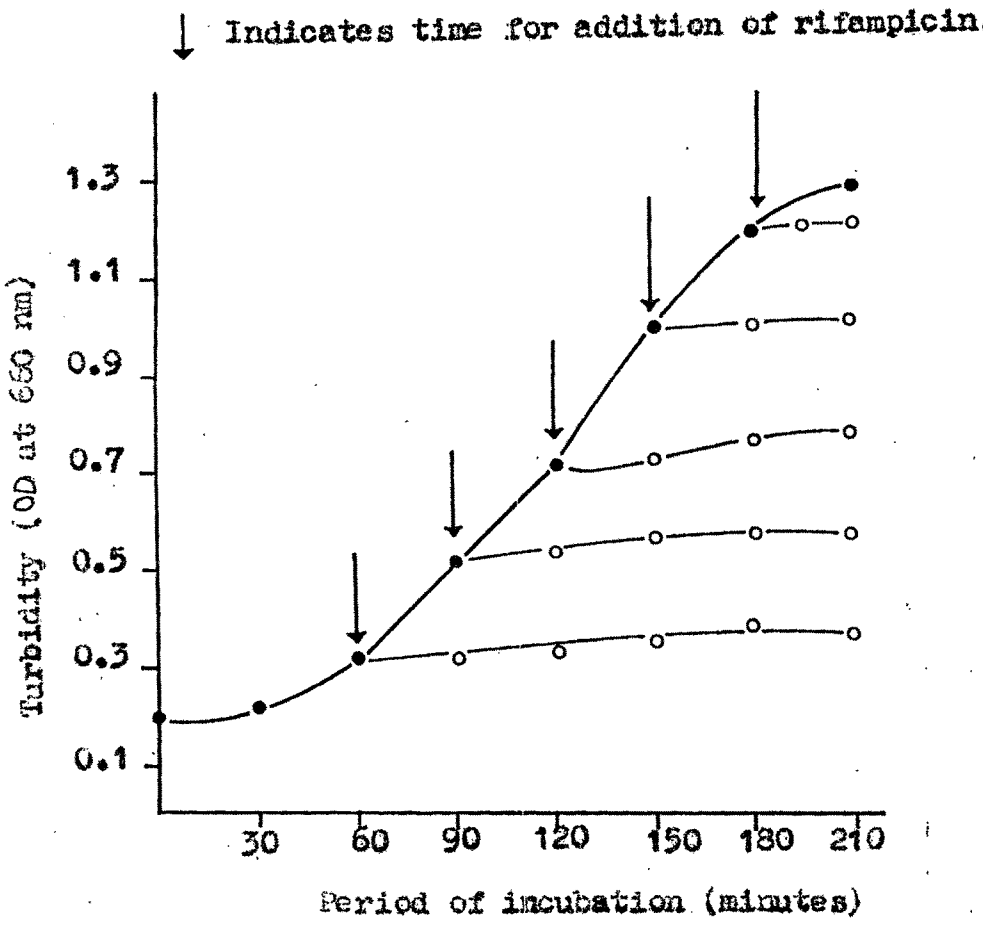


FIG.35 : Effect of addition of rifampicin on growth of Bacillus sp. I in raffinose broth. Controls without addition of rifampicin are represented as (●—●). Experimental with rifampicin (50 µg/ml of medium) are represented as (o—o).

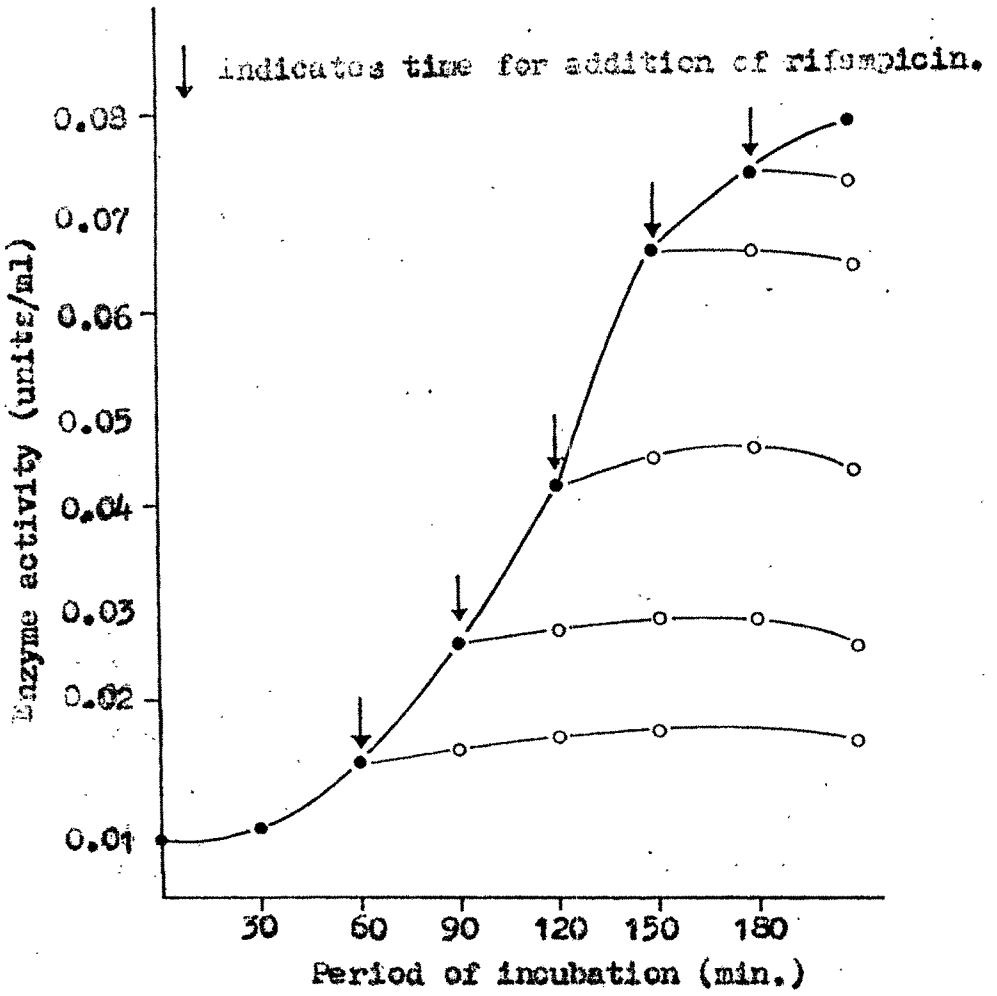


FIG.36 : Effect of addition of rifampicin on induction of ∝-galactosidase of Bacillus sp. I.
Controls without addition of rifampicin are represented as (•—•), Experimental with rifampicin (50 µg/ml of medium) are represented as (○—○).

(Hogness and Battley, 1957), Micrococcus sp. (Akiba and Horikoshi, 1976) and B. stearothermophilus (Delente et al, 1974). However, constitutive synthesis of α-galactosidase have been reported in Lactobacillus sp. (Mital et al, 1973), B. stearothermophilus NCA-2184 (Mosely, 1976), D. pneumoniae (Li et al, 1963). Alpha galactosidase from yeast S. carlsbergensis was originally described as an adaptive enzyme (Lindergren et al, 1944; Lazo et al, 1977; 1978) whose synthesis was dependent on the presence of galactose residue of α-galactosides. The present experiment also shows that galactose as well as sugars containing α-galactosides serve as inducers for the synthesis of α-galactosidase in Bacillus sp. I. Similar observations were made in yeast for the synthesis and secretion of this enzyme (Lazo et al, 1981; Gascon et al, 1973; Kew and Douglas, 1976). In S. carlsbergenesis besides galactose and the substrates of enzyme melibiose, raffinose and stachyose, D-galacturonic acid, L-arabinose, D-tagatose, methyl α-D-galactoside, lactose and isopropyl- β -D-thiogalactoside (IPTG) also induced the synthesis of α-galactosidase (Isabel et al, 1981). It was found that molecular structure similar to galactose may be required for the induction of α-galactosidase in yeast. However, Broach (1979) suggested that the actual inducer of α-galactosidase is an intermediate of galactose metabolism. Invertases in yeasts and molds are synthesized constitutively, regardless of the nature of carbon source in the medium (Davies, 1953; Metzenberg, 1962; Dodyle and Rothstein, 1964; Iizuka and Yamamoto, 1972). However, inducible nature of this enzyme has been reported in thermophilic fungus - Thermomyces lanuginosus (Maheshwari et al, 1983).

The operon model of gene regulation as conceived and developed by Jacob and Monod (1947) and their collaborators was a scheme of regulation of β-galactosidase based exclusively on a system of negative control derived from the'lac' operon in E. Coli. Subsequent work has shown that all rapidly metabolizable energy sources repress the formation of enzymes necessary for the dissimilation of energy sources that are more slowly attacked and the phenomenon now known as 'catabolite repression'.

However, in the present experiment, glucose did not have any repression effect on the synthesis of either α-galactosidase or invertase of Bacillus sp. I, suggesting that the regulation of production of these proteins might not be controlled by glucose although glucose itself could not give production of α-galactosidase. In contrast to the present findings, α-galactosidase from B. stearothermophilus (Delente et al, 1974), A. aerogenes (Hogness and Buttley,1957) and 'mel' coded α-galactosidase from E. Coli (Burstein and Kepes, 1971) are produced under the control of catabolite repression. Although, the plasmid coded α-galactosidase which is determined by 'raf' gene is not under the control of catabolite repression in E. Coli (Konishi et al, 1985). Detailed studies carried out by Burstein and Kepes (1971) reported that in E. Coli melibiose operon is an inducible system and is under the control of transient and catabolite repression and the transport of melibiose is under the control of inducer exclusion.

However, recent studies carried out by Okada et al. (1981) suggested that inducer exclusion alone is sufficient in preferential utilization of glucose over melibiose in diauxic growth of E. Coli. Regulation of ∝-galactosidase synthesis is studied in detail in yeast by Martinez et al. (1982) and showed that in the simultaneous presence of both galactose and glucose, the transcription of ∝-galactosidase mRNA is blocked, regulation is by catabolite repression. In their experiments, glucose also interfered with mRNA translation, but the degree of inhibition depended on concentration and time of addition of hexose to the induced cells. These group of workers also showed that antibiotic 'cerulenin' which inhibits synthesis of lipids also blocks secretion of exocellular ∝-galactosidase in S. cerevisiae as a consequence of changes in the cell at the level of transport or at the level of fusion of carrier vesicles with the plasms membrane.

The regulation of invertase synthesis through catabolic repression in yeast and Neurospora is well documented (Lampen, 1971; Meyer and Matile, 1974; Iglesias et al, 1980). In yeast, invertase is synthesized in the cytoplasm and after the glycosylation, it is secreted by an unknown process (Creanor and Mitchison, 1975). In S. cerevisiae, cells synthesized invertase in media containing maltose or sucrose, whereas in the presence of glucose synthesis of enzyme took place when sugar concentration was lower than 1.0% (Elorza et al, 1977). It was shown by the same group of workers that catabolic repression of the invertase synthesis produced by glucose operates at the levels

of transcription and translation and causes increase in the rate of mRNA degradation. However, later on it has been suggested that the effect of repression by glucose on invertase synthesis occurs at the level of translation (Chu and Maley, 1980). Glucose has no effect on secretion and catalytic activity of invertase in yeast (Elorza et al, 1977).

Purification, characterization and kinetic studies of α-galactosidase and invertase from Bacillus sp. I :

α-Galactosidase and invertase from various sources have been isolated by conventional methods of extraction of proteins. The common techniques used for purification of α-galactosidase include ammonium sulfate precipitation (Malhotra and Dey, 1967; Bahl and Agrawal, 1969; Suzuki et al, 1970; Dey et al, 1971), organic solvent precipitation (Petek and Dong, 1961; Li et al, 1963), heat treatment (Li et al, 1963; Suzuki et al, 1970), acidification (Dey and Pridham, 1968; 1969), ion exchange chromatography (Coleman, 1968; Bahl and Agrawal, 1969; Dey et al,1971; Schmid and Schmitt, 1976; Lazo et al, 1979), gel filtration chromatography (Hadacova and Benes, 1977; Williams et al,1978), and isoelectric focusing (Lee and Wacek, 1970).

The separation of multiple forms of plant α-galactosidases has been obtained by applying methods that resolve macromolecules according to their molecular weights, ionic characteristics, electrophoretic mobility and isoelectric pH (Dey and Campillo,1984)

Affinity chromatography has proved successful for purifying several ∝-galactosidases, melibiose sepharose and melibiose agarose were used for purification of plant ∝-galactosidase (Mapes and Sweeley, 1973; 1978; Campillo del and Shannon, 1982). In these cases the bound enzyme was eluted with solutions of D-galactose or p-nitrophenyl ∝-D-galactoside. In several instances, Con-A Sepharose was employed as an affinity adsorbent (Dey et al, 1982; 1983), which retarded those glycoprotein ∝-galactosidases with glucose/mannose termini on the protein. The eluting medium in these cases was methyl- ∝-D-mannoside or methyl- ∝-D-glucoside. One of the steps in purification of Pneumococcal ∝-galactosidases involved adsorption of the enzyme on human blood cells, where enzyme showed an affinity for the ∝-D-galactosyl residues of blood group 'B' substance (Li and Shetlar, 1964).

Some microbial invertases also have been extensively purified to homogeneity, mainly from yeast (Gascon and Lampen, 1968; Nisizawa and Hashimoto, 1970; Lampen, 1971) and Fusarium enzyme has been crystallized (Onodera and Maruyama, 1979). In yeast, a number of enzyme forms were detected in the culture fluid in which the cells were grown (Berggren, 1970; Colonna et al, 1975). Many studies on S. cerevisiae and Kluyveromyces marxianus have been shown that invertase is synthesized within the cell and exported across the plasmalemma. Most of the invertases of fresh baker's yeast (Arnold, 1972) or of brewer's yeast (Williams and Wiseman, 1974) are released into solution by mechanical disruption of the cells. Only partial purification of some of the plant invertases has been reported

(Arnold, 1965; 1966; Kato and Kubota, 1978). The glycoprotein nature of some plant invertases made it possible to demonstrate microheterogeneity by using affinity difference toward immobilized lectins (Faye, 1981; Faye et al, 1982).

As described earlier, in E. Coli, plasmid coded α-galactosidase had different properties from that coded by chromosomal DNA (Schmid and Schmitt, 1976). In the present investigation, since a strain of Bacillus sp. I isolated from stored soybean grains could utilise stachyose, raffinose and melibiose with concomitant production of α-galactosidase and invertase, it was of interest to purify and characterize these two enzymes. Therefore, during the present studies a culture was grown in raffinose broth and both the enzymes were purified to homogeneity as described under 'Materials and Methods'. Later on the properties of raffinose induced α-galactosidase were compared with α-galactosidase induced by melibiose.

The purification of α-galactosidase and invertase from Bacillus sp. I is summarized in Tables-100 & 101. Using different purification techniques viz. ammonium sulfate precipitation, gel filtration through Sephadex G-200 and ion-exchange chromatography using DEAE cellulose, hydroxylapatite and QAE Sephadex columns, α-galactosidase and invertase were purified from a strain of Bacillus sp. I to 304 and 232 folds respectively over the crude homogenate. The detail procedures for the purification are described under 'Materials and Methods'. Data given in Tables-100 & 101 show that the

TABLE-100 : Purification of ∝-galactosidase from the cell-free extract of Bacillus sp. I.

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Crude homogenate (cell-free extract)	460	1300	0.354	100	1
2. Streptomycin sulfate precipitation	445	1285	0.346	97	1
3. Ammonium sulfate precipiation (50-70% fraction)	280	260	1.077	61	3
4. Gel filtration on Sephadex G-200	235	80	2.938	51	8
5. DEAE-cellulose chromatography-I	188	13.2	14.24	41	40
6. Hydroxylapatite chromatography	124	5.0	23.2	26	70
7. DEAE-cellulose chromatography-II	109	3.0	45.42	24	103
8. QAE Sephadex chromatography	99	0.92	107.6	22	304

Enzyme units are expressed as amount of enzyme required to liberate one micromole of paranitrophenol per minute under assay conditions.

TABLE-101 : Purification of invertase from the cell-free extract of Bacillus sp. I.

	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
1. Crude-homogenate (cell-free extract)	278	1300	0.214	100	1
2. Streptomycin sulphate precipitation	273	1285	0.213	98	1
3. Ammonium sulphate precipitation (50-70% fraction)	155	260	0.596	56	3
4. Gel filtration by Sephadex G-200	137	28.60	4.790	49	22
5. DEAE Cellulose chromatography-I	110	5.24	20.99	40	98
6. Hydroxyapatite chromatography	86	2.02	42.57	31	199
7. DEAE Cellulose chromatography-II	77	1.55	49.68	28	232

Enzyme units are expressed as amount of enzyme required to liberate one micromole of glucose per minute under assay conditions.

recovery of ∝-galactosidase was 22% and that of invertase was 28% after purification.

Fig.37 shows the elution pattern of both the enzymes when the enzyme preparation was loaded on Sephadex G-200 column after precipitation with ammonium sulfate. It can be seen from the figure that during present investigation it was possible to separate out ∝-galactosidase and invertase from the same extract. The enzymes were separately pooled from this step and purified further. These findings are interesting because Lazo et al.(1977) could not separate external invertase and ∝-galactosidase from the same crude extract preparation of S. carlsbergenesis using Sephadex G-200. During their experiments, the ratio of ∝-galactosidase to invertase remained constant throughout their purification procedure and only after the partial degradation of invertase, the contamination of invertase was reduced. In the present investigation since both the enzymes were not copurified, it was clear that there could be a significant difference between the molecular weights of ∝-galactosidase and invertase of Bacillus sp. I.

Figs. 38 & 39 show the elution pattern of ∝-galactosidase and invertase when they were loaded on DEAE cellulose columns respectively. Both the enzymes were eluted out at 0.3M NaCl when a linear gradient of 0.1M to 1.0M NaCl was applied onto the columns to elute out the enzymes. Hydroxylapatite column also could bind both the enzymes and they were eluted out at 0.2M sodium phosphate buffer (pH 7.0) as shown in Figs.40 & 41.

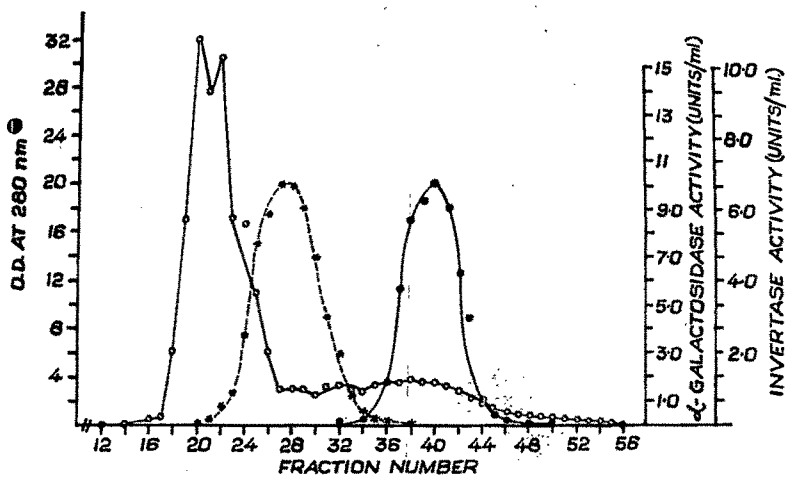


FIG. 3 SEPHADEX G-200 GEL FILTRATION PROFILE OF α-GALACTOSIDOSE AND INVERTASE FROM BACILLUS Sp I (o—o) ABSORBANCE (O.D.) AT 280 nm; (•—•) α-GALACTOSIDASE ACTIVITY, (o—o) INVERTASE ACTIVITY.
● EACH FRACTION WAS DILUTED APROPRIATELY TO READ ABSORPTION AT 280 nm, AND THEN THE READING WAS MULTIPLIED WITH THE DILUTION FACTOR.

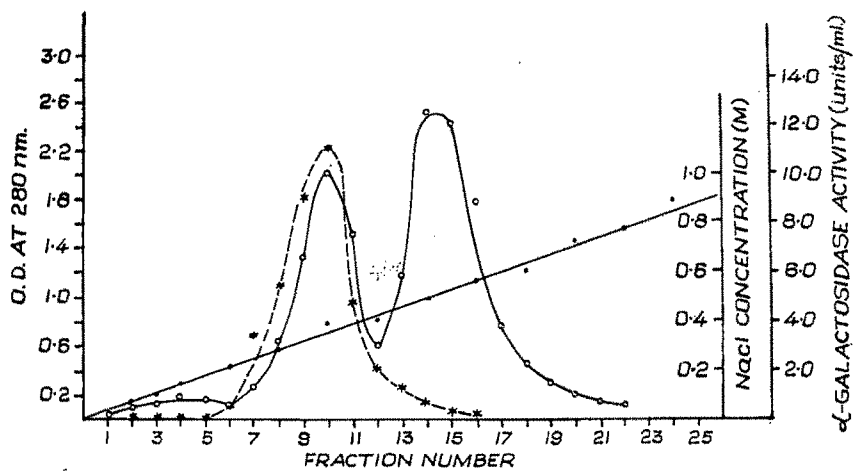


FIG. 38 DEAE CELLULOSE CHROMATOGRAPHY-I OF α-GALACTOSIDASE FROM BACILLUS Sp.-I. (o—o) ABSORBANCE AT 280 nm; (•—•) α-GALACTOSIDASE ACTIVITY; (—•)NaCl GRADIENT.

Fig: 38

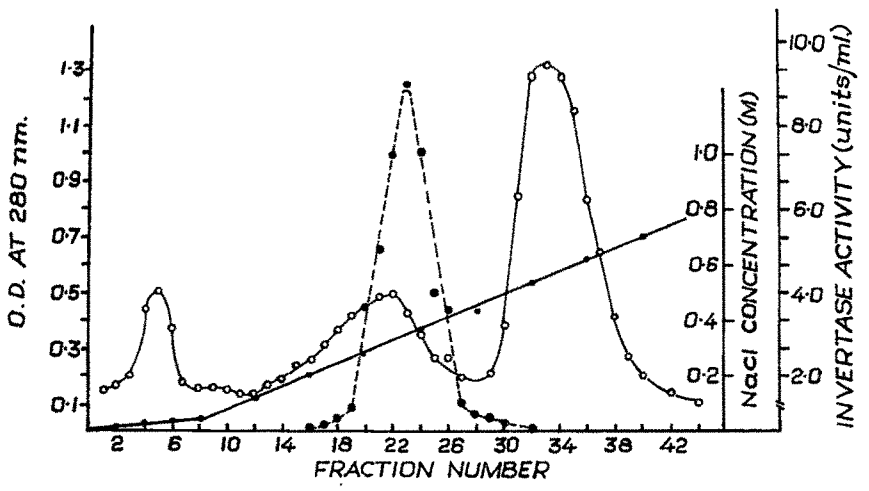


FIG. 39 DEAE-CELLULOSE CHROMATOGRAPHY-I OF INVERTASE FROM BACILLUS Sp. I. (o—o) ABSORBANCE AT 280 nm.; (•—•) INVERTASE ACTIVITY; (—•) NaCl GRADIENT.

Fig: 39

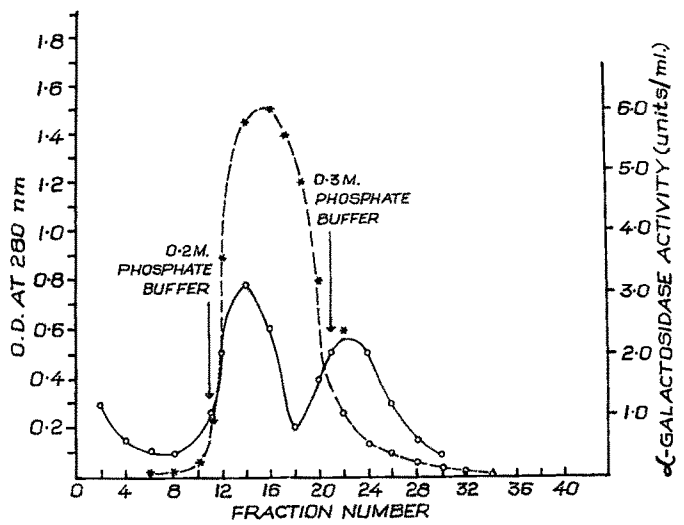


FIG: 40 HYDROXYLAPATITE CHROMATOGRAPHY OF α-GALACTOSIDASE FROM BACILLUS Sp.-I. (o—o) ABSORBANCE AT 280 nm; (•—•) α-GALACTOSIDASE ACTIVITY.

Fig: 40

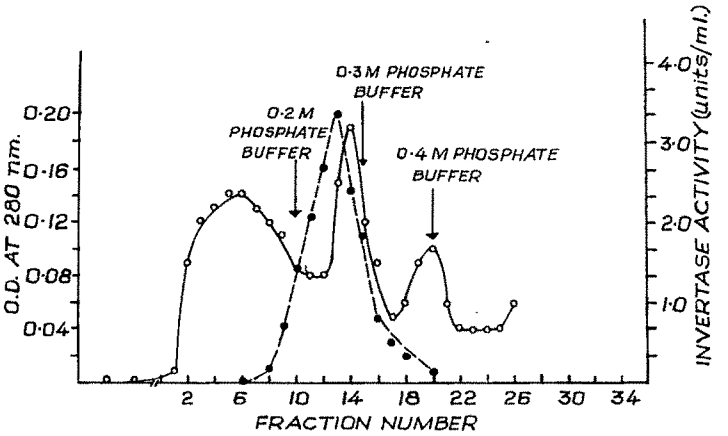


FIG: 41: HYDROXYLAPATITE CHROMATOGRAPHY OF INVERTASE FROM BACILLUS Sp. I. (o—o) ABSORBANCE AT 280 nm; (•—•) INVERTASE ACTIVITY.

Fig: 41

Figs. 42 & 43 show the elution pattern of ∝-galactosidase and invertase when they are loaded on second DEAE-cellulose columns.

∝-Galactosidase purification was further achieved by loading partially purified preparation onto QAE Sephadex column. Elution pattern of ∝-galactosidase on QAE Sephadex shows that bound enzyme was eluted out with 0.4M NaCl when a linear gradient of 0.1M to 1.0M NaCl was applied onto the column (Fig.44). The purified enzymes were further dialyzed against 50% glycerol and concentrated and stored at -20°C for further use.

Kinetic properties of the purified enzymes :

Data given in Fig.45 indicate that ∝-galactosidase and invertase isolated from Bacillus sp. I exhibit maximum activity at pH 7.0, in 50 mM sodium phosphate buffer when ∝-PNPG was used as a substrate, eventhough there was 10-25% of activity at acidic pH. This pH profile of enzymes show that they were most stable at neutral pH in 50 mM sodium phosphate buffer at 37°C. At the same time, Tris-HCl buffer 50 mM pH 7.0 inhibited activity of both the enzymes. There are very few bacterial ∝-galactosidases reported with neutral pH optimum viz. from E. Coli (Schmid and Schmitt, 1976); B. stearothermophilus (Pederson and Goodman, 1980), Micrococcocus sp. (Akiba and Horikoshi, 1976), C. rolfsii (Kaji and Yashihara, 1972), E. Coli subsp. communior (Kawamura et al, 1976) and Bacteroides ovatus (Gherardini et al, 1985). Most of the ∝-galactosidases

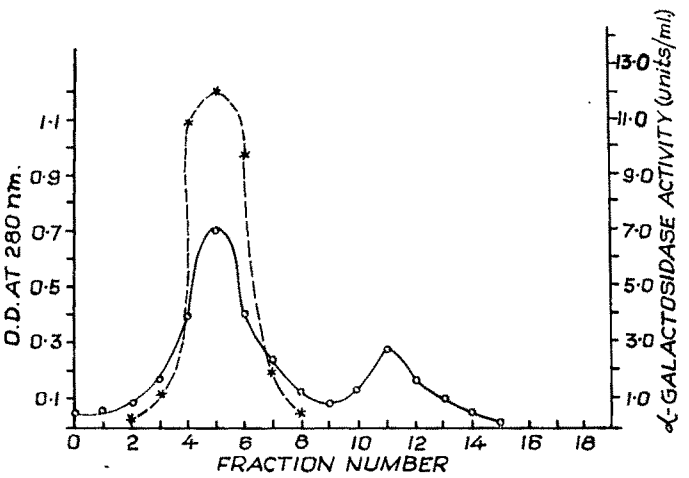


FIG.: 42 DEAE CELLULOSE CHROMATOGRAPHY-II OF α-GALACTOSIDASE FROM BACILLUS Sp.-I. (o—o) ABSORBANCE AT 280 nm; (*—*) α-GALACTOSIDASE ACTIVITY.

Fig: 42

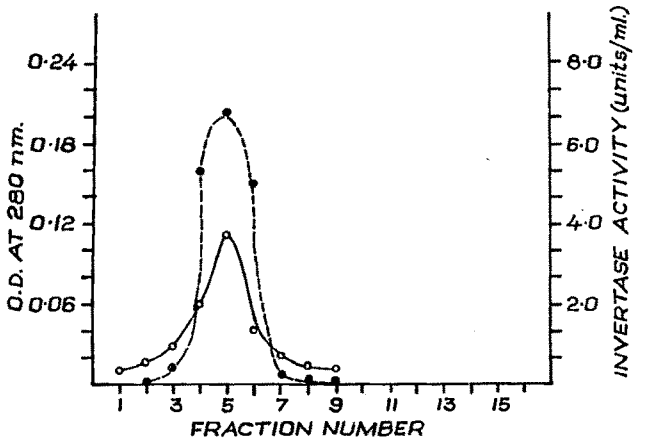


FIG: 43 DEAE CELLULOSE-II CHROMATOGRAPHY OF INVERTASE FROM BACILLUS Sp.I. (o—o) ABSORBANCE AT 280 nm; (•—•) INVERTASE ACTIVITY.

Fig 43

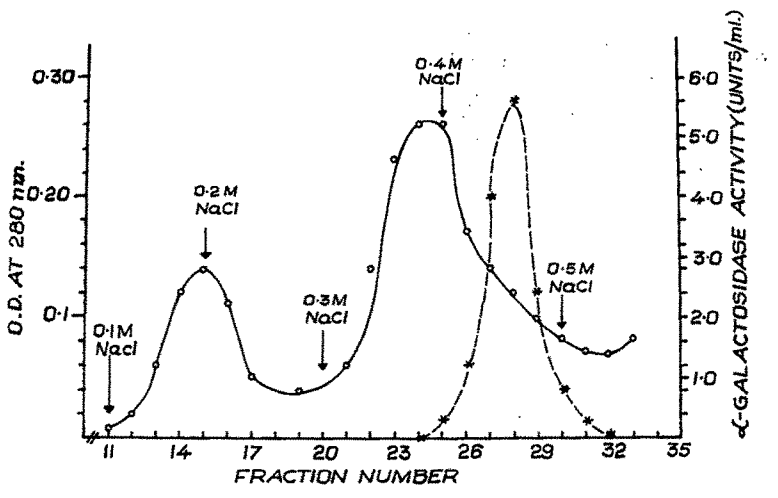
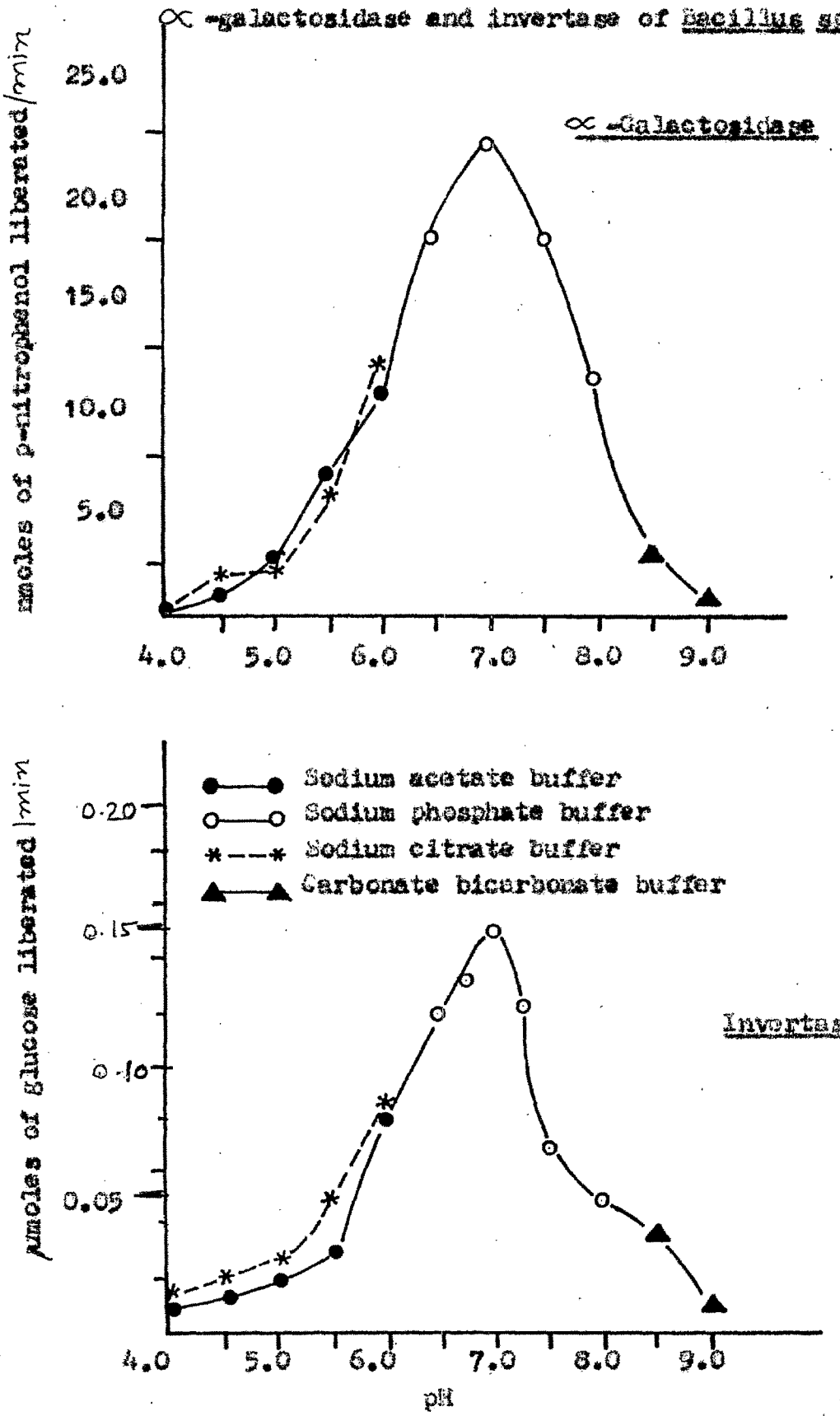


FIG: 44 QAE SEPHADEX CHROMATOGRAPHY OF α-GALACTOSIDASE FROM BACILLUS Sp. I. (o—o) ABSORBANCE AT 280 nm; (*—*) α-GALACTOSIDASE ACTIVITY.

FIG.45 : Effect of pH on the activity of purified ∝-galactosidase and invertase of Bacillus sp.1.



isolated from bacteria, yeasts and molds show optimum pH in the range of 3-6 e.g. α-galactosidase from Cladosporium cladosporoides (Cruz et al, 1981); Aspergillus oryzae (Cruz and Parks, 1982), lactobacilli (Mital et al, 1973), Pycnoporus sp. (Ohtakara et al, 1984), S. carlsbergensis (Lazo et al, 1978) and Monascus pilosus (Wong et al, 1986). α-Galactosidase from plants and animals also have pH optimum in acidic range e.g. in Phaseolus seeds (Agrawal and Bahl, 1968), Vicia faba (Dey and Pridham, 1969b; Dey et al, 1984), Alfalfa seeds (Takatoshi et al, 1979), rat brain (Subba Rao and Pieringer, 1970), rat kidney (Gillman et al, 1970) and rat uterus (Coleman, 1968). Because of the stability of plant α-galactosidases at lower pH, lowering the pH of the crude extract to 3-4 is often used to precipitate storage proteins in the initial stages of purification.

Invertases from most of the microorganisms have pH optimum in acidic range (Dey and Campillo, 1984). Invertase of N. crassa has broad pH optimum from pH 4.5 to 6.0 (Lampen, 1971). In yeast, the pH stability curves for the external and internal invertase forms differ markedly (Gascon et al, 1968). The external enzyme is inactivated at pH 8.0 and above, but it is stable at pH 3.0, whereas the internal invertase form is stable at pH 9.0, but it undergoes reversible inactivation at pH 5.0 or less. The pH optima of the multiple forms of the acid invertases from plants are generally close to each other and similar situation exists for the alkaline forms (Dey and Campillo, 1984). Dey (1986) has reported high levels of alkaline invertase activity in dormant mung bean seeds, which

during germination decreases rapidly and is replaced by high 'acid' invertase activity.

Fig.46 shows that both the enzyme activities increased with the increase in temperature and were maximum at 37°. Above 37°, the activity decreased as the temperature was raised further. The optimum temperature for most α-galactosidases is in the range of 37-40° (Ulezlo and Zaprometova, 1982). α-Galactosidases isolated from E. Coli, S. bovis, Diplococcus pneumoniae, Trifolium repens and leaves of cucurbita show optimum temperature 37° for their enzyme activity (Bailey, 1963; Li et al, 1963; Kawamura et al, 1976; Schmid and Schmitt, 1976; Thomas and Webb, 1977; Williams et al, 1978).

The thermal stability of α-galactosidase from Bacillus Sp. I as shown in Fig.47, indicates that the enzyme is fully active at 37° and retained 55-60% activity upto 40°. Above this temperature, the enzyme was extremely heat labile and was completely inactivated at 50°, within 10 minutes. This is in agreement with α-galactosidase isolated from E. Coli, Micrococcus sp. and Bacillus sp. which were also completely inactivated at 50° within 10-15 min (Akiba and Horikoshi, 1976; Kawamura et al, 1976) whereas α-galactosidases reported from Bacillus stearothermophilus and Pycnoporcus cinnabarinus were thermostable at 75-80° (Delente et al, 1974; Ohtakara et al, 1984). α-Galactosidase from Aspergillus niger (Lee and Ulacek, 1970); Diplococcus pneumoniae (Li et al, 1963), Vicia faba (Dey and Pridham, 1969), Alfalfa seeds (Takatoshi et al, 1979),

Fig. 3 : Effect of temperature on the activity of purified ∝-galactosidase and invertase of Bacillus sp. I.

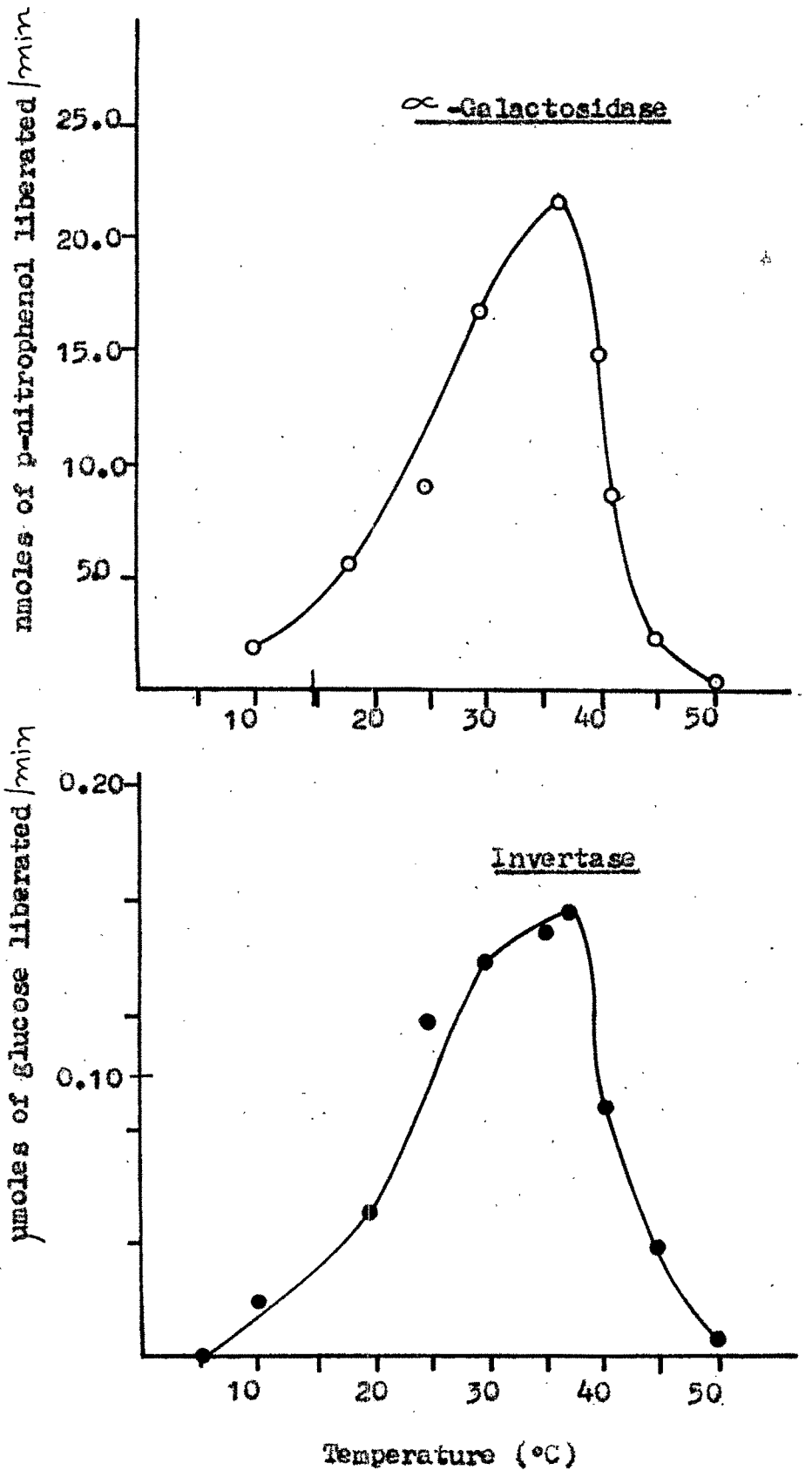
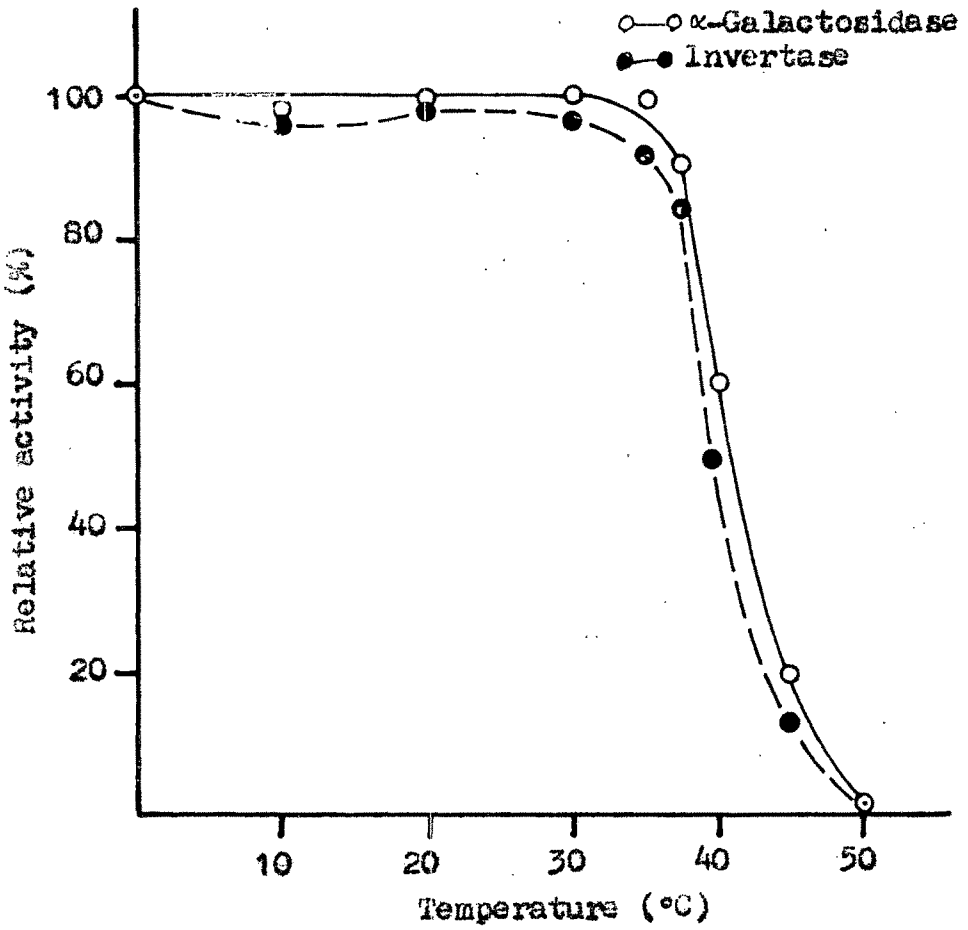


FIG.47 : Thermal stability of purified ∝-galactosidase and invertase of Bacillus sp. I.
Enzymes were incubated at various temperatures for 10 min., cooled and then used for assay.



Saccharomyces carlsbergenesis (Lazo et al, 1978), Cladosporium cladosporiodes (Cruz et al, 1981), Streptomyces olivaceus (Suzuki et al, 1966), Morterella vinacea (Suzuki et al, 1970) and Monascus pilosus (Wong et al, 1986) were quite stable at 50-60°.

During the present investigation, the activity of ∝-galactosidase increased proportionately upto 0.2 µg of purified protein (Fig.48) and upto 10 minutes of incubation (Fig.49) with∝-PNPG as substrate. When melibiose was used as substrate, the activity increased linearly upto 1 µg of protein (Fig.50) and upto 30 minutes of incubation (Fig.52). When raffinose or stachyose was used as a substrate, the activity increased linearly upto 4.0 µg protein (Fig.51) and upto 30 minutes of incubation (Fig.53).

Figs.54 to 58a show the lineweaver Burk plots of ∝-galactosidase from Bacillus sp. I with different substrates from which the calculated Km were 0.5, 8, 36, 66 and 20 mM and Vmax values were 83, 250, 285, 300 and 76 µmoles of product liberated per minute per mg protein for para-nitrophenyl-∝-D-galactoside, melibiose, raffinose, stachyose and p-nitrophenyl-∝-D-fucoside respectively. When the products of raffinose, stachyose and melibiose hydrolysis by purified∝-galactosidase from Bacillus sp.I were detected using TLC (Fig.58b), it was found that raffinose and stachyose were hydrolysed into sucrose glucose and galactose, when incubated with the enzyme. When effect of various sugars was studied on catalytic activity of ∝-galactosidase from Bacillus sp.I, using ∝-PNPG as substrate, it was found that inositol, arabinose, galactose, melibiose and raffinose were powerful inhibitors of this enzyme in this bacteria at 1-50 mM concentration, whereas

FIG.48 : Effect of enzyme concentration on the activity of purified ∝ -galactosidase and invertase of Bacillus sp. I.

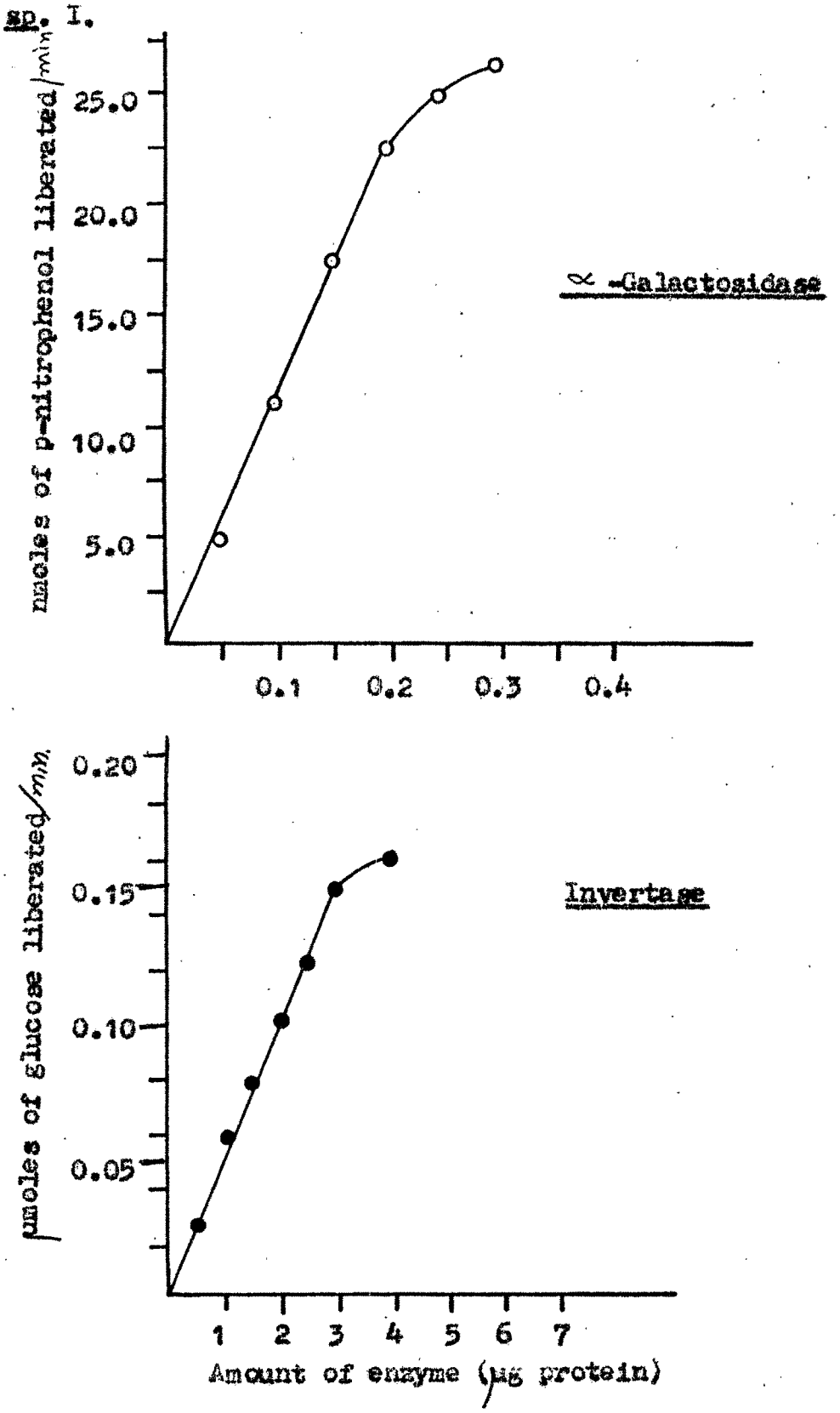


FIG.49 : Effect of period of incubation on the activity of purified ∝-galactosidase and invertase of Bacillus sp.I.

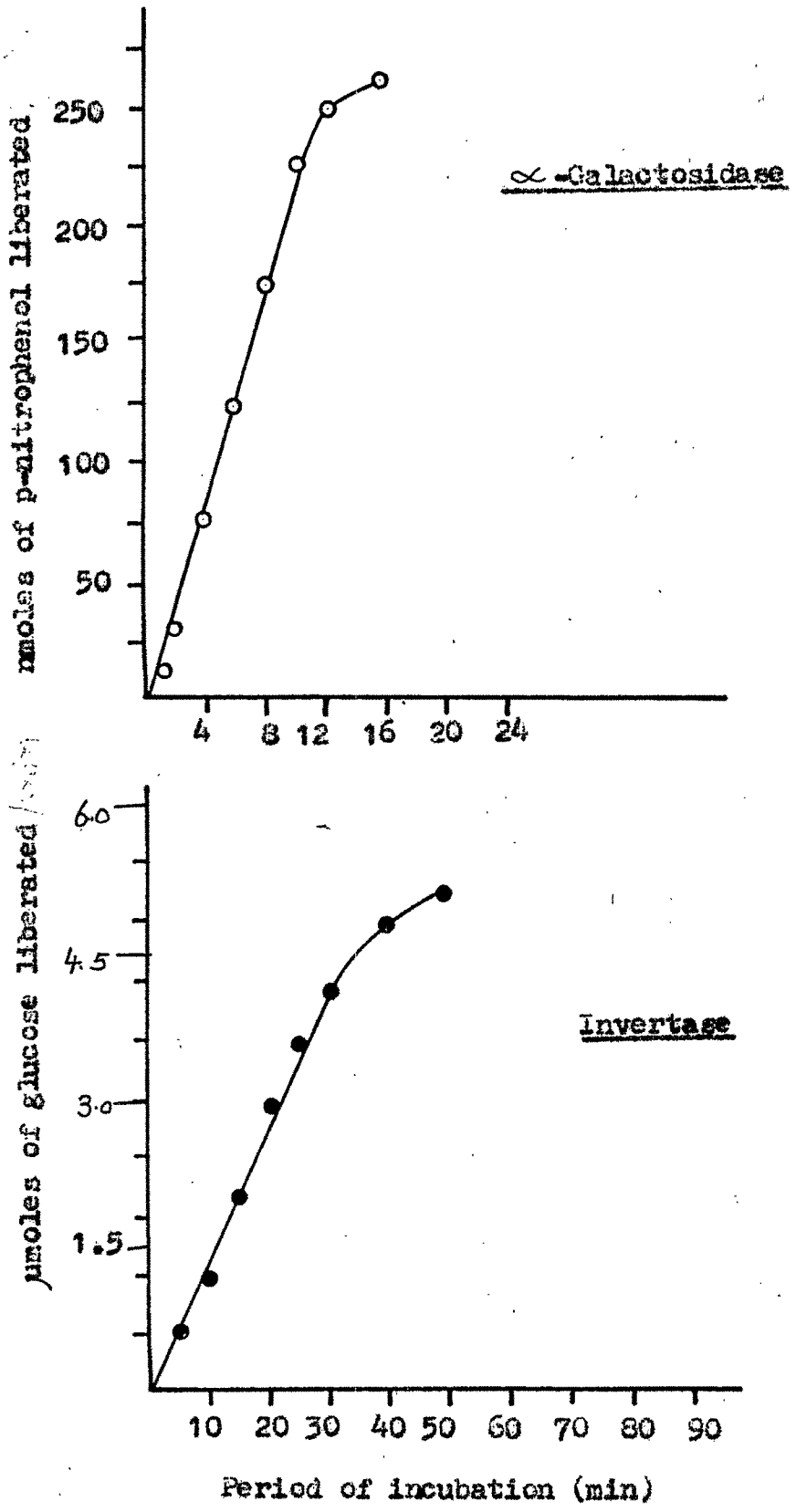


FIG.50 : Effect of purified ∝-galactosidase concentration on hydrolysis of melibiose.

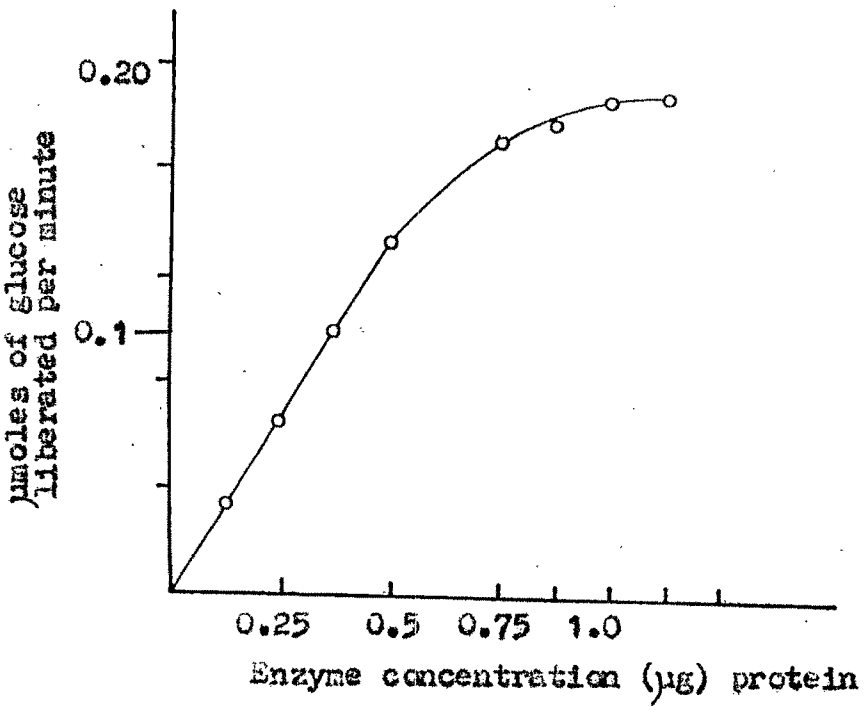


FIG.51 : Effect of purified ∝-galactosidase concentration on hydrolysis of raffinose and stachyose.

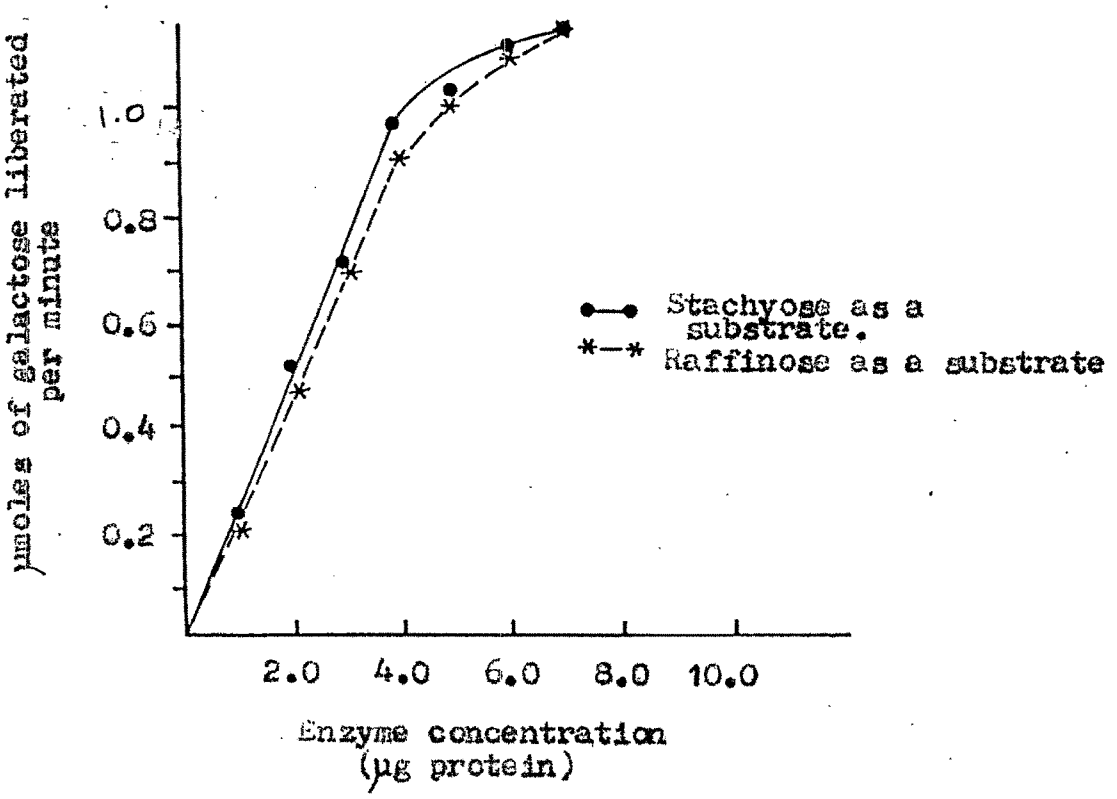


FIG.52 : Effect of period of incubation on the hydrolysis of melibiose by purified α-galactosidase from Bacillus sp. I.

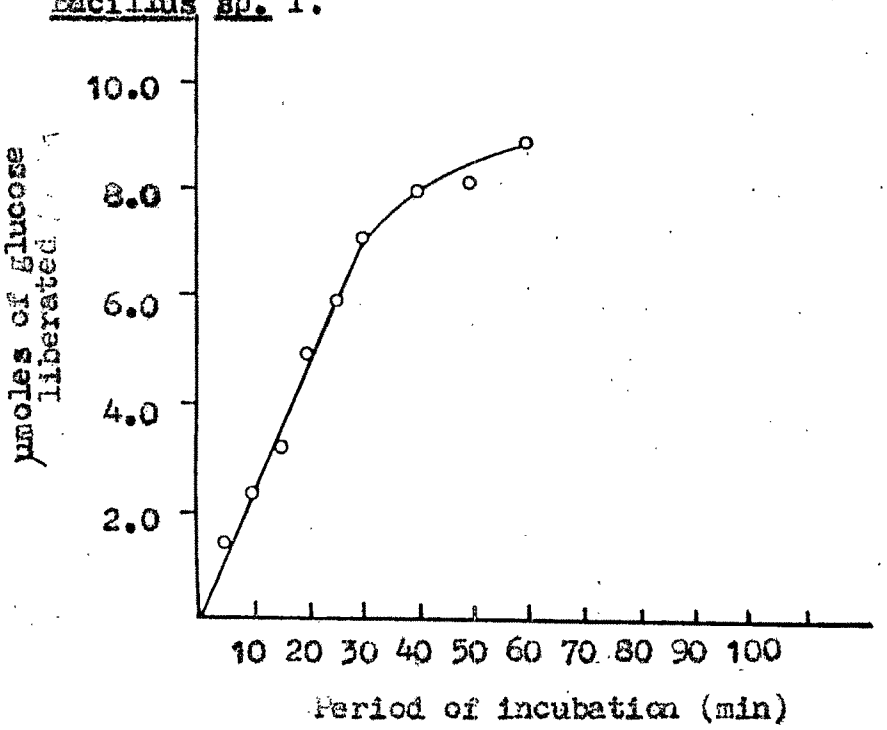
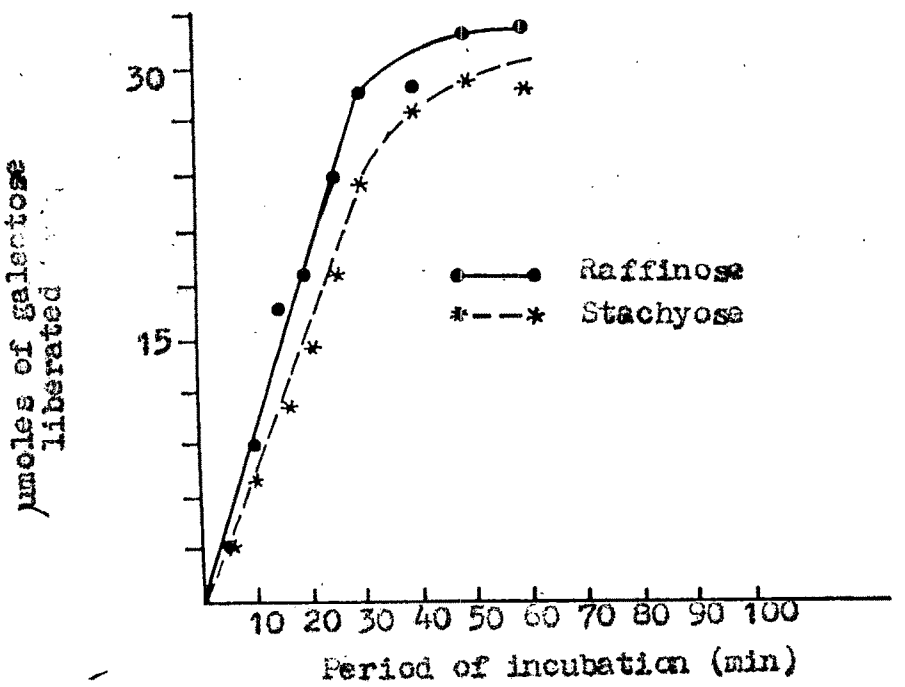


FIG.53 : Effect of period of incubation on the raffinose on the hydrolysis of raffinose and stachyose by purified α-galactosidase from Bacillus sp. I.



glucose and stachyose were less inhibitory (Table-102). Lactose and cellobiose at 1-50 mM did not affect the catalytic activity of this enzyme. Figs.59 & 60 shows that galactose and melibiose were competetive inhibitors of α-galactosidase in the present investigations, whose KI values were 0.5 mM and 10 mM respectively calculated from Dixon plot (Figs. 61 & 62). KI values for raffinose was 28 mM for the hydrolysis of α-PNPG (Fig.63). Thus KI values for these inhibitors, viz. melibiose, raffinose and stachyose are in agreement with the Km values of the same.

Thus substrate studies with α-galactosidase of Bacillus sp. I show that it can hydrolyse α-PNPG, melibiose, raffinose, stachyose and p-nitrophenyl-α-D-fucoside. The affinity for α-PNPG was the highest and for paranitrophenyl α-D-fucoside it was lowest among all the synthetic substrates studied (Table-103)

Data given in Table-103 indicate the hydrolysis of various substrates, from which it can be concluded that α-galactosidase from Bacillus sp. I is a highly specific enzyme for α-D-glycosides and does not act on β-D-galactosides. The quantitative evaluation of glycon and aglycon specificity has been carried out with α-galactosidases from sweet almond (Malhotra and Dey, 1967) and V. faba (Dey and Pridham, 1969b) which showed that the affinity (1/Km) of the enzymes for the substrates seems to depend largely on the structural changes in the glycon moeity and follows the order : α-D-galactosides > α-D-fucosides > β-L-arabinoside. This suggests that one of the

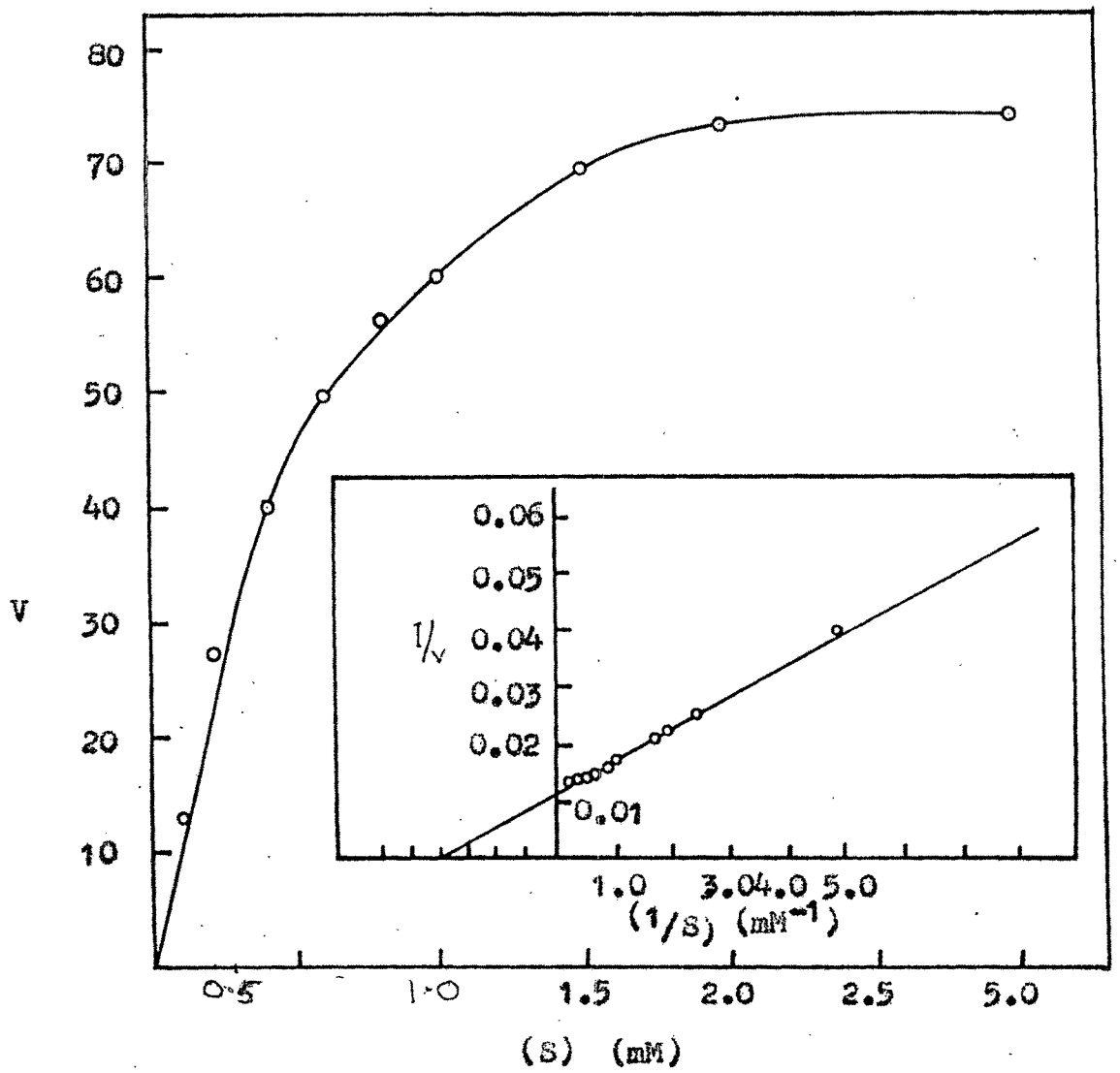


FIG.54 : Effect of α-PNPG concentration on the rate of its hydrolysis ~~of~~ by α-galactosidase from Bacillus sp. I.

The assay mixture contained α-PNPG (desired concentration), 2-mercaptoethanol (1 mM) and purified α-galactosidase (0.2 µg protein) in 50 mM sodium phosphate buffer, pH 7.0 (Total 1.0 ml assay system) at 37°. Reaction was started by addition of enzyme. The rate of reaction (V) is expressed as umoles of paranitrophenol liberated/min/mg protein. Inset shows double-reciprocal plot of the data.

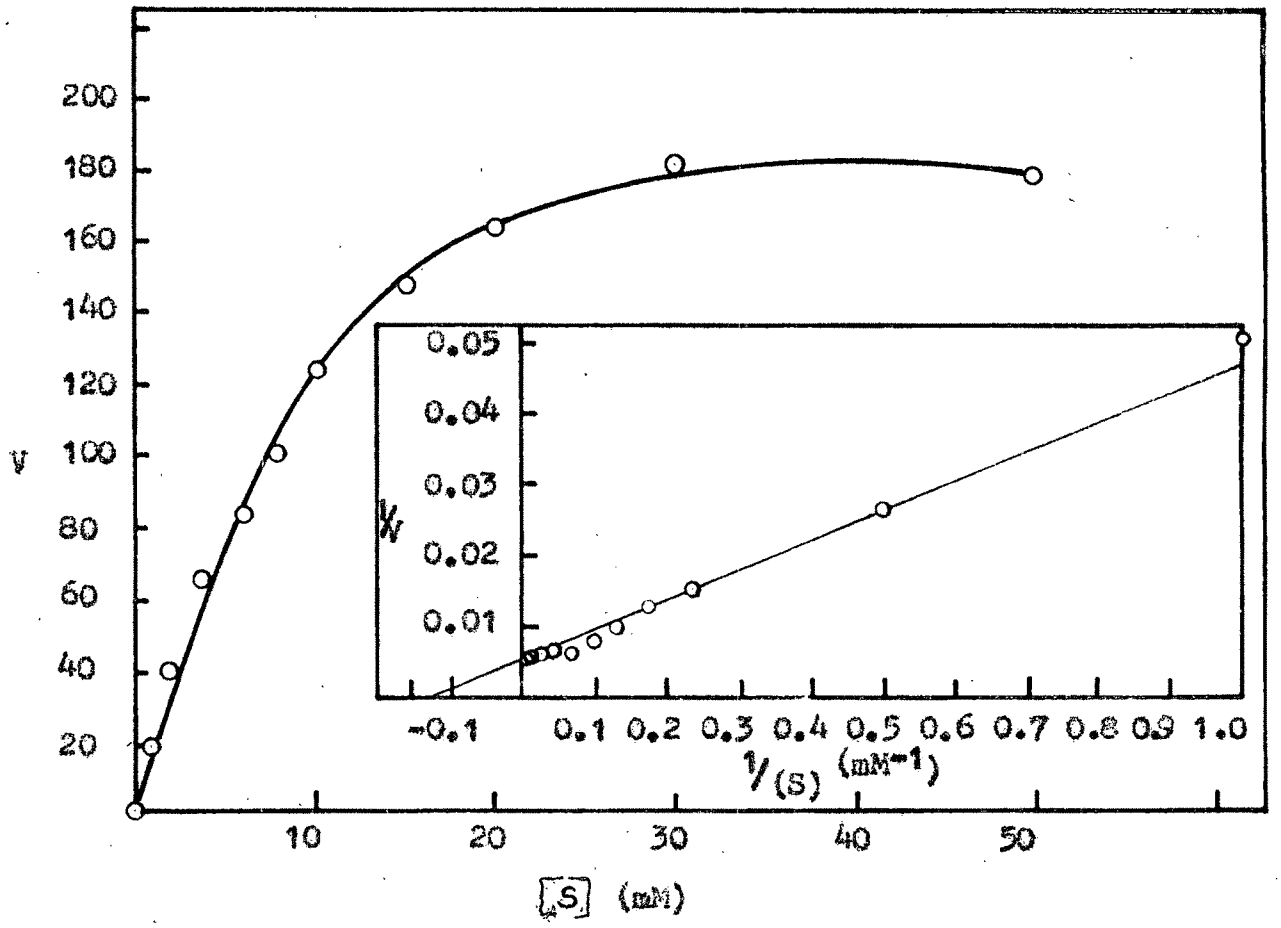


FIG.55 : Effect of melibiose concentration on the rate of its hydrolysis by ∝-galactosidase from Bacillus sp. I.

The assay mixture contained melibiose (desired concentration), 2-mercaptoethanol (1 mM) and purified ∝-galactosidase (1 µg protein) in 50 mM sodium phosphate buffer, pH 7.0.(Total 1.0 ml assay system) at 37°. The reaction was started by the addition of enzyme. The rate of reaction (V) is expressed as umoles of glucose liberated/min/mg protein.

Inset shows double-reciprocal plot of the data.

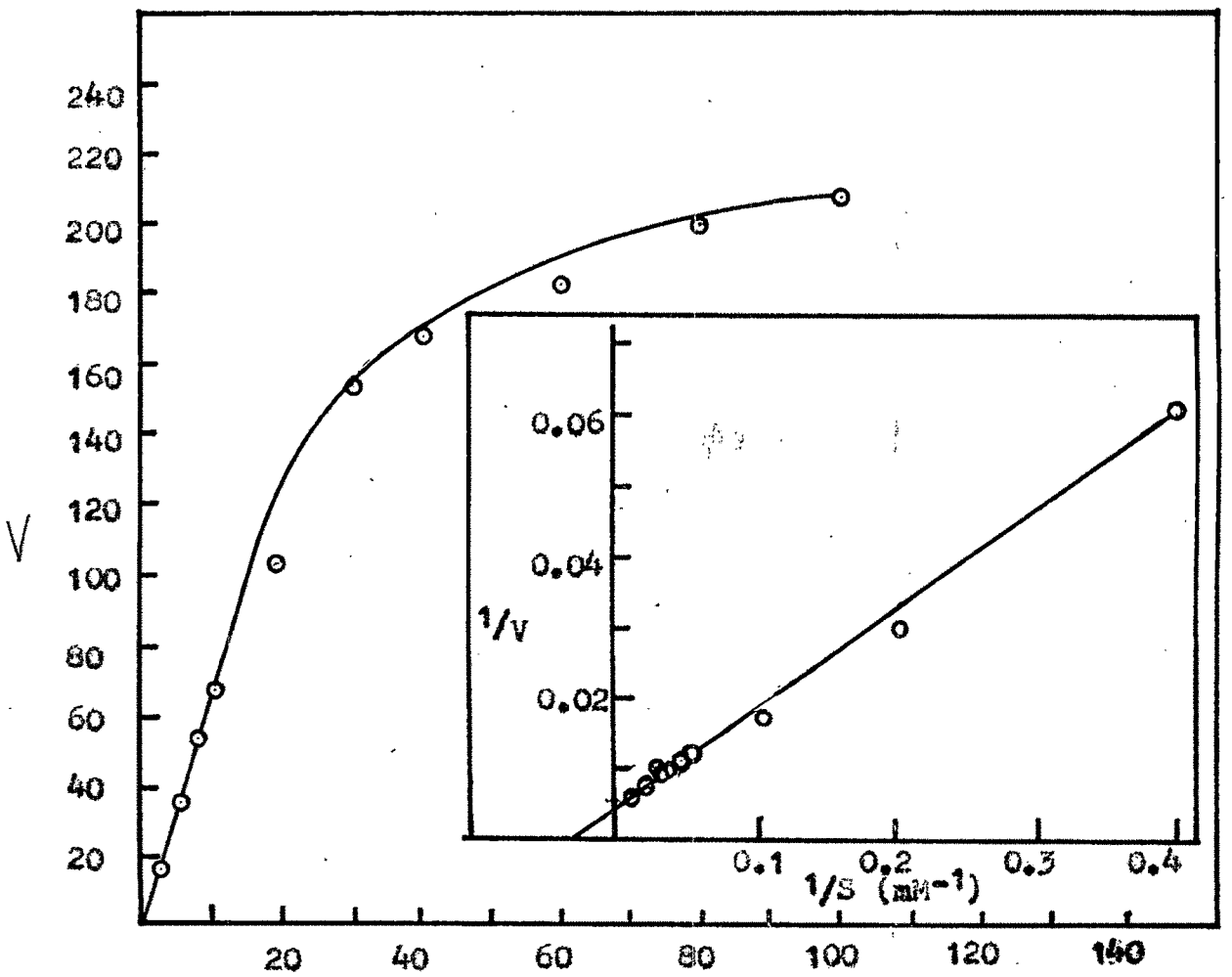


FIG.56 : Effect of raffinose concentration on the rate of its hydrolysis by ∝-galactosidase from Bacillus sp. I.

The assay mixture contained raffinose (desired concentration), 2-mercaptoethanol (1 mM) and purified ∝-galactosidase (4 µg protein) in 50 mM sodium phosphate buffer, pH 7.0. (Total 1.5 ml assay system) at 37°. Reaction was started by addition of enzyme. The rate of reaction (V) is expressed as µmoles of galactose liberated/min/mg protein. Inset shows double reciprocal plot of the data.

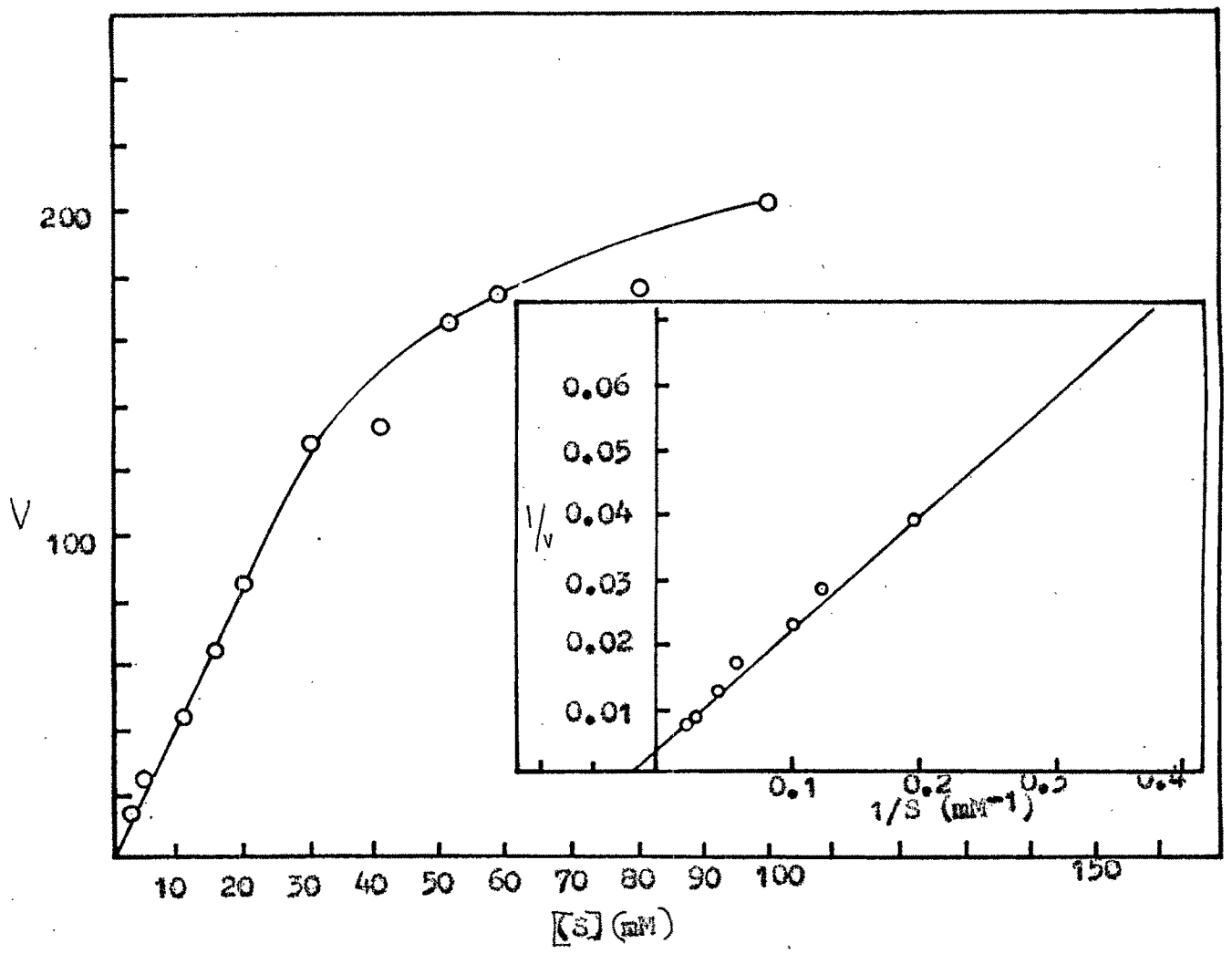


FIG.57 : Effect of stachyose concentration on the rate of its hydrolysis by ∝-galactosidase from Bacillus sp. I.

The assay mixture contained stachyose (desired concentration), 2-mercaptoethanol (1 mM) and purified ∝-galactosidase (4 µg protein) in 50 mM sodium phosphate buffer, pH 7.0. (Total 1.5 ml assay system) at 37°. Reaction was started by addition of enzyme. The rate of reaction (V) is expressed as µmoles of galactose liberated/min/mg protein. Inset shows double-reciprocal plot of the data.

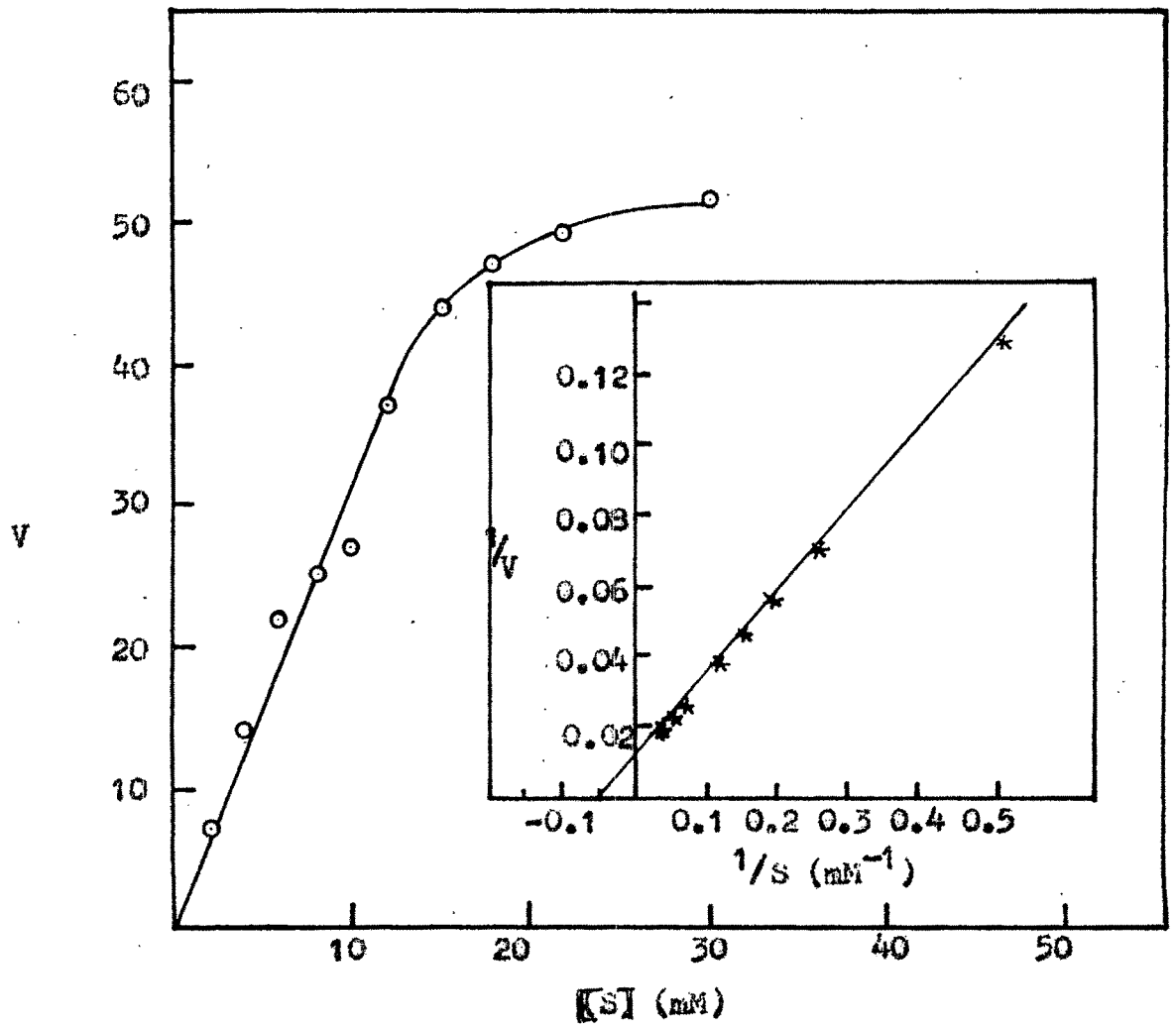


FIG.58a: Effect of paranitrophenyl- ∝ -D-fucoside concentration on the rate of tis hydrolysis by ∝-galactosidase from Bacillus sp. I.

The assay mixture contained p-nitrophenyl- ∝ -D-fucoside (desired concentration), 2-mercaptoethanol (1 mM) and purified ∝-galactosidase (0·2 µg protein) in 50 mM sodium phosphate buffer, pH 7.0 (Total 1.0 ml assay system) at 37°. Reaction was started by addition of enzyme. The rate of reaction (V) is expressed as µmoles of p-nitrophenol liberated/min/mg protein. Inset shows double reciprocal plot of the data.

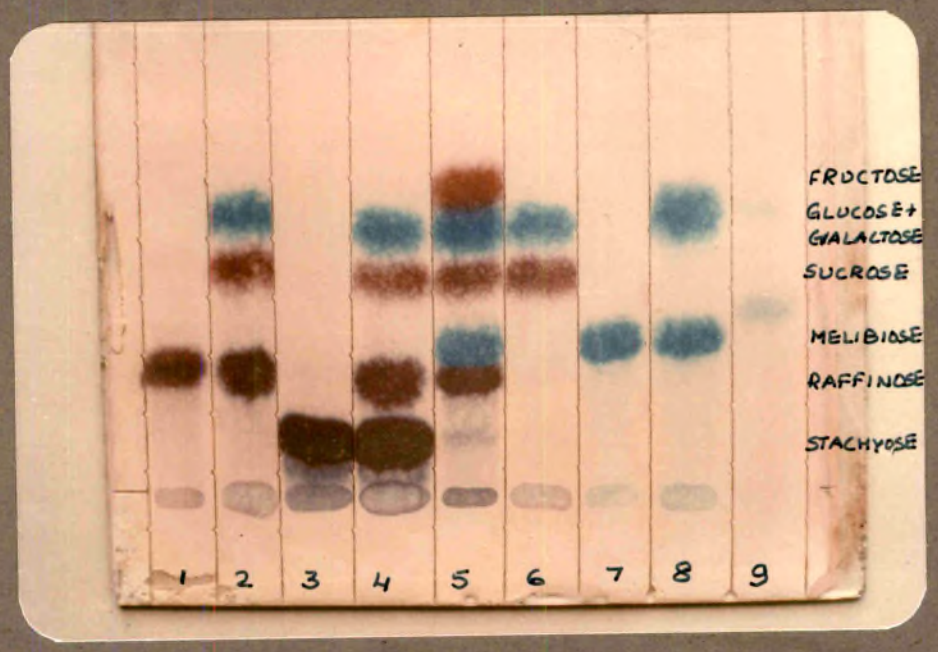


FIG.58b : Detection of products of stachyose, raffinose and melibiose hydrolysis by purified α-galactosidase from Bacillus sp. I (analysed by TLC method).

1. Enzyme Incubated with raffinose for 0 Hour. (Contr
2. Enzyme Incubated with raffinose for 1 Hour.
3. Enzyme Incubated with Stachyose for 0 Hour.
4. Enzyme Incubated with Stachyose for 1 Hour.
5. Standard Sugars.
6. Enzyme Incubated with Stachyose for 4 Hour.
7. Enzyme Incubated with Melibiose 0 Hour.
8. Enzyme Incubated with Melubiose for 1 Hour.

TABLE-102 : Effect of various sugars on ∝ -galactosidase activity of Bacillus sp. I.

	0.1 mM**		1.0 mM		10 mM		50 mM	
	Enzyme * activity	% Residual activity control	Enzyme activity	% Residual activity	Enzyme activity	% Residual activity	Enzyme activity	% Residual activity
Control	25.2	100	25.2	100	25.2	100	25.2	100
Sucrose	26.0	103	24.9	99	22.1	88	20.7	82
Melibiose	21.4	85	13.9	55	9.0	36	3.8	15
Glucose	25.4	101	23.7	94	19.0	75	12.6	50
Galactose	20.2	80	7.1	28	2.5	10	3.3	13
Inositol	24.9	99	8.5	34	6.0	24	2.5	10
Maltose	29.9	119	2.75	109	23.9	95	22.1	88
Raffinose	19.6	78	9.6	38	8.1	32	3.8	15
L-arabinose	17.6	70	8.0	32	7.1	28	2.6	10
Cellobiose	24.4	97	25.7	102	23.9	95	25.7	102
Fructose	26.5	105	21.2	84	17.1	68	13.9	55
Stachyose	23.7	94	27.9	111	13.9	55	6.3	25
Lactose	25.7	102	25.2	100	23.9	95	23.1	92
Mannose	21.7	86	23.1	92	24.2	96	20.2	80

*Enzyme units are expressed as amount of enzyme required to liberate one nanomole of paranitrophenol per minute under assay conditions.

** Sugars were added during the assay of enzyme.

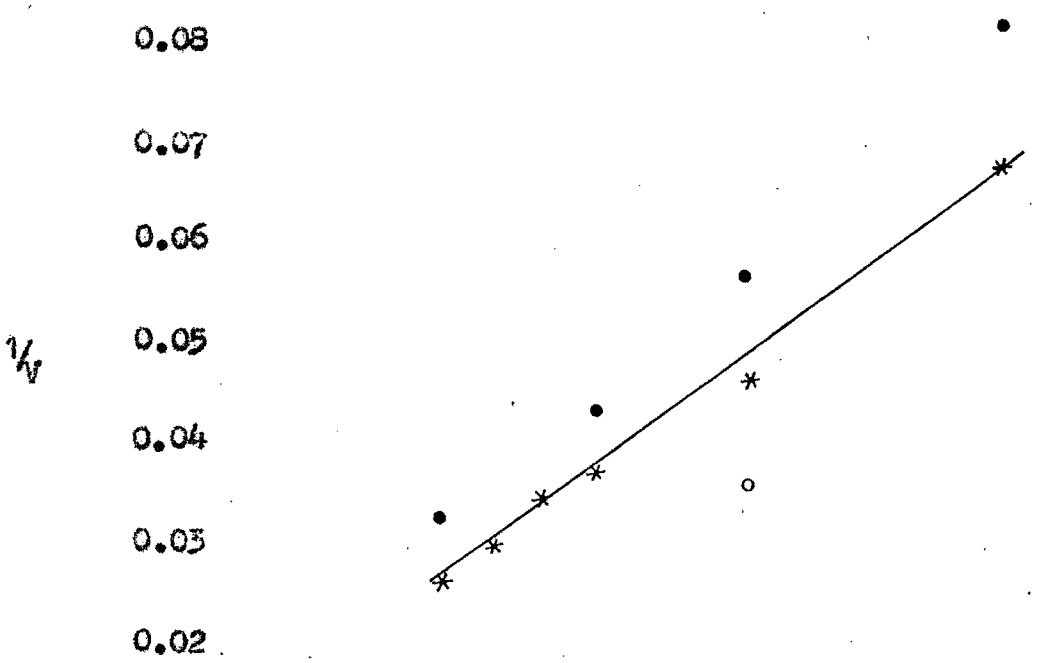


FIG.59 : Lineweaver Burk plot of ∝-galactosidase for the hydrolysis of ∝-PNPG in the presence of hx inhibitor (galactose).

The reaction mixture consisting of 0.2 — 4.0 mM ∝-PNPG, 50 mM sodium phosphate buffer, pH 7.0, 1 mM mercaptoethanol appropriate amount of inhibitor (galactose) and enzyme (in total volume of 1.0 ml assay mixture) was incubated at 37°C for 10 min, — ○ — no inhibitor; — ✳ — ∝-galactose (0.5 mM); — ● — galactose (2.0 mM).

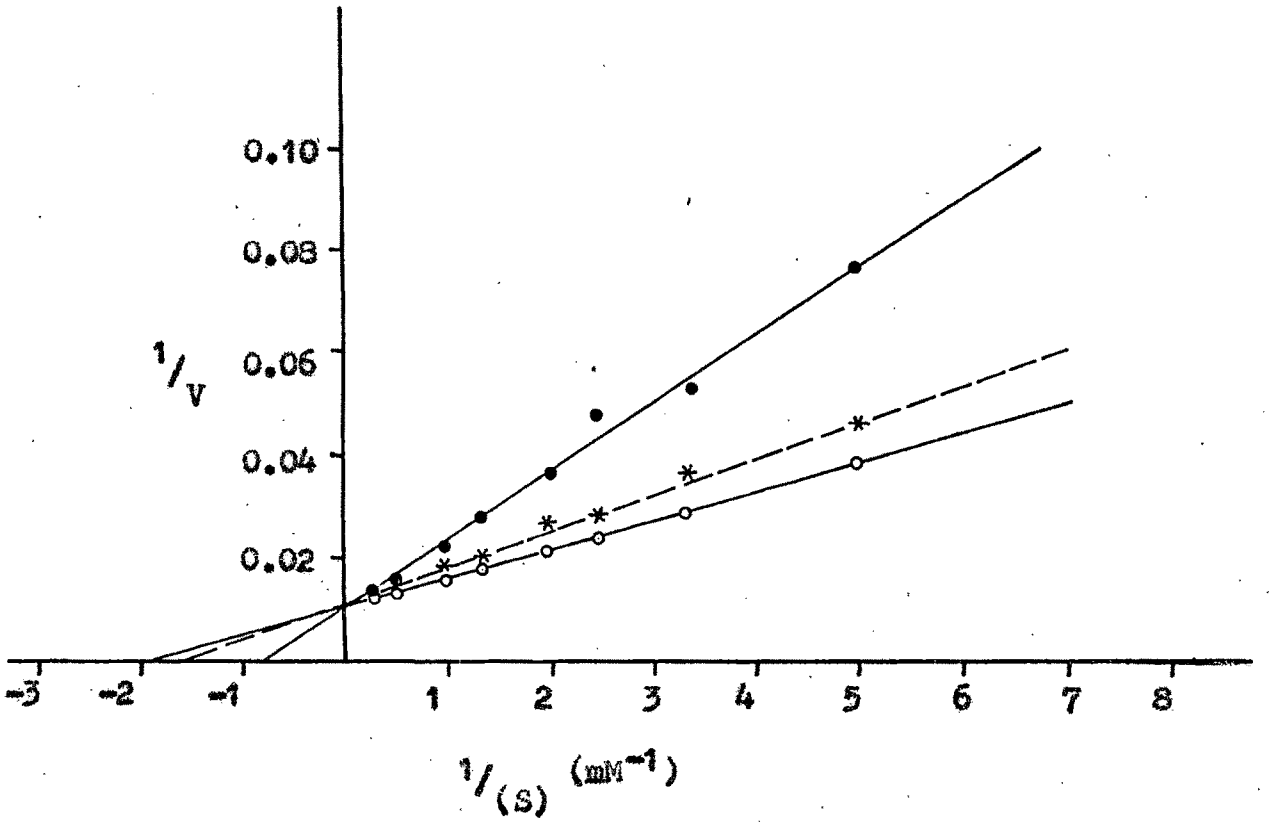


FIG.60 : Lineweaver Burk plot for the hydrolysis of α-PNPG in the presence of inhibitor (melibiose).

The reaction mixture consisting of 0.2 ~ 4.0 mM α-PNPG, 50 mM sodium phosphate buffer, pH 7.0, 1 mM mercaptoethanol, appropriate amount of inhibitor (melibiose) and enzyme (total volume of assay mixture = 1.0 ml) was incubated at 37°C for 10 min, —○— ,no inhibitor; —✳— , melibiose (1mM); —●— , melibiose (10 mM).

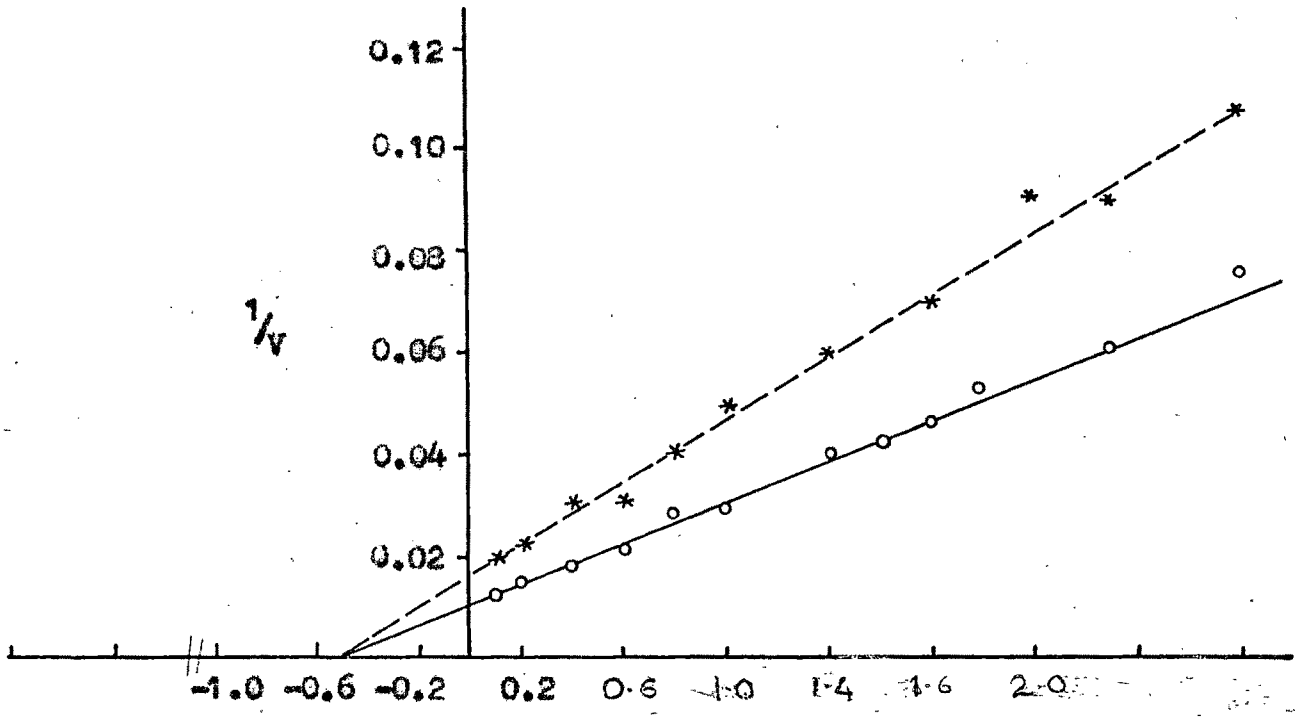
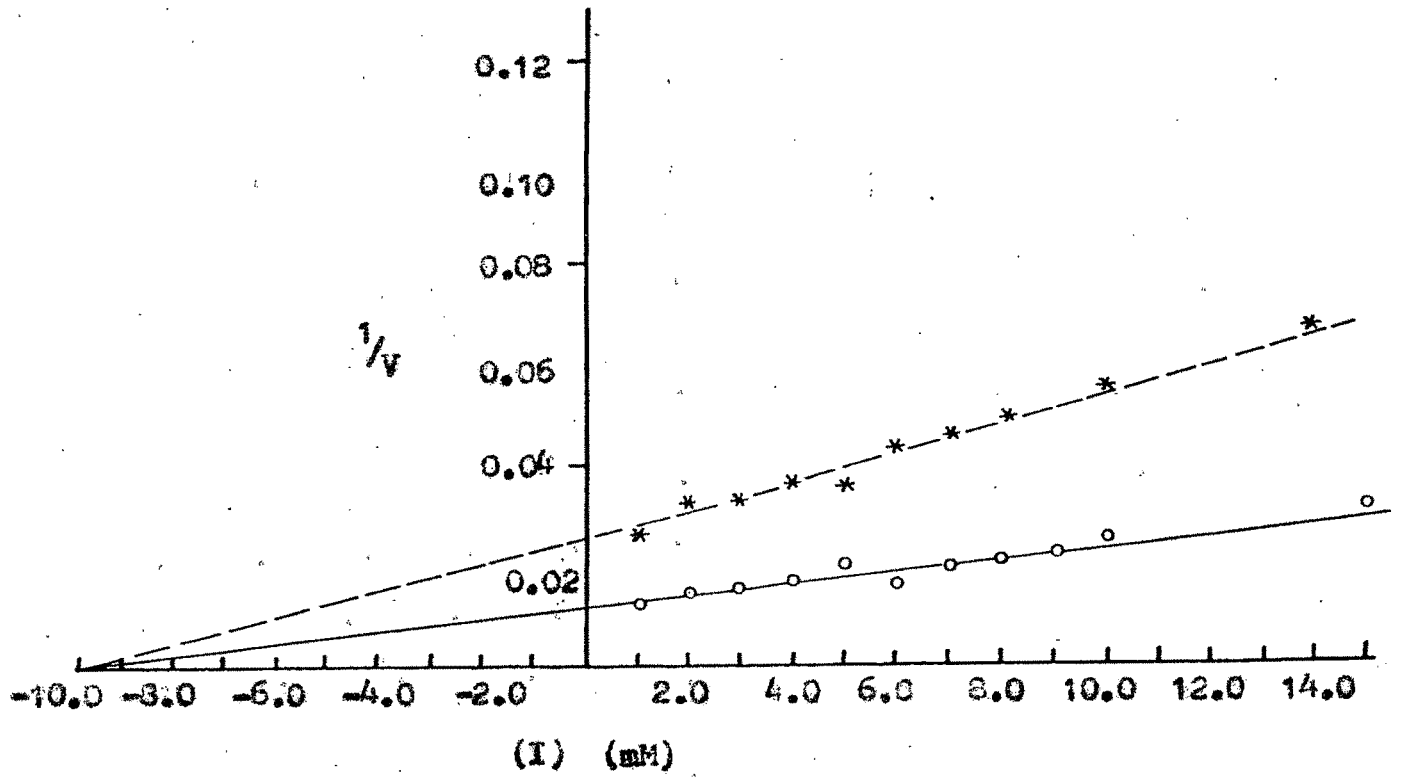


FIG.61 : Dixon plot of ∝-galactosidase of Bacillus sp. I for the inhibition by galactose.

The reaction mixture consisting of 0.1 ~ 3.0 mM galactose, ∝-PNPG (desired concentration), 50 mM sodium phosphate buffer, pH 7.0, 1 mM 2-mercaptoethanol and ∝-galactosidase (Total volume of the assay mixture = 1.0 ml) was incubated at 37°C for 10 minutes.

—O— , 1 mM ∝-PNPG; --X-- 0.5 mM ∝-PNPG.

FIG.62 : Dixon plot of α-galactosidase of Bacillus sp. I for the inhibition by melib
Details of the experiment were same as described in legend of Fig.61, except melibiose was used as an inhibitor instead of galactose.
— ○ — , 1.0 mM α-PNPG; -- * -- 0.5 mM α-PNPG)



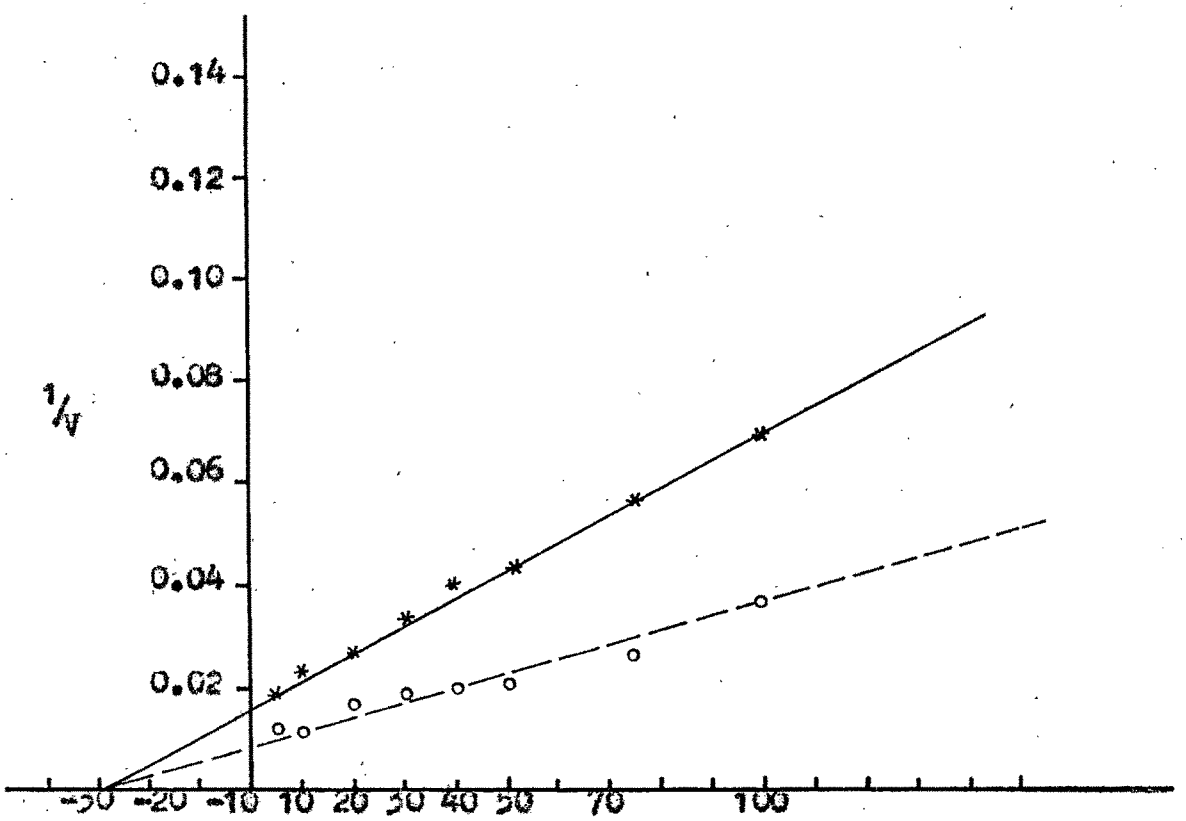


FIG.63 : Dixon plot of ∝-galactosidase of Bacillus sp. I for the inhibition by raffinose.

Details of the experiment were same as described in legend of Fig.61, except raffinose was used as an inhibitor instead of galactose.

1 mM of ∝- PNPG

0.5 mM of ∝-PNPG.

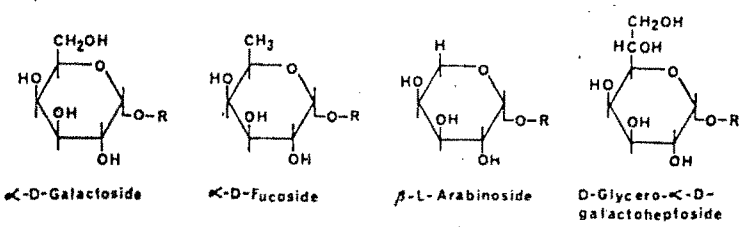
TABLE-103 : Substrate specificity of ∝-galactosidase from Bacillus Sp. I.

	Substrate	Km (mM)	Vmax (µmoles of product liberated per min per mg protein)
1)	p-nitrophenyl-∝-D-galactosidase	0.50	83
2)	p-nitrophenyl-∝-D-glucoside	(a)	(a)
3)	p-nitrophenyl-∝-D-fucoside	20	76
4)	O-Nitrophenyl-∝-D-galactoside	1.5	70
5)	m-Nitrophenyl ∝-D-galactoside	7.5	68
6)	p-Nitrophenyl--β-D-galactoside	(a)	(a)
7)	p-Nitrophenyl--β-L-arabinoside	(a)	(a)
8)	Melibiose	8.0	250
9)	Raffinose	36.0	285
10)	Stachyose	66.0	300

(a) - Not hydrolysed

specific points of attachment of the substrate to the enzyme is through the primary alcohol group of the galactose structure (Dey and Pridham, 1972; Oishi and Aida, 1976). During the present investigation on ∝-galactosidase from Bacillus sp. I also similar results were obtained viz. affinity of the enzyme for ∝-D-galactoside >∝D-fucoside. The Km and Vmax values of ∝-galactosidase during present investigation are compared with ∝-galactosidase from various sources in Table-104.

It has been known that ∝-galactosidases recognize and require for their action the specific configuration of -OH groups at the C-1, 2, 3 and 4 positions of -D-galactopyranoside. The configuration at the C-6 position is also recognized but is not necessarily required for the enzyme action (Dey and Pridham, 1972). Thus ∝-D-fucoside (Dey and Pridham, 1969a; Oishi and Aidam, 1976) and β-L-arabinoside (Dey and Pridham, 1969a; Malhotra and Dey, 1967; Suzuki et al, 1970) having D-galactose configuration are hydrolyzed by ∝-galactosidases, although some enzymes are unable to hydrolyze the latter substrates (Bailey, 1963; Li et al, 1963). The structure of ∝-D-galactopyranoside and related glycosides are as follows :-



α-D-Galactopyranoside and related glycosides.

D-Galactose, L-arabinose and inositol behaved as inhibitors of ∝-galactosidase of Bacillus sp. I when p-nitrophenyl- ∝-D-galactoside was used as a substrate, during the present studies. D-galactose is a powerful and competitive inhibitor of various other ∝-galactosidases, viz. Vicia faba (Dey and Pridham, 1969a), alfa-alfa seeds (Takatoshi et al, 1979), Aspergillus niger (Lee and Waeek, 1970), Calvatia cyanthiformis (Li and Shetlar, 1964), Diplococcus pneumoniae (Li et al, 1963), Mortirella vinaceae (Suzuki et al 1970), Bacillus stearothermophilus (Pederson and Goodman, 1980), Saccharomyces carlsbergensis (Lazo et al, 1978) etc. In contrast to this, Akiba and Horikoshi (1976) did not find inhibition of ∝-galactosidase from alkalophilic bacteria by galactose, but glucose and sucrose inhibited the enzyme reaction. The action of ∝-galactosidase in many cases is strongly inhibited by L-arabinose competitively or non-competitively (Dey and Pridham, 1969a; Dey, 1969; Lee and Wacek, 1970; Suzuki et al, 1970), ∝-galactosidases from Pycnoporus sp., Micrococcus sp., alfaalfa seeds and Streptomyces sp. are found to be inhibited by melibiose and raffinose either competitively or non-competitively (Takatoshi et al, 1979; Akiba and Horikoshi, 1976; Oishi and Aida, 1976; Mitsutomi et al, 1985), whereas ∝-galactosidases from some plants are not inhibited by these sugars (Dey and Pridham, 1971). Xylose and inositol behaved as competitive inhibitors for some plant ∝-galactosidases (Sharma, 1971; Hankins et al, 1980). A highly specific inhibition of ∝-galactosidases occurs with myo-inositol due to similarity in the orientation of -OH groups at C-2, C-3 and C-4 in myo-inositol to those at C-4, C-3 and

C-2 or C-1, C-2 and C-3 of ∝-D-galactopyranosyl residue of the substrate (Keleman and Whelan, 1966; Sharma, 1971). Glucose, fructose, mannose and D-ribose do not produce inhibition of ∝-galactosidase in the present investigation, which is similar to other ∝-galactosidases (Cf. Dey and Pridham, 1972). In the present experiment, ∝-galactosidase from Bacillus sp. I did not recognize β-galactosyl bond thus lactose and cellobiose had no effect on catalytic activity of the enzyme.

In the present investigation, the rate of hydrolysis of the substrate seem to be reduced by an increase in the chain length of oligosaccharides because Km for melibiose < raffinose < stachyose of ∝-galactosidase from Bacillus sp. I. Similar observations were made by several workers for-∝-galactosidases from various sources (Li et al, 1963; Coleman, 1968; Dey and Pridham, 1969a; Oishi and Aida, 1976; Akiba and Horikoshi, 1976; Lazo et al, 1978), but in many other ∝-galactosidases reverse is also reported to occur (Courtois et al, 1959; Bailey, 1963).

Data given in Table-105 show the effect of different divalent cations and other organic and inorganic compounds on the catalytic activities of ∝-galactosidase from Bacillus sp. I. It was found that enzyme activity was completely inhibited by Ag^{+}, Hg^{+2}, Pb^{+2} and Cu^{+2}. ∝-Galactosidase from various microbial sources have been shown to be affected by metals like Ag^{+}, Hg^{+2}, Cu^{+2}, Pb^{+2} and Fe^{+2} (Li and Shetlar, 1964; Lee and Wacek, 1970; Suzuki et al, 1966; Oishi and Aida, 1972; Schmid

TABLE-105 : Effect of various inhibitors on catalytic activity of purified -galactosidase from Bacillus sp. I.

	0.1 mM		1.0 mM		10 mM	
	Enzyme units (a)	% Residual activity	Enzyme units	% Residual activity	Enzyme units	% Residual activity
Control	0.265	100	0.265	100	0.265	100
Ca^{2+}	0.294	111	0.276	104	ppts	-
NH_4^+	0.273	103	0.246	93	0.240	91
Fe^{2+}	0.262	99	0.276	104	ppts	-
Co^{2+}	0.268	101	0.233	88	"	-
Li^{2+}	0.257	97	0.248	94	"	-
Mn^{2+}	0.270	102	0.276	104	"	-
Ag^+	0.027	10	0	0	"	-
Hg^{2+}	0	0	0	0	"	-
Zn^{2+}	0.173	65	0.123	46	0.110	42
Pb^{2+}	0.080	30	0.040	15	ppts	-
Cu^{2+}	0.048	18	0	0	"	-
Sodium azide	0.241	91	0.225	85	0·164	62
Iodoacetate	0.024	09	0.085	32	0.032	12
PCMB	0.032	12	0	0	0	0

contd.

TABLE-105 (Contd...)

	0.1 mM		1.0 mM		10 mM	
	Enzyme units	% Residual activity	Enzyme units	% Residual activity	Enzyme units	% Residual activity
NEM (N-Ethyl malemide)	0.044	17	0.021	8	0	0
EDTA	0.254	96	0.246	93	0.254	96
Tris-HCl (pH 7.0)	0.199	75	0.080	30	0.006	0
Phytate	0.278	105	0.260	98	0.244	92
Trypsin inhibitor	0.255	96	0.233	88	0.217	82
Urea (0.1M)[b]	0.111	42	-	-	-	-
(0.5M)	0.021	08	-	-	-	-
(1.0M)	0.00	00	-	-	-	-
Guenidine-HCl (0.1M)[b]	0.156	59	-	-	-	-
(0.5M)	0.085	32	-	-	-	-
(1.0M)	0.00	00	-	-	-	-

(a) Enzyme units are expressed as amount of enzyme required to liberate one micromole of p-nitrophenol per minute under assay conditions.

(b) Urea and guanidine-HCl were added at the concentration given in parentheses.

Akiba and Horikoshi, 1976; Schmid and Schmitt, 1976; Williams et al, 1977; 1978; Ohtakara et al, 1984). Plant -galactosidases from Vicia faba and Prunus amygdalus (Dey, 1969; Dey and Pridham, 1969), spinach leaves (Gatt and Baker, 1970) and sweet almond (Dey and Malhotra, 1969) are also inhibited by various metal ions. ∝-Galactosidase from fungi (Ohtakara et al, 1984), legumes (Dey and Pridham, 1977) and sugar cane (Chinen et al, 1981) are usually inhibited by silver and mercury ions competitively or noncompetitively. The inhibition by silver ions may be attributed to their reaction with the carboxyl group or histidine residues, and that by mercury ions may be attributed to their binding of the thiol group of the enzyme (Dey and Pridham, 1977; Chinen et al, 1981). Bacterial ∝-galactosidases are also inhibited by lead, zinc, cobalt, magnesium and nickel ions (Olezlo and Zaprometova, 1982). Other inhibitors like PCMB and NEM also caused 100% loss of ∝-galactosidase activity during present investigation suggesting that present ∝-galactosidase require -SH groups for its activity. Contribution of sulphydryl group for the ∝-galactosidase activity is also shown in Micrococcus sp. (Akiba and Horikoshi, 1976), A. niger (Lee and Wacek, 1970), D. pneumoniae (Li et al, 1963), S. olivaceus (Suzuki et al, 1966) whereas ∝-galactosidase from Calvatia cyanthiforms (Li and Shetlar, 1964), spinach leaves (Gatt and Baker, 1970), sweet almond and Vicia faba (Dey and Pridham, 1969; Dey, 1969) were not inhibited by sulphydryl binding reagents.

Studies were also carried out to find out the effect of various metal ions and other known inhibitors on catalytic activity of invertase of Bacillus sp. I. Data given in Table-106 shows that the enzyme activity was inhibited by Ag^{+}, Hg^{+2}, Zn^{+2}, Cu^{+2} and iodide. β -D-fructofuranosidases from N. crassa, yeast and plants are also found to be inhibited by metals like Zn^{+2}, Cu^{+2}, Hg^{+2} and Ag^{+} (Lampen, 1974; Hawker, 1980; Pressey and Avants, 1980). However, Mn^{+2} had a protective effect on sugarcane enzyme (Guiroga et al, 1977). PCMB and NEM also completely inhibited the catalytic activity of the enzyme at 1-10 mM suggesting the requirement of -SH group in its active site. The thiol group requirement of the enzyme from Bacillus sp. I is similar with external invertase of N. crassa, which also requires '-SH' group at an active site, whereas it is different from yeast external invertase, which does not require '-SH' group (Neumann and Lampen, 1969). Urea and guanidine-HCl at 1-50 mM did not affect the enzyme activity, which indicates that the present enzyme might not be existing in a multimeric form. Pyridoxal phosphate which inhibited invertase of potato (Pressey, 1967) did not affect the present enzyme as shown in Table-106.

α -Galactosidase isolated in the present investigation was completely inhibited by 10 mM Tris-HCl buffer, as shown in Table-105. Competitive inhibition of glycosidases by 'Tris' is reported by other workers also (Dahlquist, 1961; Jorgensen and Jorgensen 1967; Schmalreck et al, 1975; Pederson and

TABLE-106 : Effect of various inhibitors on the catalytic activity of purified invertase from Bacillus sp. I.

	1 mM		10 mM		0.1 mM	
	Enzyme units(a)	% Residual activity	Enzyme units	% Residual activity	Enzyme units	% Residu activity
ontrol	0.160	100	0.160	100	0.160	100
Ca^{2+}	0.182	114	ppts	-	0.162	101
NH_4^+	0.168	105	0.122	76	0.168	105
Fe^{2+}	0.160	100	ppts	-	0.163	102
Co^{2+}	0.147	92	ppts	-	0.153	94
Li^{2+}	0.152	95	"	-	0.156	98
Cu^{2+}	0.016	10	"	-	0.040	25
Mn^{2+}	0.160	100	"	-	0.168	105
Ag^{2+}	0	0	"	-	0.015	09
Hg^{2+}	0	0	"	-	0	0
Mg^{2+}	0.157	98	0.173	108	0.156	98
Zn^{2+}	0.114	71	ppts	-	0.128	80
Pb^{2+}	0.064	40	"	-	0.104	65
KI	0.05	32	0.019	-	0.142	89
Iodo acetate	0.035	22	0	0	0.089	56
pCMB	0	0	0	0	0.008	05
NEM(N-Ethyl Malemide)	0.012	8	0	0	0.027	17
Sodium azide	0.126	79	0.089	12	0.158	99
EDTA	0.142	89	0.149	93	0.156	98
Tris	0.076	48	0.006	04	0.072	45

contd.

TABLE-106 (Contd.....)

	1 mM		10 mM		0.1 mM	
	Enzyme units	% Residual activity	Enzyme units	% Residual activity	Enzyme units	% Residual activity
Pyridoxal hosphate	0.163	102	0.152	95	0.162	1
Urea (0.1M)[6]	0.142	89				
(0.5M)	0.138	86				
(1.0M)	0.128	80				
Guanidine HCl (0.1M)[b]	0.158	99				
(0.5M)	0.134	84				
(1.0M)	0.130	81				

(a) Enzyme units are expressed as amount of enzyme required to liberate one micromole of glucose per minute under assay conditions.

(b) Urea and guanidine HCl were added at concentration given in parentheses.

Goodman, 1980) and Burstein and Kepes (1971) have also reported that an ∝ -galactosidase of E. Coli loses its activity, if the enzyme is stored in Tris-HCl buffer. The present enzyme does not require any cofactor or divalent cations for its catalytic action unlike 'mel' coded ∝ -galactosidase of E. Coli (Schmitt and Rotman, 1966; Burstein and Kepes, 1971).

During present investigation, when substrate studies with β-D-fructofuranosidase from Bacillus sp. I were carried out it was found that Km for sucrose, raffinose and maltose were 5 mM, 22 mM and 20 mM respectively as shown in Figs.64 & 65. Trehalose and melilotriose were not attacked by the purified enzyme preparation, which suggest that it is a very specific enzyme. Thus substrate specificity studies on invertase of Bacillus sp. I shows that the affinity of the enzyme is highest for sucrose among all the substrates studied. Invertase from N. crassa (Lampen, 1971) and S. cerevisiae (Barnett ., 1980) seem to have similar properties whereas Grootwassink and Fleming (1980) reported a higher rate of hydrolysis of stachyose > raffinose > sucrose for a commercial preparation of this enzyme from xx other Saccharomyces species. Purified β-D-fructofuranosidase from S. cerevisiae hydrolyzed ethyl β -D-fructofuranoside and p-nitrophenyl- β -D-furanosylamine according to Baser and Shall (1971) who suggested that later compound might be a useful chromogenic substrate for the assay. Since this compound was not available easily, the hydrolysis of the same by β -D-fructofuranosidase from Bacillus sp. I was not studied in the present

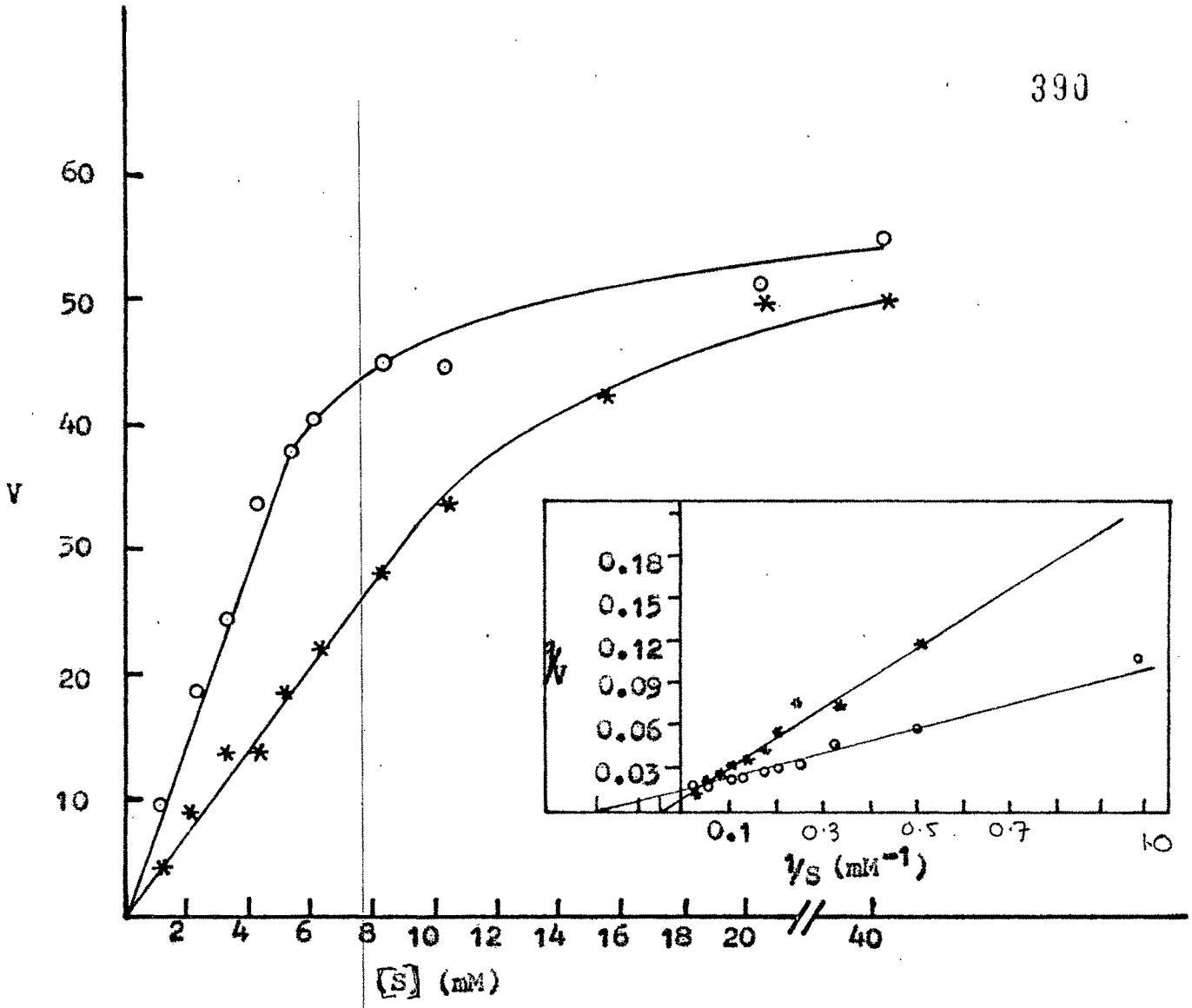


FIG.64 : Effect of substrate (sucrose O—O ; and maltose *—*) concentration on the rate of its hydrolysis by invertase of Bacillus sp. I. The assay mixture contained substrate (desired concentration), mercaptoethanol (1 mM), 0.05 M sodium phosphate buffer, pH 7.0, purified invertase (3.0 µg) in total volume 1.5 ml, at 37°. Reaction was started by the addition of enzyme and the time dependent rate of glucose liberated was measured. The rate is expressed as micromoles of glucose liberated in per minute per mg protein. Inset shows double reciprocal plot of the data.

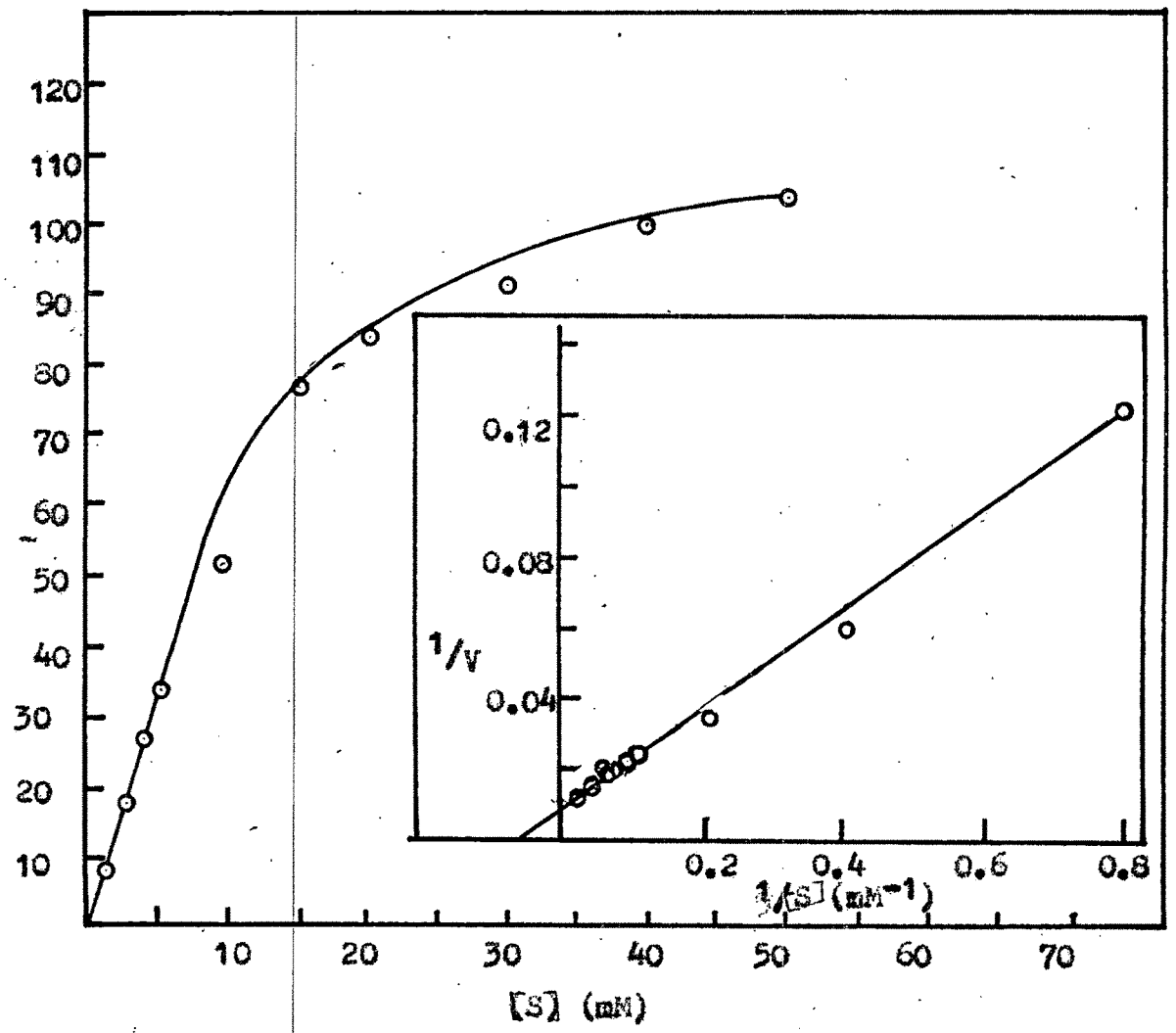


FIG. 65 : Effect of raffinose concentration on the rate of its hydrolysis by purified invertase from Bacillus sp. I.

The assay mixture contained raffinose (desired concentration), 2-mercaptoethanol (1 mM), and purified invertase ($\leq$ µg protein) in 50 mM sodium phosphate buffer, pH 7.0. (Total 1.5 ml assay system) at 37°. Reaction was started by addition of the enzyme. The rate of reaction 'V' is expressed as umoles of reducing sugar liberated per min per mg protein. Inset shows double-reciprocal plot of the data.

investigation. The Km (⟶) values of β-D-fructofuranosidase for various substrates in different micro-organisms are compared in Table-107. The β-D-fructofuranosidase isolated in the present study was inhibited in 10 mM Tris-HCl buffer, pH 7.0 resembling French beans invertase (Hawker, 1980) which was also inhibited by Tris buffer.

The characteristics of invertases from Bacillus sp. I are compared in Table-107 with invertase isolated from various other sources.

Molecular properties of purified α-galactosidase and invertase of Bacillus sp. I :

Purity of the final enzyme preparations was tested by dodecyl sulfate gel electrophoresis after boiling the sample with 1% SDS and 1% 2-mercaptoethanol in sample buffer (Shapiro, 1967) as described under 'Materials and Methods'. A single band of each enzyme preparation was obtained at protein concentration ranging from 20-100 µg protein/gel indicating a high degree of homogeneity as shown in Figs. 66 & 67.

Specific staining of gels for glycoprotein using a method of Zacharias et al. (1969) gave no positive reaction indicating the lack of detectable carbohydrate moiety in both α-galactosidase and invertase in the present investigation. This is possible because only eukaryotic α-galactosidases and

TABLE-107 : Comparison of characteristics of inverteses from various sources.

Source	Optimum pH	Km (mM) for Sucrose	Km (mM) for Raffinose	Molecular weight	Inhibitors	Reference
Saccharomyces sp.						
Smaller enzyme	9.0	16-40	81-292	1,35,000	Ag^+, Hg^{2+}, Zn^{2+}, urea, PCMB, Iodine, NH_4^+	Gascon and Lampen (19[illegible]; Lampen (1971); Baseer and Shall (1971).
Larger enzyme	3.0			2,70,000		
Saccharomyces pastorianus	5.0	38	-	-	-	Toda and Shoda (1978)
Neurospora sp.						
Heavy	4.5 - 6.0	6.1	6.5	2,10,000	Zn^{2+}, Cu^{2+}, aniline, PCMB	Lampen (1971)
Light	"	6.1	6.5	51,500		
French bean	5.0	7.5	-	-	-	
(Leaf)	7.5	27.0	-	-	Tris	Hawker (1980)
Sugar cane I	5.0-5.5	2.84	-	-	-	Shampietro et al.(1980)
(leaf sheath) II	4.5	23.0	-	-	Metal ions	

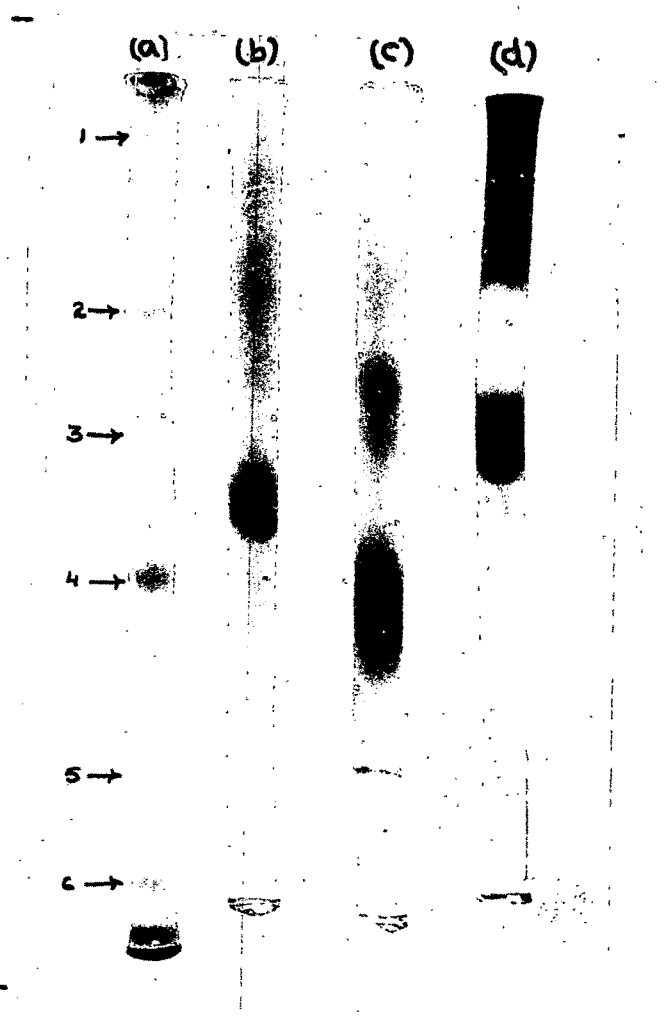


FIG.66 : SDS-polyacrylamide disc gel electrophoresis in 7.5% acrylamide gels of purified α-galactosidase from Bacillus sp. I and of standard proteins :

(a) Standard proteins: 1) Myosin L^H chain - MW = 2,00,000, 2) Phosphorylase - b - MW - 97,000, 3) Bovine serum albumin - MW - 68,000, 4) Ovalbumin - MW - 43,000, 5) α-Chymotrypsinogen - MW - 25,700, 6) β-lactoglobulin - MW - 18,400;

(b) Purified α-galactosidase from Bacillus sp. I;

(c) Standard protein - (ovalbumin - MW - 43,000);

(d) Standard protein (bovine serum albumin - MW - 68,000).

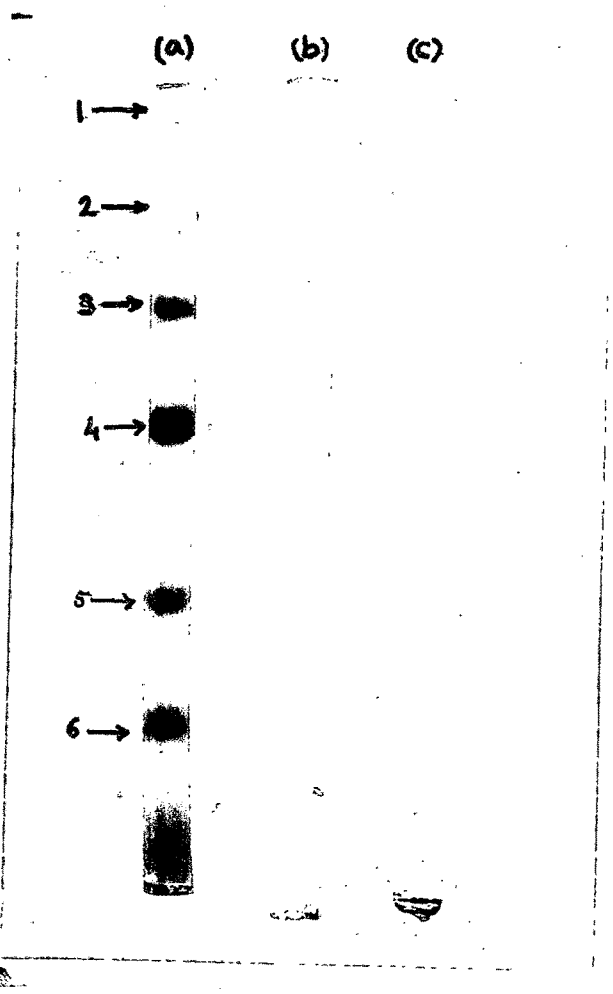


FIG.67 : SDS-polyacrylamide disc gel electrophoresis in 7.5% acrylamide gel of purified invertase from Bacillus sp.I and of standard proteins;

(a) Standard proteins(Same as in fig 66).
(b) Other standard proteins;
(c) Purified invertase from Bacillus sp. I

invertases are reported to be glycoproteins (Malhotra and Dey, 1967; Adya and Elbein, 1977; Lazo et al, 1978; Dey et al, 1982; Dey et al, 1983).

The molecular weight of native purified ∝ -galactosidase from Bacillus sp. I as determined by gel filtration technique (details are given in 'Materials and Methods') is found to be approximately 1,90,000 (Fig.68a).

Data given in Table-105 show the effect of urea, and guanidine-HCl ⟶ on the activity of purified ∝ -galactosidase from Bacillus sp. I. All the three compounds inactivated the enzyme as the concentrations of each were increased, suggesting that the protein must be having oligomeric structure. When the mobility of denatured enzyme in SDS PAGE was compared to those of proteins of known molecular weights, it appears to have molecular weight of 48,000 as shown in Fig.66. Thus the molecular weight determined by SDS-PAGE and gel filtration proved that ∝ -galactosidase of Bacillus sp. I is having a tetramer structure with each subunit of 48,000.

With respect to molecular weight ∝ -galactosidase from Bacillus sp. I, it resembles other microbial ∝ -galactosidases from Py. cinnabariuis MW 2,10,000 (Ohtakara et al,1984), E. Coli MW 2,00,000 (Burstein and Kepes, 1971), whereas ∝ -galactosidase having oligomeric structure and higher molecular weights have also been reported from other sources viz.

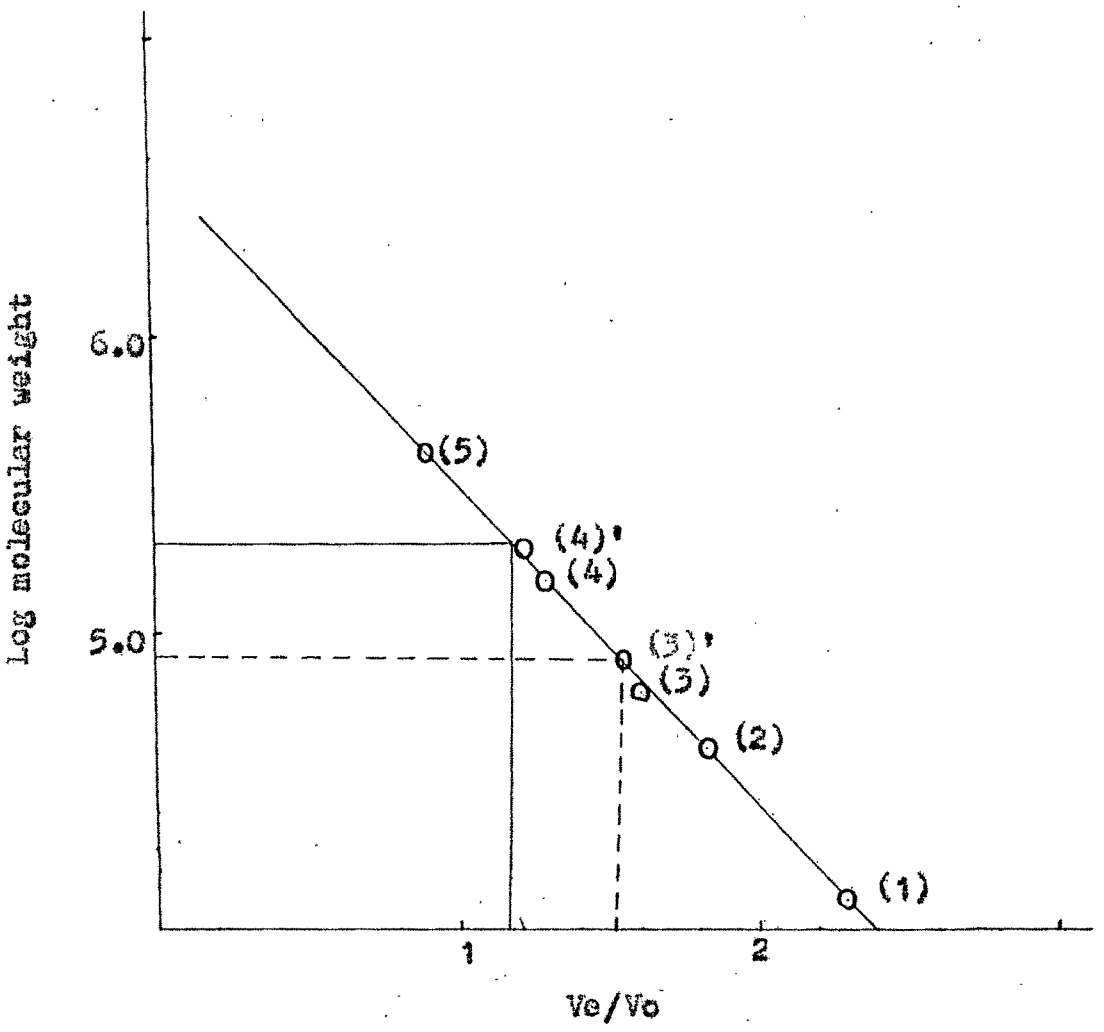


FIG.68a : Molecular weight determination of the native ∝-galactosidase and invertase by analytical gel filtration using Sephadex G-200.

An aliquot of purified enzyme was filtered through a column of Sephadex G-200 equilibrated with 0.05M phosphate buffer, pH 7.0, and their elution compared to those of (1) Cytochromose-C (MW - 13,000); (2) Ovalbumin (MW - 43,000); (3) Bovine serum albumin (MW - 68,000); (4) Yeast alcohol dehydrogenase (MW - 1,50,000), and (5) Horse spleen ferritin (MW - 4,40,000).

(3)' Invertase from Bacillus sp. I.

(4)' ∝-Galactosidase from Bacillus sp. I.

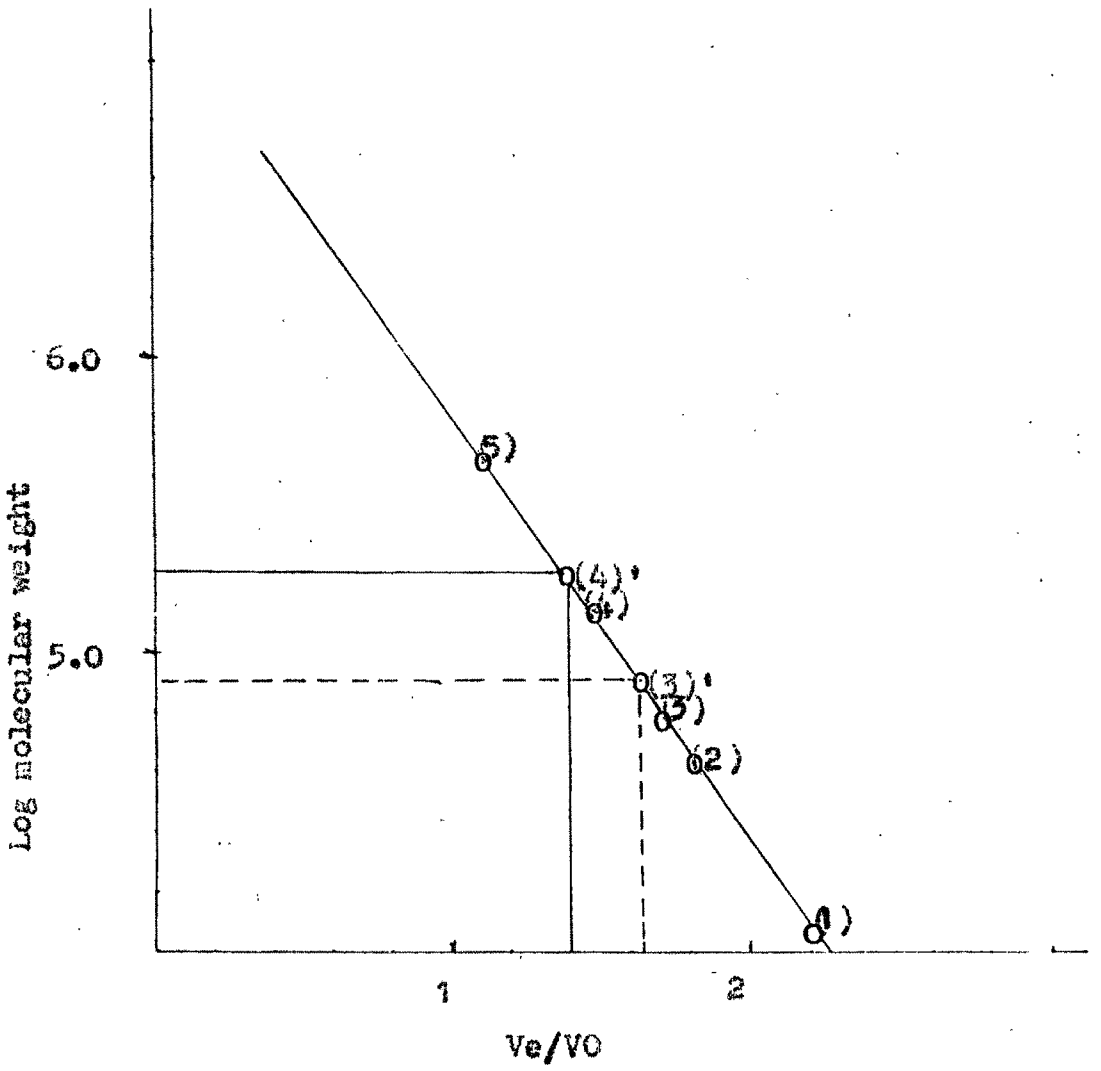


FIG.68b : Molecular weight determination of the native ∝-galactosidase and invertase by analytical gel filtration using Sepharose-6B.

An aliquot of purified enzyme was filtered through a column of Sepharose-6B equilibrated with 0.05M phosphate buffer, pH 7.0, and their elution compared to those of (1) Cytochromose-C (MW - 13,000); (2) Ovalbumin (MW -43, 000);(3) Bovine serum albumin (MW - 68,000); (4) Yeast alcohol dehydrogenase (MW - 1,50,000), and (5) Horse spleen ferritin (MW - 4,40,000).

(3)' Invertase from Bacillus sp.I.

(4)' -Galactosidase from Bacillus sp.I.

S. carlsbergenesis, MW 3,00,000 (Lazo et al, 1978), B. stearothermophilus MW I - 2,80,000, II - 3,25,000 (Pederson and Goodman, 1980) and E. Coli harboring PRSD$_1$ plasmid MW 3,00,000 (Schmid and Schmitt, 1976). The present enzyme for Bacillus sp. I is not having any molecular forms unlike those which are reported in thermophilic B. stearothermophilus by Pederson and Goodman (1980). The existence of multimolecular forms of α-galactosidase has been reported in various seeds as discussed im Section-I. Dey et al. (1982) have found that α-galactosidase of Vicia faba is separated by gel filtration using Sephadex G-100 into two forms, form I with molecular weight 1,60,000 and II of 40,000. Later on, they resolved Form-II into two ionic forms II^1 and II^2, and also shown that form I (1,60,000) is a tetramer consisting of subunits II^2.

The molecular weights and other properties of α-galactosidase from various other sources are compared with that of Bacillus Sp. I, in Table-108.

The molecular weight of purified invertase from Bacillus sp. I as determined by gel filtration was found to be 80,000 as shown in Fig.68b. When determination of molecular weight was carried out using SDS-PAGE, it was estimated to be 75,000 (Fig.67). Thus invertase from Bacillus sp. I is a smaller enzyme compared to those of eukaryotic sources as described in Table-107. In the present investigation, the enzyme was not found to be glycoprotein and the multimolecular forms were

also absent unlike those present in eukaryotic sources. The molecular forms of yeast invertase differ in their molecular weights and cellular location (Iglesias et al, 1980). The heavy enzyme is a glycoprotein (MW 2,70,000) with 50% mannose and 3% glucosamine, whereas the light enzyme (MW 1,35,000) has no carbohydrates. Many plant invertase have been also characterized as glycoproteins, e.g. those from grapes (Arnold, 1966), barley (Prentice and Robbins, 1976), Convolvulus (Klis and Akster, 1974) and raddish (Faye and Berjohneau, 1979). Invertase from N. crassa is also a glycoprotein containing 11% mannose and 3% glucosamine covalently linked to the protein moiety (Lampen, 1971).

Section - IV

Studies on bacterial degradation of Neurotoxin (ODAP)

The results of the investigation on capacity of various bacterial isolates to degrade neurotoxin (ODAP), mode of breakdown of ODAP and optimization of cultural conditions for the maximum production of ODAP hydrolysing enzymes in a bacterial isolate and the effect of fermentation on ODAP content of L. sativus dhal are presented and discussed in this section.

For these studies, it was necessary to have a large quantity of pure neurotoxin (ODAP). The method of isolation of ODAP from seeds as reported by Rao et al. (1964) was time consuming and further the yield was very low. Therefore a chemical method for synthesizing the toxin from diaminopropionic acid was reported later on by the same worker. He prepared a copper complex of diaminopropionic acid to protect the α-amino group and introduced the oxalyl group in the `$\beta$' position, by treating the complex with oxalylchloride. It was possible to avoid this step and introduce oxalyl group in the `β' position by treating diaminopropionic acid with dimethyl oxalate in the synthesis method reported by Ramchand et al. (1983). By this method, oxalyl group enters only in the `β' position of diamino-

propionic acid, essentially due to electromeric interaction of C-O and C-N bonds. Hence for the present investigation, ODAP was prepared using method of Ramchand et al. (1983), as described under 'Materials and Methods'. ODAP was also extracted and purified from L. sativus seeds using method of Rao et al. (1964) for comparative studies.

Many investigators have used labelled ODAP for understanding its mechanism of action in animals. Rao (1975b) has reported a method for the synthesis of (^{3}H) ODAP containing tritium at 2 and 3 positions. Mehta et al. (1976) have reported a method for the synthesis of (^{14}C) oxalyl labelled ODAP. In the present studies, labelled ODAP was required for metabolic studies in bacteria, hence an attempt was made to synthesize oxalyl labelled ODAP using ^{14}C-labelled oxalic acid as described under "Materials and Methods".

Tables-109 & 110 gives the percentage recovery of radioactivity in the intermediates and final product viz. dimethyl oxalate and ODAP obtained during the synthesis of labelled ODAP. This labelled ODAP having specific activity 14.2 µCi/mmole was used in the enzyme assay as a substrate to assay the bacterial enzymes, after checking the biological and chemical properties as described below :

TABLE-109 : Recovery of radioactivity during conversion of ^{14}C-oxalic acid into ^{14}C-dimethyl oxalate.

Radioactivity of ^{14}C-oxalate used			Concentration of carrier oxalate added (mmoles)	Specific activity after the addition of carrier (µCi/mmole)	Activity of labelled dimethyl oxalate			% Recovery of radioactivity
Total activity (µCi)	Specific activity (µCi/mmole)	Total CPM			Total activity (µCi)	Specific activity (µCi/mmoles)	CPM	
50	5000	9.4×10^7	5.0	10	30	14.2	5.6×10^7	60

TABLE-110 : Recovery of radioactivity during conversion of ^{14}C-dimethyl oxalate into oxalyl labelled ODAP.

^{14}C-dimethyl oxalate used			Concentration of diamino-propionic acid used (mmoles)	Oxalyl labelled ODAP formed			Recovery of radioactivity (%)
Total activity (μCi)	Specific activity (μCi/mmole)	Total CPM		Total activity (μCi)	Specific activity (μCi/mmoles)	Total CPM	
30	14.2	5.6×10^7	2.0	21.6	22.0	4.15×10^7	72

Biological and chemical properties of different ODAP preparations :

Chemical properties of the ODAP preparation were studied with respect to melting point, IR spectra, as well as mobility in the thin layer chromatography (Table-111). The IR spectral analysis of labelled ODAP, cold synthetic ODAP and ODAP extracted from seeds was found to be the same which is given in Fig.69. The chromatographic separation of all the three preparations is given in Fig.70 which indicate that all the three had same Rf values and they did not contain free diaminopropionic acid as impurity.

Since ODAP is found to be toxic to the rats when injected intraperitoneally, the toxicity of the compounds prepared were also tested during present investigation. When ODAP preparations at a level of 50-60 mg/100g body weight were injected into adult rats intraperitoneally, the typical symptoms of paralysis and death were observed after 10 to 15 minutes of injection (Table-112). These experiments showed that all the three preparations had neurotoxic effects on rats. When the precursors of the ODAP synthesis viz. dimethyl oxalate and diaminopropionic acid were injected they did not show any symptoms of the toxicity, thereby suggesting that the toxicity is due to ODAP only and not the intermediates/precursors.

Since the biological and chemical properties of the ODAP prepared in the laboratory were same as reported ones, this ODAP was used for further experiments.

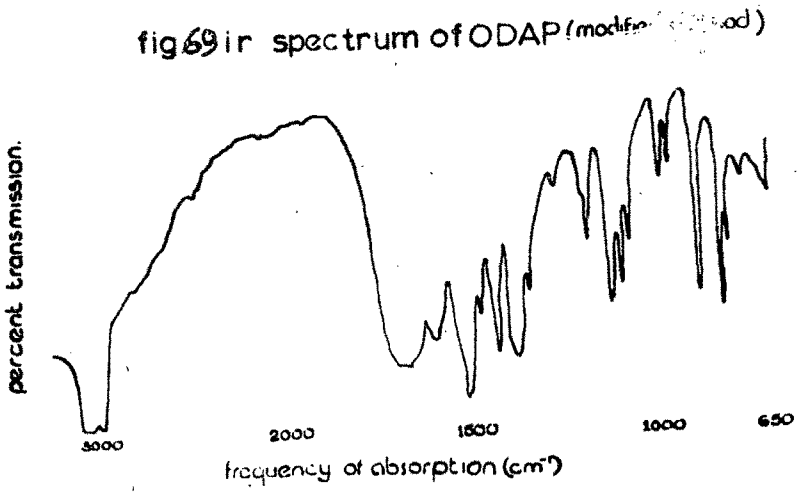


FIG.70 : TLC of different neurotoxin (ODAP) preparations:
(a) Standard diaminopropionic acid,
(b) ODAP extracted from L. sativus seeds,
(c) ODAP, synthesized using the chemical method of Ramchand et al.(1983),
(d) C-^{14} Oxalyl-labelled ODAP, synthesized using the chemical method of Ramchand et al. (1983).

(a) (b) (c) (d)

ODAP.

DAPA.

Fig: 9

TABLE-111 : Characteristics of ODAP prepared by different methods.

Mode of preparation of neurotoxin	Melting Point (°C)	'Rf' value in thin layer chromatography	Yield of the compound (%)
1. ODAP isolated from L. sativus seeds by the method of Rao et al. (1964).	205	0.80	40 - 45
2. ODAP synthesized from diamino-propionic acid using a method of Ramchand et al. (1983).	205	0.80	20 - 30
3. Oxalyl labelled ODAP synthesized by chemical method of Ramchand et al. (1983).	205	0.80	20 - 30

TABLE-112 : Effect of intraperitoneal injection of ODAP (prepared by different methods) on adult albino rats.

Time period after injection (min)	Behaviour of rat injected with saline	Behaviour of rats when injected with ODAP		
		Isolated from *L. sativus* seeds	Synthesized by the method of Ramchand et al.(1984)	Labelled ODAP synthesized by the method of Ramchand *et al.* (1984).
0	Normal	Normal	Normal	Normal
5	Normal	Irritable to touch convulsions started, increased rate of respiration, difficulty in walking.	Irritable to touch, increased rate of respiration, convulsions started.	Irritable to touch, convulsions started, increased rate of respiration.
10	Normal	Convulsions, head retraction, the head went down, hind leg stretched.	Head retraction, difficulty in walking, head went down, hind leg stretched.	Head retraction, difficulty in walking, hind leg stretched.
15	Normal	Symptoms of convulsions increased, death of animal, with severe convulsions.	Symptoms of convulsions increased, death of animal, with severe convulsions.	Symptoms of convulsions increased, head went down, death of animal with severe convulsions.

ODAP was injected at a concentration of 60 mg/100g body weight.

Control animals were injected with 1.0 ml of 0.9% saline.

Screening of bacterial isolates (from fermented foods and stored grains) for their capacity to breakdown ODAP:

As discussed earlier, fermentation of legumes as well as a mixture of legumes + cereals bring about favourable biochemical changes along with the degradation of anti-nutritional compounds, and detoxification of certain toxins. The preliminary studies carried out on fermentation of L. sativus dhal showed 20-30% decrease in neurotoxin (ODAP) content (Table-115). Therefore the experiments were carried out to isolate the bacteria from fermented foods as well as from grains from which they are being prepared which have the capacity to degrade ODAP. For these studies, various bacteria isolated from fermented soyidli and L. sativus dhal as well as from stored grains were screened for their capacity to breakdown ODAP. The preparation of culture media containing ODAP (ODAP broth) as sole source of 'C' and 'N' and the methods involved in the estimation of ODAP have been described under 'Materials and Methods'. Table-113 indicate the data on the change in pH, growth in terms of turbidity and the amount of ODAP present in the culture filtrate and cells. It was observed that among all the bacteria studied, Streptococcus sp. I and Bacillus sp. II isolated from stored L. sativus grains were efficient in degrading neurotoxin. They showed highest growth in ODAP broth in terms of turbidity measured at 660 nm. The cells did not show any accumulation of ODAP after the growth, suggesting that it is taken up in the cells and

utilized during the growth. The pH of the medium increased from 7.0 to 8.0 during the growth of bacteria, due to accumulation of alkaline metabolic end products, mainly NH_3. Since there were no other 'C' as well as 'N' sources added in the medium except ODAP, it can be concluded that bacteria could utilize ODAP as sole 'C' and 'N' source. Klebsiella aerogenes isolated from stored bajra grains did show 45% degradation of ODAP, which is much less than that by two other bacterial isolates viz. Streptococcus sp. I and Bacillus sp. II.
It can be seen in the Table-113 that bacteria isolated from fermented foods were not efficient in degrading ODAP.
L. fermenti, L. mesenteroids and Pediococcus sp. which are dominant flora during fermentation of L. sativus dhal might be responsible for bringing about other biochemical changes during fermentation.

When bacterial isolates Strentococcus sp. I and Bacillus sp. II were grown in ODAP broth (containing ODAP obtained from natural source - L. sativus seeds), as sole source of carbon and nitrogen, it was found that these bacteria could utilize this ODAP also (Table-114). The growth in terms of turbidity was more when seed ODAP was used as a substrate as compared to that of synthetic ODAP. In the preliminary experiment on TLC analysis of the culture filtrates of these bacteria (during the growth in ODAP broth), diaminopropionic acid and oxalic acid were detected as the degradative products of ODAP utilization. Since DAPA and oxalic acid were found to be the intermediates of ODAP degradation, attempts

were also made to study the efficiency of bacteria to utilize these compounds.

Figs. 71 & 72 show the growth curve of two bacterial isolates in ODAP broth and DAPA broth respectively. Streptococcus sp. I grew faster than Bacillus sp. I in ODAP broth. Complete degradation of ODAP was observed after 24 h and 48 h. of growth in the case of Streptococcus sp. I and Bacillus sp.II respectively (Fig. 71). It was found that when bacteria were grown in DAPA broth they exhibited similar growth and degradation pattern as in ODAP broth (Fig. 72).

It can be seen from Fig.73 that Bacillus sp. II and Streptococcus sp. I which can hydrolyse ODAP also have capacity to grow in oxalate broth. This suggests that these bacteria can utilize oxalic acid as 'C' source which is one of the metabolites during the degradation of ODAP. Several other aerobic species of bacteria viz. Pseudomonas sp., Thiobacillus sp. and Alcaligenes sp. are known to be capable of utilizing oxalic acid (Bhat and Baker, 1948; Jakoby and Bhat, 1959; Cromack et al, 1977; Chandra and Sethna, 1975). Anaerobic oxalate degradation occurs within the gastrointestinal tracts of certain herbivores (James et al, 1967; Allison et al, 1979; Allison and Cook, 1981) and recently an anaerobic bacterium which decomposed oxalate to formate and CO_2 was isolated from a sheep rumen (Dawson et al, 1980). Anaerobic microbial degradation of oxalate in aquatic sediments is also reported (Smith and Cremland, 1983).

FIG. : 71 : GROWTH OF ODAP HYDROLYSING BACTERIA IN ODAP BROTH.

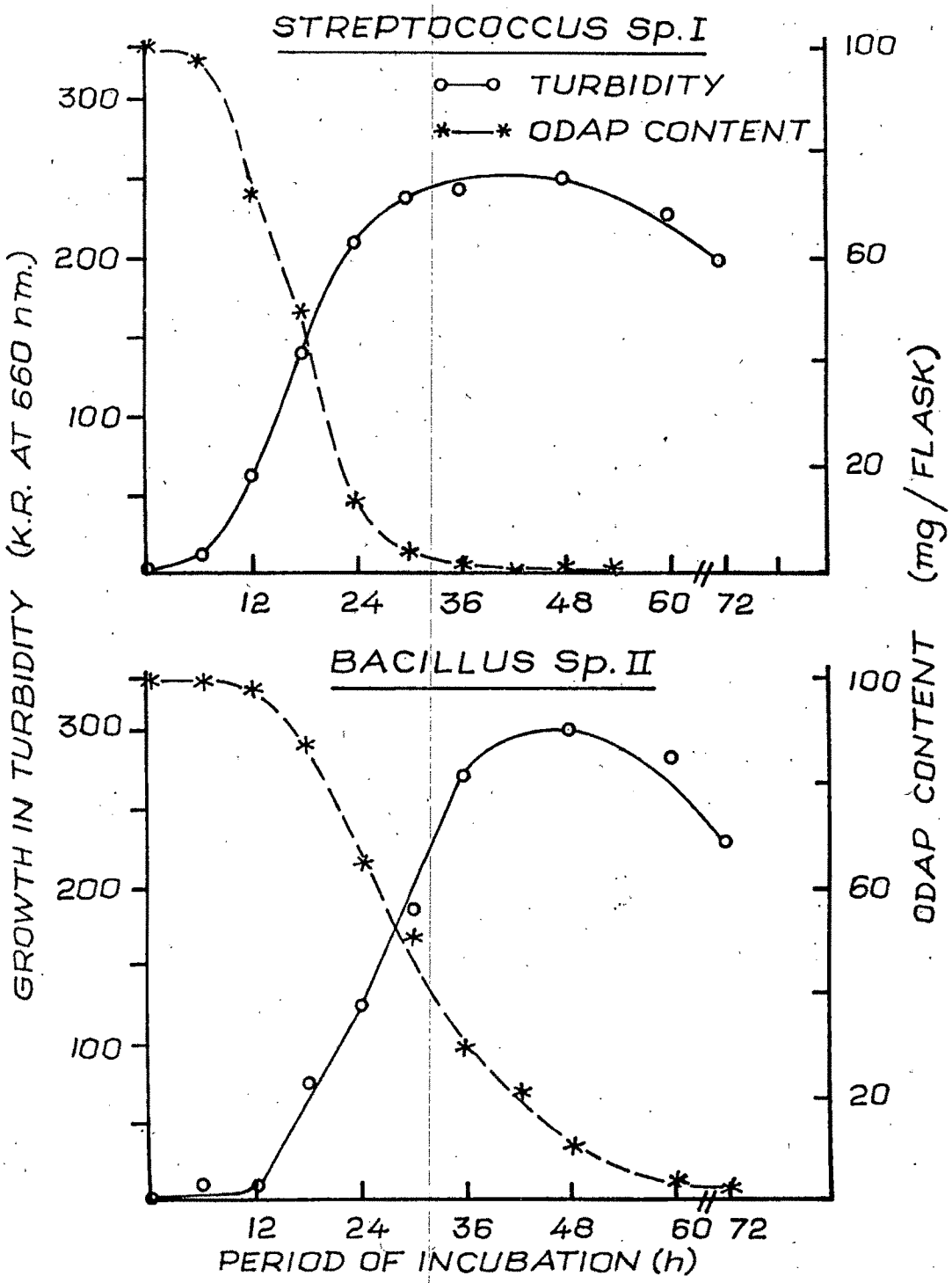


FIG.: 72. GROWTH OF ODAP HYDROLYSING BACTERIA IN DAPA BROTH.

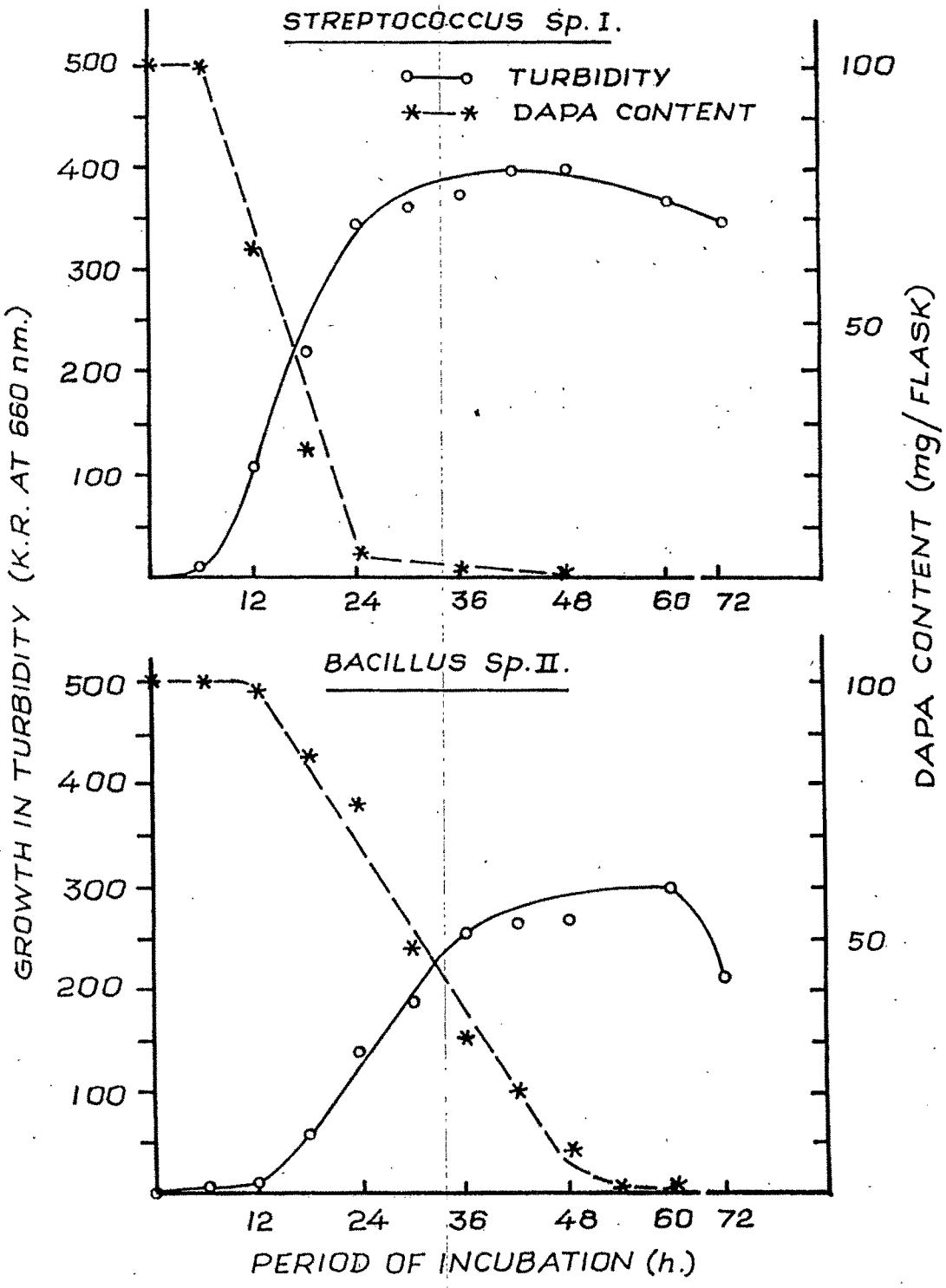
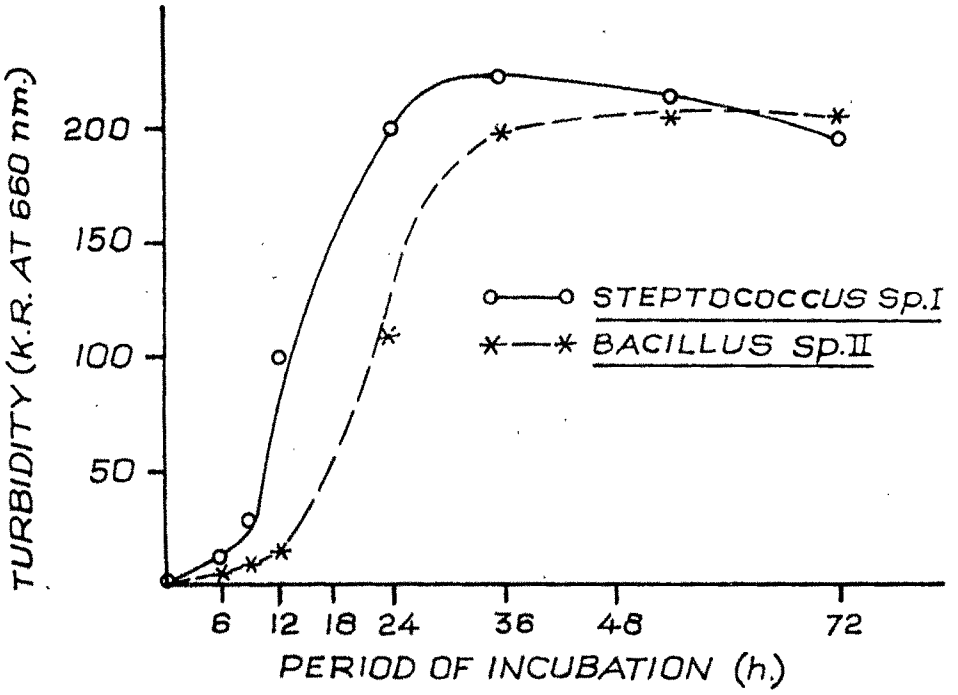


FIG. 73: GROWTH OF ODAP HYDROLYSING BACTERIA IN OXALATE BROTH.



Oxalic acid is assimilated into microbial cells either by the glycerate or by the serine pathway (Blackmore and Quayle, 1970). The necessary energy for growth on oxalate is derived from a series of catabolic reactions involving formation of oxaly CoA, formyl-CoA and formate as intermediates (Blackmore and Quayle, 1970). Oxalate is metabolized by the glycerate pathway involving glycoxylate carboligase in Alcaligenes Lox and Pseudomonas-Ko_x and by the serine pathway involving hydroxypyruvate reductase in other Pseudomonas Mo_x strains (Chandra and Shethna, 1975).

Thus Bacillus sp. II and Streptococcus Sp.I, which can degrade neurotoxin, are also capable of utilizing oxalic acid as sole 'C' source, and thereby can assimilate ODAP into their cellular metabolites.

The present experiments on degradation of ODAP and DAPA and growth on oxalate broth suggested that the two bacterial isolates i.e. Streptoccus sp. I and Bacillus sp. II (isolated from stored kesari dhal grains) could utilize neurotoxin (ODAP) and its metabolites for their growth.

Fermentation of L. sativus batter by a mixture of L. mesinteroides, L. fermenti and ODAP degrading isolates :

Earlier reports suggested that reduction of ODAP content in L. sativus seeds by various methods such as steeping, parboiling, roasting and use of mutagens cause either loss of

vitamins or involves cumbersome technology. Therefore studies were carried out on the better method of detoxification of the toxin present in seeds viz. fermentation during present investgation.

During preliminary investigations when a fermented product of L. sativus dhal was analysed for ODAP content, it was found that during natural fermentation only 20-30% reduction of ODAP occurs (Table-115). The dominant bacteria isolated during fermentation of L. sativus dhal were L. mesenteroides and L. fermenti as discussed previously. Studies carried out by Rao (1978) on experimental fermentation of 'soyidli' showed that when sterilized (autoclaved) rice-soyidli batter was fermented with a mixture of dominant bacteria viz. L.mesenteroides, L. fermenti and L. mesenteroides (MA) showed the same changes in the volume and pH as naturally fermented one and hemagglutinin was completely hydrolysed. Since Streptococcus sp. I and Bacillus sp. II isolated from stored kesari dhal grains were able to utilize ODAP as sole 'C' and 'N' source during their growth, it was of interest to see whether these bacteria can bring about degradation of ODAP during fermentation when inoculated along with the dominant bacteria of fermentation. Therefore during the present experiment, experimental fermentation of sterilized L. sativus flour was carried out using L. mesenteroides, L. fermenti and Bacillus sp. II or Streptococcus sp. I.

TABLE-115 : Changes in physical characteristics and neurotoxin (ODAP) content in L. sativus batter during natural fermentations and experimental fermentation of L. sativus dhal with pure cultures [a].

Type of fermentation	Batter characteristics			Sensory acceptibility of steamed product (mean score)(9)			ODAP concentration (mg/100g of flour)	Degradation of ODAP (%)
	pH		Increase in batter volume (%)	Taste	Texture	Colour		
	Control	Experimental						
1. Control (autoclaved batter)	6.5 ± 0.08	6.5 ± 0.08	0.0	1	0	1	275 ± 10	00
2. Naturally fermented batter (16 h) [b]	6.5 ± 0.08	4.4 ± 0.19	116 ± 8.4	3	2.5	2.5	220 ± 8.0	20
3. Experimentally [c] fermented - I (16h)	6.48 ± 0.07	4.95 ± 0.35	84 ± 6.6	2.0	2.0	2.0	49.5 ± 3.8	82
4. Natural + [d] experimentally fermented - I (16 h)	6.5 ± 0.08	4.23 ± 0.35	119 ± 10.5	3.0	2.5	2.0	27.5 ± 2.5	90
5. Experimentally [e] fermented - II	6.6 ± 0.08	4.85 ± 0.20	75 ± 5.0	2.0	2.0	2.0	159.50 ± 8.6	42
6. Natural + [f] experimentally fermented	6.5 ± 0.07	4.20 ± 0.35	110 ± 9.5	3.0	2.0	2.0	135 ± 10.5	51

contd..

TABLE-115 (Contd...)

(a) Average of four trials ± S.E.

(b) Ingredients steeped in water, wet ground and allowed to ferment for 16 hr at 30°.

(c) During experimental fermentation, the optimum concentration of bacteria (L. mesenteroides and L. fermenti) required to bring about fermentation was standardized and (as described under "Materials and Methods"), the following bacteria were added :

L. mesenteroides	-	4×10^6 cells/gm of batter
L. fermenti	-	2×10^6 cells/gm
Streptococcus sp. I	-	5×10^6 cells/gm

(d) Unautoclaved flour was mixed with bacteria described in 'c'.

(e) Instead of Streptococcus sp. I, Bacillus sp. II were inoculated to the autoclaved batter 5×10^6 cells/gm alongwith L. fermenti and Leuconostoc mesenteroides.

(f) Unautoclaved flour was mixed with bacteria described in 'e'.

(g) 3= Very good; 2 = good; 1 = acceptable 0 = not acceptable.

Data presented in Table-115 show that when experimental fermentation was carried out using these bacteria, experimentally fermented batter resembled naturally fermented batter with respect to increase in volume, acceptability and pH. Significant decrease viz. 82% in the content of ODAP was found during experimental fermentation with Streptococcus sp.I. During natural plus experimental fermentation also 90% degradation of ODAP were observed with Streptococcus sp. I. When the experimental fermentation was carried out using Bacillus sp. II, there was 42 to 51% degradation of ODAP which is less compared to that brought about by Streptococcus sp. I. This may be due to inability of Bacillus sp. II to grow, in presence of other bacteria during fermentation. Another explanation which can be given is the inability of these bacteria to degrade ODAP in presence of other constituents of L. sativus.

Thus these studies have shown that when L. sativus batter was fermented for 16 h with a mixture of dominant bacteria and Streptococcus sp. I, neurotoxin concentration decreased significantly compared to natural fermentation. This proves that the bacterial isolates Streptococcus sp. I could grow during the fermentation in presence of other bacteria and degrade neurotoxin to the extent of around 80-90%.

Studies on enzyme system involved in the hydrolysis of ODAP by Streptococcus sp. I :

Preliminary experiments (described earlier) showed that Streptococcus sp. I and Bacillus sp. II were able to degrade ODAP completely within 48 h during their growth in ODAP broth. These observations suggest that bacteria possess enzymes which hydrolyse ODAP into their cellular metabolites. Studies were therefore carried out to investigate the capacity of the culture filtrate and cell free extract of bacteria to hydrolyse ODAP in vitro. Data given in Table-116 indicate that both the bacterial isolates produce an intracellular enzyme system to hydrolyse ODAP, while the culture filtrates of both the bacteria did not show any ODAP hydrolysing enzyme.

As mentioned earlier, ODAP is synthesized from oxalic acid and DL -2,3-diaminopropionic acid. Malathi et al.(1968) indicated that (U-^{14}C)-oxalic acid is incorporated as a unit into ODAP in the seedling of L. sativus. Further during the growth of bacterial isolates in ODAP broth, diaminopropionic acid and oxalic acid were detected in the culture filtrate.

The presence of oxalic acid as one of the product during ODAP hydrolysis by the enzyme extract was also checked using C^{14}-oxalyl labelled ODAP as a substrate. The cell free extract of Streptococcus sp. I was incubated with the labelled ODAP for different periods of incubation and the liberated C^{14}-oxalic acid was estimated (the details of extraction of

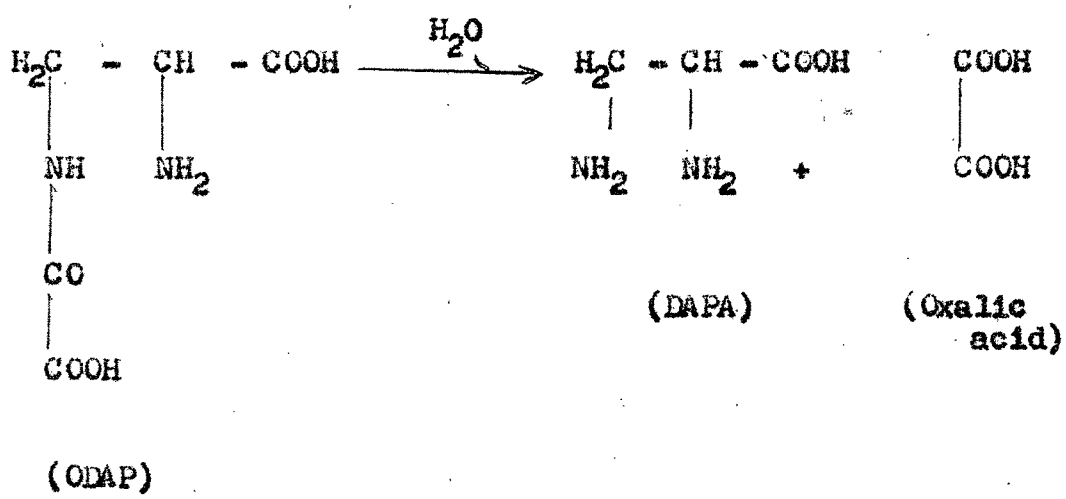
TABLE-116 : Detection of ODAP hydrolysing enzyme system in bacterial isolates.

Period of growth (h)	ODAP hydrolysing enzyme* (enzyme units/flask)			
	Bacillus Sp. I.		Streptococcus Sp. II	
	Culture filtrate	Cell free extract	Culture filtrate	Cell free extract
24	0	5.04	0	11.5
48	0	8.55	0	13.6

* Enzyme units are defined as µmoles of ODAP disappeared under per minute under assay conditions.

Cells were grown in 100 ml ODAP broth for 24 h and 48 h ar 30°, on shaker (140 After the incubation, culture was centrifuged at 8000 xg for 10 min and the supernatant (culture filtrate) was collected. Pellet was dissolved in appropriate amount of 0.05M potassium phosphate buffer, pH 8.0 and the cells were sonicated. This cell homogenate was centrifuged at 20,000 xg for 30 min and the supernatant (cell-free extract) was collected. Culture filtrate and cell-free extract were assayed for enzyme activity as described under 'Materials and Methods'.

C^{14}-oxalic acid are given in 'Materials and Methods'). Data given in Table-117 show the ODAP hydrolase activity in terms of C^{14}-oxalate liberated and from this it is also evident that there is Linear increase in counts of C^{14} oxalic acid during the assay of the enzyme. On this basis it was assumed that the first degradative products of ODAP breakdown by bacteria could be oxalic acid and DAPA by the 'hydrolase' enzyme as follows :-



Oxalic acid and diaminopropionic acid can be further assimilated into cells after conversion into their metabolites. Diaminopropionic acid can be further metabolized into pyruvate and ammonia by DAPA-ammonie lyase as reported in Pseudomonas sp. (Rajagopal Rao, 1976).

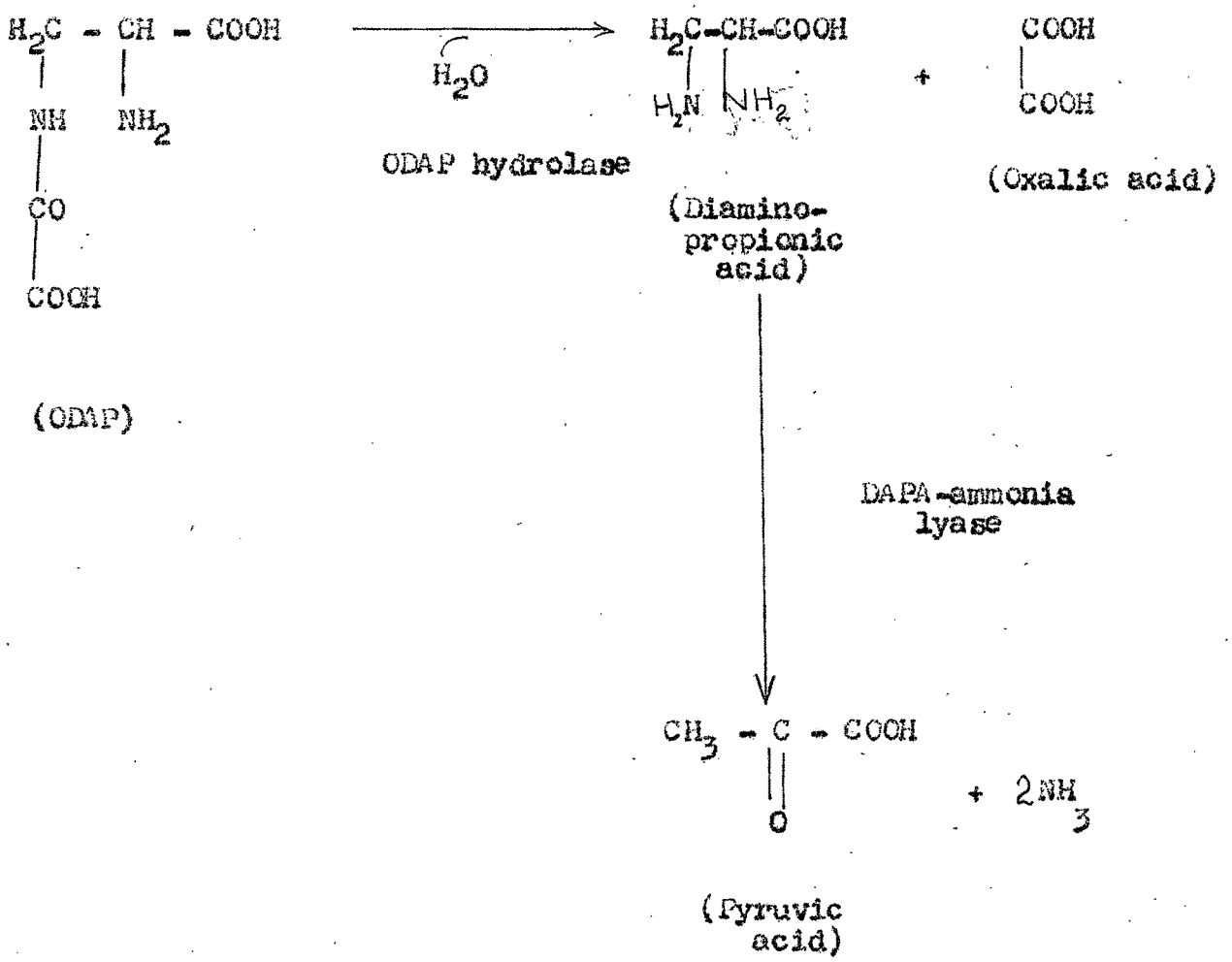
When ODAP was incubated with crude cell free extract DAPA and oxalic acid were detected as products. Therefore it is proposed that the following pathway of ODAP degradation might be operating in these bacteria (Fig. 74). Data given

TABLE-117 : Measurement ODAP hydrolase activity of Streptococcus sp. I in terms of oxalate liberated.

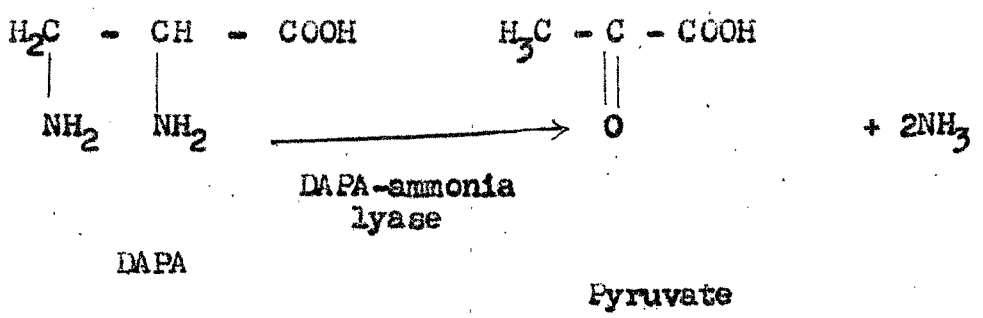
Period of assay (min)	cpm of C^{14} oxalate liberated		
	Blank (B)	Experimental (E)	Enzyme activity (E - B)
0	6200	6420	220
5	5800	16680	10,880
10	6998	28892	21,894
15	8108	41392	33,284
20	6280	44976	38,696
25	5930	52060	46,130
30	5938	57220	51,282
45	6120	62600	56,480
60	7980	71160	63,180

Cell free extract of Streptococcus sp. I having 500 ug of crude protein was incubated with oxalyl labelled ODAP (20 µmoles, having specific activity 22 µCi/mmoles) with 150 µmoles of potassium phosphate buffer (pH 8.0) and 0.1 mM pyridoxal phosphate for various time intervals. The enzymic hydrolysis of ODAP was measured in terms of amount of C^{14}-labelled oxalate formed. Details of the assay system and separation of substrate and product are given in 'Materials and Methods'.

FIG.74 : Postulated pathway for the degradation of neurotoxin (ODAP) by bacteria (Streptococcus sp. I).



in Table-118 indicate that the amount of first intermediate (DAPA) formed is not proportional to the amount of ODAP disappeared. However, DAPA is converted into pyruvate at a faster rate. This indicates that the rate of second enzyme reaction will be faster than that of first. Hence the activities of both the enzymes were measured in terms of pyruvate liberated. In the present investigation, for detecting the activity of first enzyme, the crude enzyme extract was incubated with ODAP as a substrate, and for detecting the activity of second enzyme the extract was incubated with DAPA as a substrate. The first enzyme reaction is reported as "ODAP hydrolase" in the further studies, eventhough during the assay the first and second enzymes were assayed together. The second enzyme is reported as "DAPA-ammonia lyase" (EC.4.3.1.___) the reaction carried out by this enzyme is as follows :-



ODAP hydrolase activity was not measured using oxalyl labelled ODAP for the further studies, because of very low yield of the labelled compound during its preparation.

TABLE-118 : Analysis of products formed when cell-free extract of Streptococcus sp. I incubated with ODAP as a substrate.

Period of incubation for enzyme assay (min)	Amount of ODAP disappeared (µmoles)	Amount of DAPA detected (µmoles)	Amount of pyruvate formed (µmoles)
0	0	0	0
5	0.815	0.07	0.755
10	1.850	0.25	1.500
20	3.750	0.44	5.208
30	5.550	0.72	6.285
45	8.510	0.95	6.80
60	8.995	1.05	6.80
90	9.218	1.05	7.0

Cell free extract of Streptococcus sp. I (500 µg protein) was incubated with 20 umoles of ODAP and 50 µmoles of potassium phosphate buffer, pH 8.0 (in 1.5 ml volume) and at various time intervals enzyme reaction was terminated by the addition of 0.5 ml 10% TC centrifuged and the supernatant was assayed for ODAP, DAPA and pyruvate contents, as described under "Materials and Methods".

Kinetic properties of ODAP hydrolysing enzymes of Streptococcus sp. I :

Streptococcus sp. I was selected for further studies on kinetic properties of enzymes degrading neurotoxin. The enzymatic degradation of ODAP was brought about by cell free extract and not by culture filtrate (Table-116) which implied that the enzymes - ODAP hydrolase and DAPA ammonia lyase are intracellular in Streptococcus sp. I. The crude enzyme preparations were carried out as described under "Materials and Methods" after growing the cells in ODAP broth. The optimum assay conditions for both the enzymes from Streptococcus sp. I are given in Figs. 75 to 79. ODAP hydrolase activity was proportional upto 500 µg of crude enzyme protein whereas DAPA-ammonia lyase was proportional upto 200 ug of enzyme protein (Fig.75). The enzyme activity was proportional upto 45 minutes incubation in the case of ODAP hydrolase and 10 minutes incubation in the case of DAPA-ammonia lyase (Fig.76). The optimum substrate concentration required for ODAP hydrolase and DAPA-ammonia lyase was same for both the enzymes viz. 20 µmoles of ODAP or DAPA (Fig.77). ODAP hydrolase and DAPA-ammonia lyase both had sharp pH optimum at pH 8.0 in presence of 50 mM potassium phosphate buffer. Tris HCl buffer at the same pH, inhibited the activities of both the enzymes (Fig.78). Therefore for further studies potassium phosphate buffer was used. The optimum temperature required for activities of both the enzymes was 37°C as shown in Fig.79.

FIG.75 : Effect of crude enzyme concentration on activity of ODAP hydrolysing enzymes of Streptococcus sp.I.

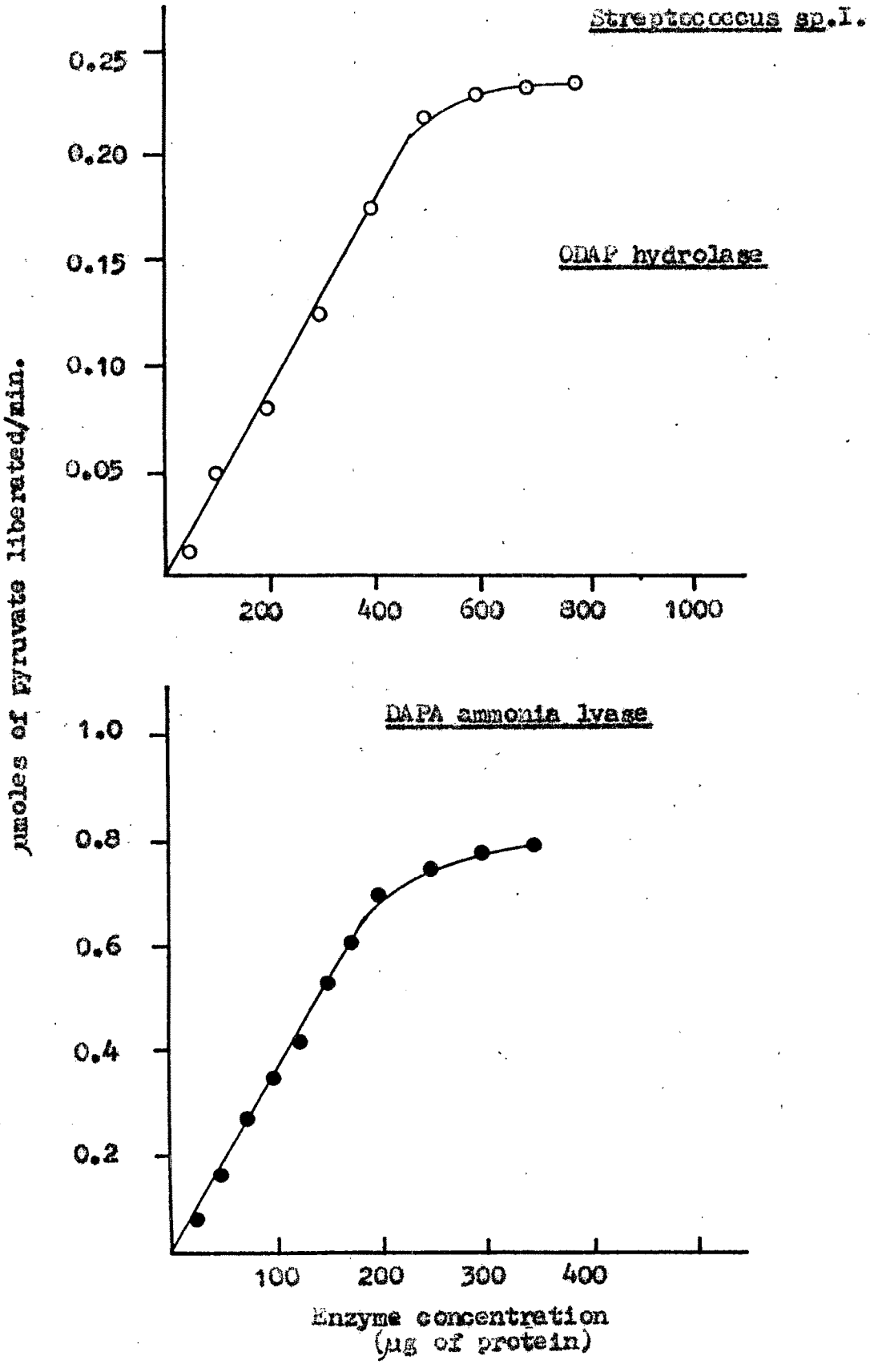


FIG.76 : Effect of period of incubation on the activity of ODAP hydrolysing enzymes of Streptococcus sp. I.

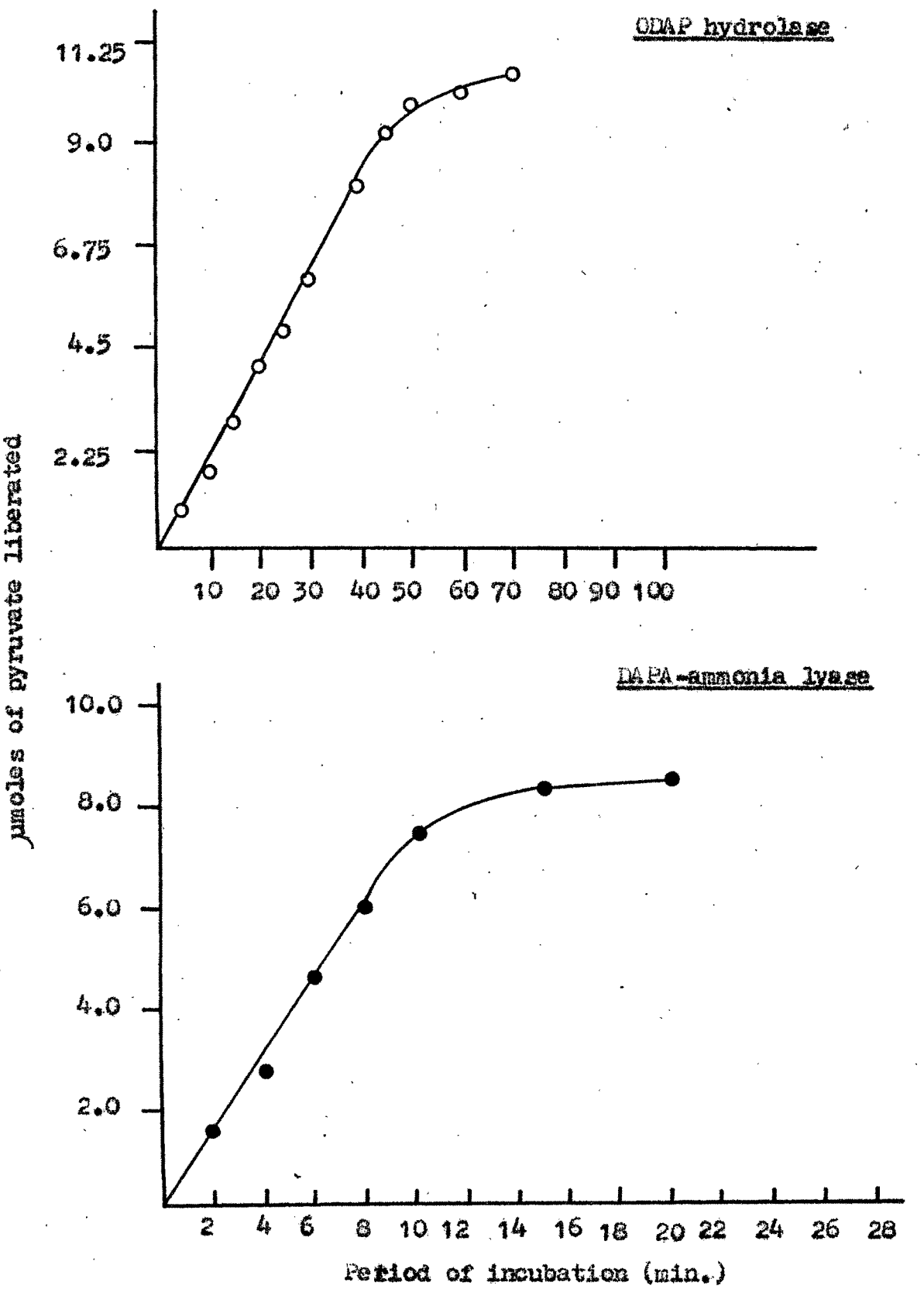


FIG.17 : Effect of substrate concentration on activity of crude ODAP hydrolysing enzymes of Streptococcus sp. I.

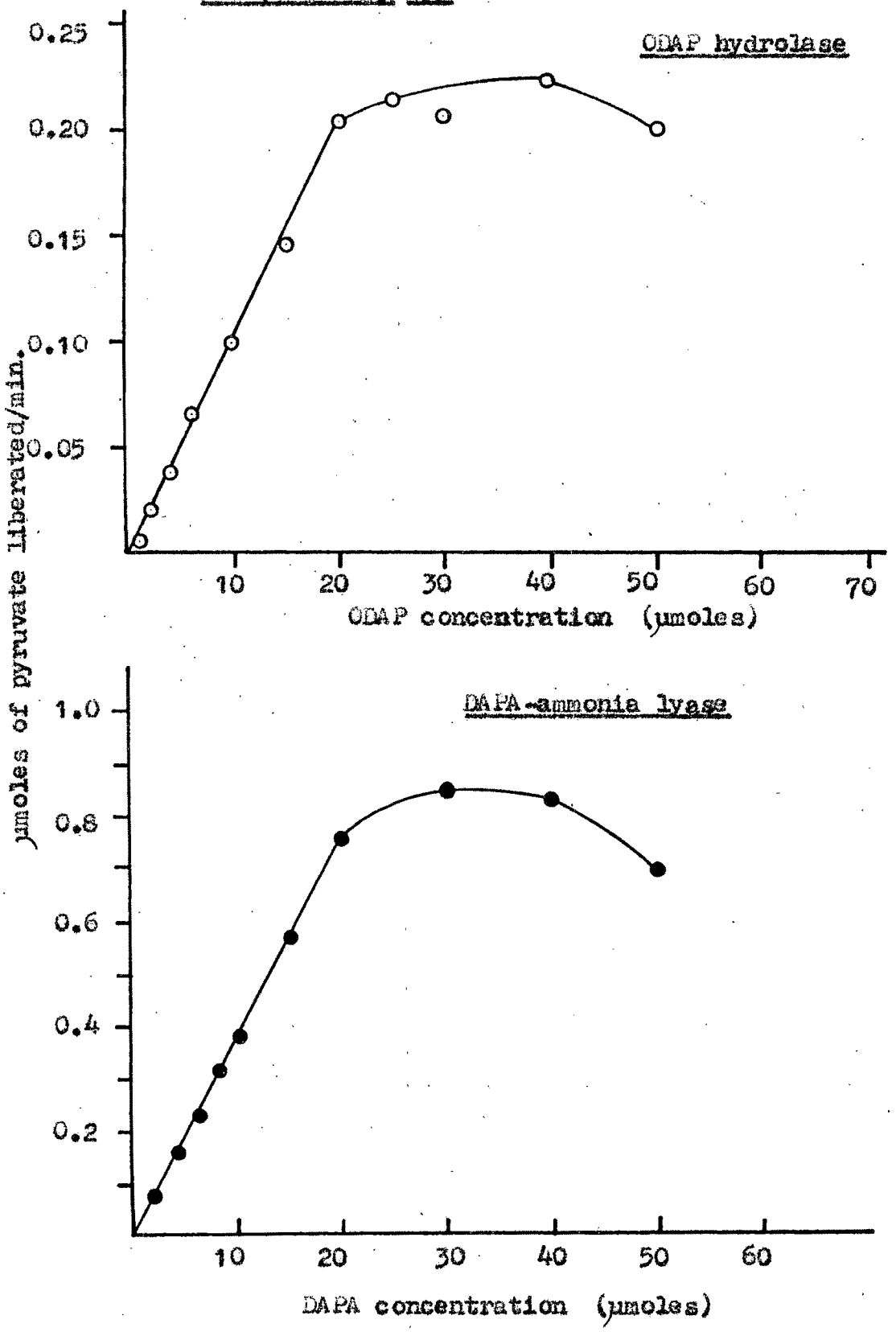
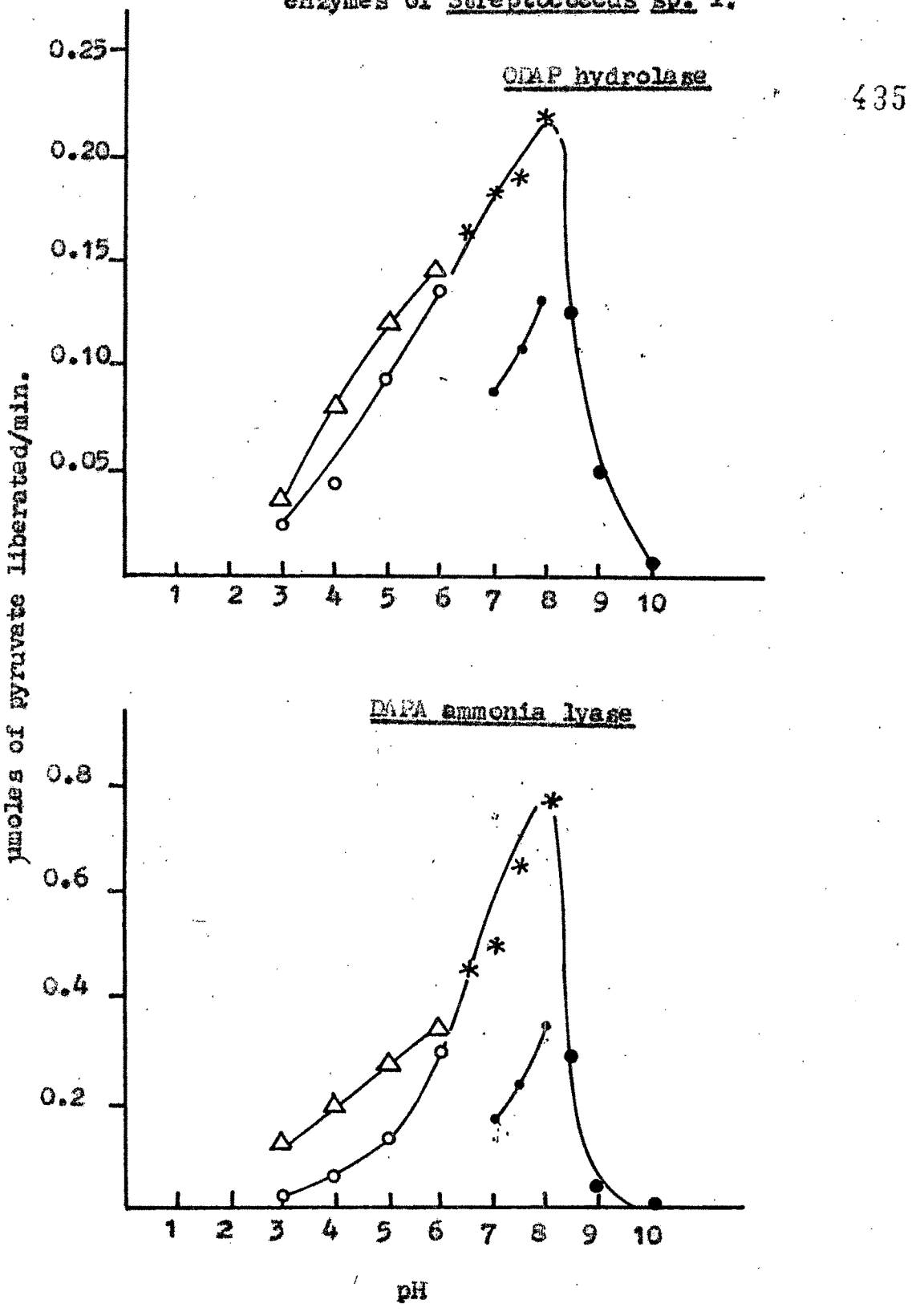


FIG.78 : Effect of pH on the activity of crude ODAP hydrolysing enzymes of Streptococcus sp. I.



○—○ Sod. citrate buffer; △—△ sod acetate buffer
✳—✳ pot. phosphate buffer; ●—● Carbonate bicarbonate buffer
•—• Tris-HCl buffer

FIG.79 : Effect of temperature on the activity of crude ODAP hydrolysing enzymes of Streptococcus sp. I.

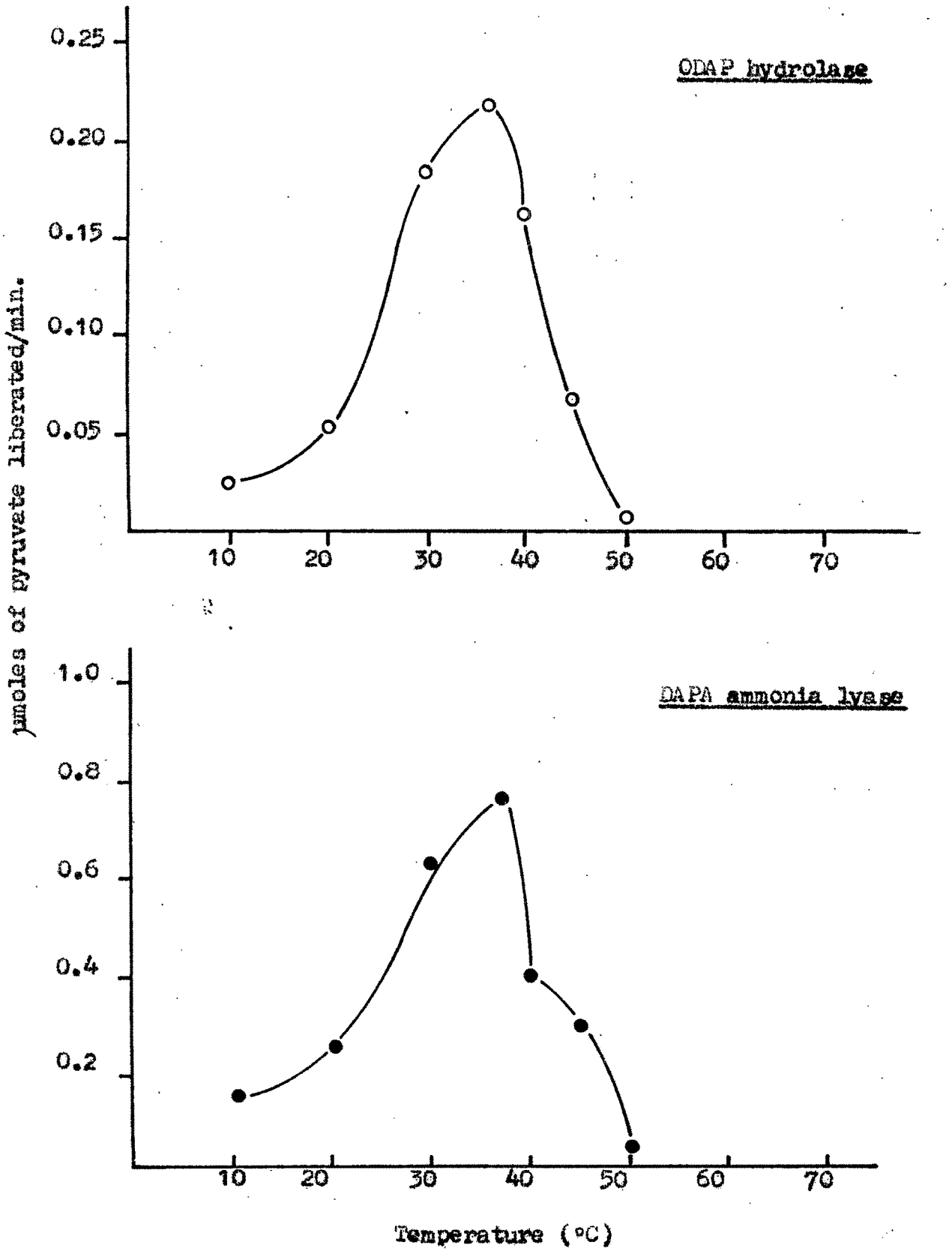


TABLE-119 : Optimum conditions for the activity of ODAP hydrolysing enzymes of *Streptococcus* sp. I.

	ODAP hydrolase	DAPA-ammonia lyase
Optimum pH	8.0	8.0
Optimum temperature (°C)	37	37
Period of incubation for assay (min.)	45	10
Optimum enzyme concentration. (µg crude protein)	500	200
Optimum substrate concentration (µmoles)	20	20
Cofactor requirement	Pyridoxal phosphate, 0.1 µmole.	Pyridoxal phosphate, 0.1 µmole

the measurement of growth of bacteria, ODAP content and ODAP hydrolysing enzymes, have been already described under 'Materials and Methods'.

The results of the studies carried out on the various factors controlling the enzyme production and growth of the organism are presented in Tables-126 to 134. Data given in Table-126 show that growth of bacteria, ODAP breakdown as well as production of ODAP hydrolysing enzymes were maximum when the initial pH of the medium was 7.0. As far as the effect of the amount of inoculum added is concerned, it showed linear increase in growth and enzyme production upto inoculum size of 60 x 10^6 cells/flask. Inoculum size more than this did not show any significant increase in growth and enzyme production (Table-127). Data on the effect of substrate concentration on growth and production of ODAP hydrolysing enzymes are given in Table-128. Exponential increase in growth and production of enzymes were observed till 0.4% ODAP in the medium. When the bacteria were grown in shaking condition, they showed significantly higher growth as well as higher production of enzymes compared to stationary conditions as shown in Table-129.

Data given in Fig.82 show that when the culture was grown in basal medium containing 0.4% ODAP and 0.1% glucose, the growth curve did not exhibit diauxic pattern of growth. It can be also seen from the figure that the presence of glucose during growth did not affect the breakdown of ODAP

TABLE-126 : Effect of initial pH on growth, degradation of ODAP and production of ODAP hydrolysing enzymes of *Streptococcus sp.* I.

pH of the medium		Turbidity (KR at 660nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme activity (units/flask)	
Control	Experimental		Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
2.0	2.0	0	100	100	0	0	0
3.0	3.0	08	100	100	0	0	0
4.0	4.1	50	100	78	22	0.75	7.4
5.0	5.8	135	100	45	55	2.20	25.2
5.5	6.2	152	100	37	63	2.50	30.4
6.0	7.0	175	100	22	88	2.80	33.0
6.5	8.2	170	100	23	87	3.9	40.0
7.0	8.2	225	100	80	92	4.1	42.5
7.5	8.4	155	100	31	69	3.7	35.2
8.0	8.5	150	100	40	60	3.1	25.0
9.0	9.0	35	100	95	5	10	1.02
10.0	10.0	0	100	100	0	0	0

./.

TABLE-126 (Contd.....)

25 ml of basal medium (composition of basal medium is given in Table-22) were taken i 100 ml conical flask and the pH of the medium was adjusted to desired values using either 1N NaOH or 1N HCl. The medium was then sterilized at 15 psi by autoclaving at 121° for 15 min. ODAP solution (20%) was sterilized separately and then added to eac flask to final concentration of 0.4%. The medium was the inoculated with bacteria (50×10^6 cells) and incubated at room temperature for 48 h. (Two types of controls were used for this experiment. One set were incubated at 2° and growth in experiment flask was measured against controls maintained at 2°. Second set of controls were incubated at 30° without bacteria). After the incubation, culture was centrifuged an culture filtrate as well as cells were collected. Neurotoxin was estimated from cult filtrate as described under "Materials and Methods". Cells were suspended in appropr quantity of 50 mM potassium phosphate buffer, pH 8.0 and sonicated to get cell-free extract. ODAP hydrolysing enzymes were assayed from the cell-free extract as describ in "Materials and Methods".

TABLE-127 : Effect of different amount of inoculum on growth, degradation of ODAP and production of ODAP hydrolysing enzymes by Streptococcus sp. I.

Amount of bacteria added No. of cells/flask	pH of the medium		Turbidity (KR at 660 nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme units/flask	
	Control	Experimental		Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
0	7.0	7.0	0	100	100	0	0	0
5×10^6	7.0	7.0	15	100	98.6	1.0	0	0
10×10^6	7.0	7.0	38	100	80	20	0.66	6.66
20×10^6	7.0	7.2	76	100	59.5	40	1.19	12.3
30×10^6	7.0	7.2	105	100	49	51	1.56	21.0
40×10^6	7.0	7.8	144	100	42	58	2.06	26.5
50×10^6	7.0	8.0	175	100	24	76	3.00	33.5
60×10^6	7.0	8.2	225	100	06	94	3.96	41.9
80×10^6	7.0	8.4	245	100	04	96	4.11	43.5
100×10^6	7.0	8.4	252	100	04	96	4.22	44.0
200×10^6	7.0	8.4	265	100	02	98	4.28	45.0

Details of the experiment are same as in Table-126 except for the various in inoculum size (Viz. number of cells inoculated).

TABLE-128 : Effect of concentration of ODAP in the medium on growth and production of ODAP hydrolysing enzymes by *Streptococcus* sp. I.

Concentration of ODAP in the medium (%)	Final pH of the medium	Turbidity (KR at 660 nm)		ODAP content		Degradation of ODAP (%)	Enzyme units/flask	
		Control	Experimental	Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
0.05	7.0	0	18	12.5	2.64	78	0.0	0.0
0.1	7.0	0	38	25	0.5	80	0.56	7.0
0.2	7.2	0	75	50	7.5	85	1.20	17.2
0.3	7.4	0	145	75	13.4	82	1.96	25.0
0.4	8.0	0	215	100	8.0	92	3.89	41.0
0.5	8.2	0	225	125	6.3	95	4.18	42.2
0.75	8.2	0	235	188	53.0	72	4.10	42.5
1.00	8.4	0	250	250	100	60	4.20	41.0

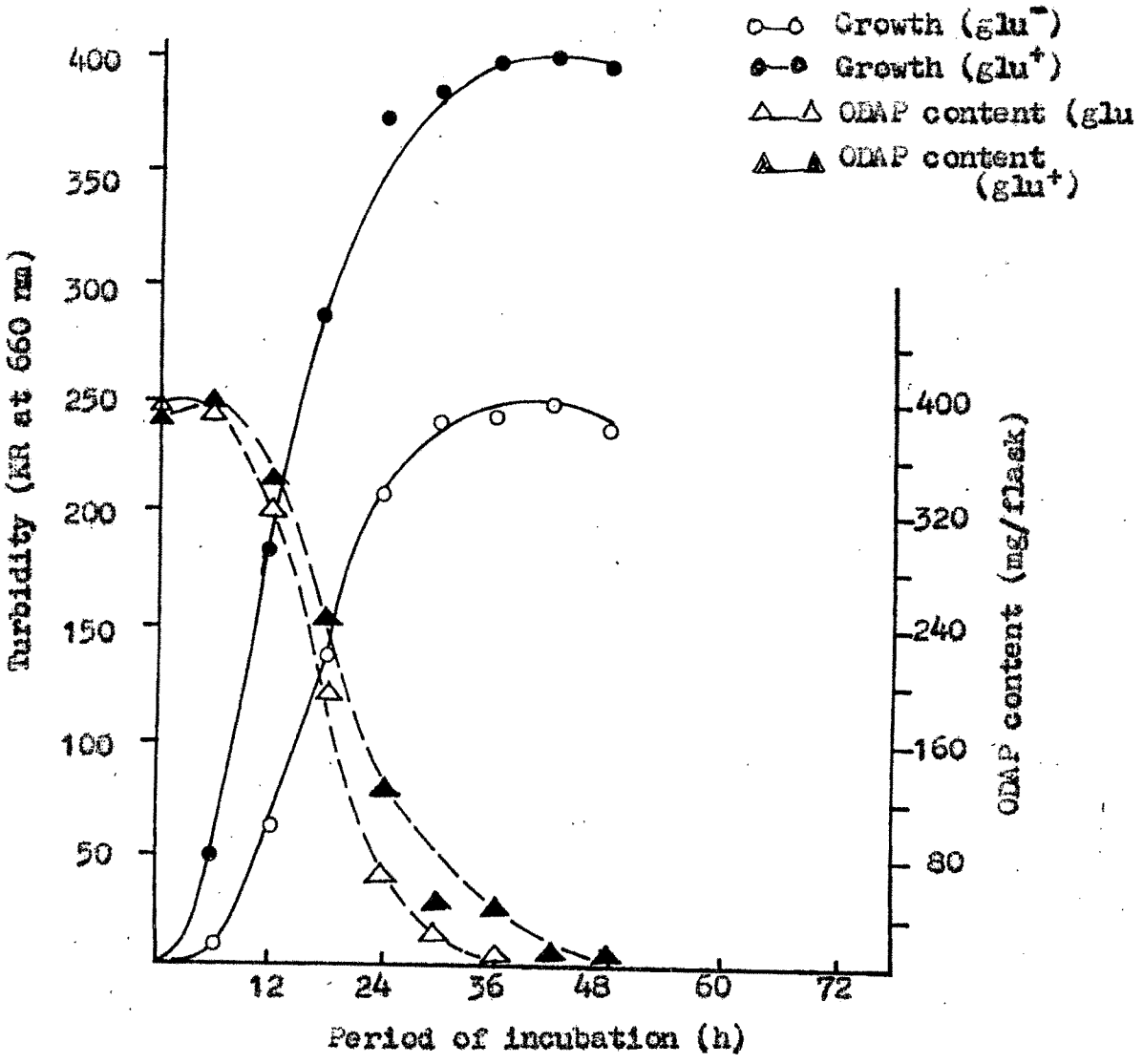
Details of the experiment are same as in Table- 126, except for the variation in the content of ODAP added into the medium.

TABLE-129 : Effect of aeration on growth degradation of ODAP and production of ODAP hydrolysing enzymes by *Streptococcus sp.* I.

	pH of the medium		Turbidity (KR at 660 nm)		ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme activity (units/fla	
	Control	Experimental	Control	Experimental	Control	Experiment		ODAP hydrolase	DA amidy
Stationary culture	7.0	7.2	0	85	100	52	48	1.29	17
Shake culture	7.0	8.0	0	230	100	06	94	3.78	39

100 ml conical flasks containing 25 ml of ODAP broth containing 0.4% ODAP were inoculated with 60×10^6 cells and incubated on shaker (140 rpm) and stationary condition for 48 hr. controls were incubated at 0°. Other experimental conditions are same as in Table-12.6 .

FIG.3@ : Effect of glucose on growth and degradation of ODAP by Streptococcus sp. I.



Bacteria were grown in 500 ml side armed conical flasks containing 100 ml ODAP broth (with and without glucose). At different intervals during incubation, growth in terms of turbidity was measured and 2.0 ml aliquate was withdrawn from the flask for estimation of ODAP. Growth is represented as (o—o) as ODAP content is represented as (△—△) when the bacteria were grown in a medium containing ODAP alone (glu^-). When the bacteria were grown in a medium containing ODAP + 0.5% glucose (glu^+) growth is represented as (●—●) and ODAP content is represented as (▲—▲).

as well as production of ODAP hydrolysing enzymes. When the effect of different glucose concentrations was studied on the breakdown of ODAP, it was found that glucose concentration upto 0.5% did not affect the degradation of ODAP but at higher concentration it caused decreased production of ODAP hydrolysing enzymes (Table-130). Eventhough, high concentration of glucose increased the growth of bacteria with decrease in pH of the medium.

Data given in Table-131 show the effect of different sugars (which are present in L. sativus seeds) on growth and breakdown of ODAP. Addition of different sugars, viz. mono-, oligo- and polysaccharides (at 0.5% level) did not affect the production of enzymes as well as degradation of ODAP significantly. pH of the medium decreased and turbidity increased during the growth of bacteria in presence of glucose, galactose, fructose, sucrose, maltose or starch. However, melibiose, raffinose, stachyose cellulose and cellobiose were not utilizable by the bacteria.

When the effect of different vitamins was studied, it was found that biotin and pyridoxine significantly increased the growth of bacteria as well as production of ODAP hydrolysing enzymes (Table-132). Increase in production of enzymes can be attributed to the requirement of pyridoxal phosphate as a cofactor for these enzymes as discussed earlier. Table-133 gives the data on effect of addition of different

TABLE-130 : Effect of different amount of glucose on growth, degradation of ODAP and ODAP hydrolysing enzymes by Streptococcus sp. I.

Glucose concentration (%)	pH of the medium		Turbidity (KR at 660 nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme activity (units/flask)	
	Control	Experimental		Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
Control*	7.0	8.1	212	100	9.5	90.5	3.76	38.5
0.1	7.0	8.0	235	100	7.0	93	4.10	39.0
0.2	7.0	8.0	275	100	6.0	94	4.20	40.2
0.3	7.0	7.3	295	100	4.0	96	4.3	41.2
0.4	7.0	6.8	345	100	6.0	94	4.5	42.0
0.5	7.0	6.2	375	100	10.0	90	4.7	43.0
0.75	7.0	6.0	425	100	21.0	79	4.1	37.5
1.0	7.0	5.8	500	100	40.0	60	3.0	28.5
1.5	7.0	5.5	520	100	88.0	22	0.56	6.9
2.0	7.0	5.5	550	100	82.0	28	0.62	6.5

* Control flask did not contain glucose.

Details of the experiment are same as described under Table-126 ; except the ODAP broth contained different concentrations of glucose.

TABLE-131 : Effect of different sugars on growth, ODAP degradation and ODAP hydrolysing enzymes by *Streptococcus sp.* I.

Type of sugar	pH of the medium		Turbidity (KR at 660 nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme activity (units/flask)	
	Control	Experimental		Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
Control*	7.0	8.2	220	100	08	92	3.9	39.5
Glucose	7.0	6.0	365	100	3.5	96.5	4.1	40.2
Galactose	7.0	6.3	345	100	14.0	86	3.7	38.0
Fructose	7.0	6.0	305	100	20.0	80	3.4	37.5
Sucrose	7.0	6.2	420	100	04	96	4.7	42.5
Maltose	7.0	5.8	350	100	23.5	76.5	3.3	35.5
Melibiose	7.0	8.0	240	100	8.2	91.8	3.8	39.0
Raffinose	7.0	8.2	210	100	12.0	88	3.7	38.1
Stachyose	7.0	8.0	235	100	10.0	90	3.3	37.5
Starch	7.0	5.8	325	100	13.5	86.5	3.4	35.5
Cellulose	7.0	8.0	195	100	40	60	2.5	20.2
Cellobiose	7.0	8.2	245	100	15	85	3.7	38.0

* Control did not contain any sugar in the medium.

Details of the experiment are same as in Table-126, except the basal medium contained 0.4% ODAP and 0.5% of various sugars.

TABLE-132 : Effect of different vitamins of growth, degradation of ODAP and production of ODAP hydrolysing enzymes of Streptococcus sp. I.

Type of vitamin	pH of the medium		Turbidity (KR at 660 nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme(units/fla	
	Control	Experimental		Control	Experiment		ODAP hydrolase	DA ammo lya
Control (Vit.)	7.0	8.0	220	100	05	95	3.9	41.(
Carotene	7.0	8.0	220	100	05	95	3.9	40.5
Thiamine	7.0	8.2	235	100	04	96	4.1	42.5
Riboflavin	7.0	8.2	255	100	02	96	4.1	42.2
Niacin	7.0	8.0	198	100	15	85	3.67	38.8
Biotin	7.0	8.4	310	100	0	100	4.4	49.5
Pyridoxine	7.0	8.4	345	100	0	100	5.6	55.(
Pantothenic acid	7.0	7.8	200	100	10	90	3.8	40.8
Inositol	7.0	8.0	205	100	10	90	3.7	39.2
Folic acid	7.0	8.0	225	100	05	95	3.96	40.(

Details of the experiment are same as that of Table-126 except ODAP broth contained different vitamins at concentrations of 0.01%.

TABLE-133 : Effect of different amino acids on growth, degradation and production of ODAP hydrolysing enzymes by *Streptococcus* sp. I.

Type of amino acid	pH of the medium		Growth in turbidity (KR at 660 nm)	ODAP content (mg/flask)		Degradation of ODAP (%)	Enzyme units/flask	
	Control	Experimental		Control	Experimental		ODAP hydrolase	DAPA-ammonia lyase
Control (a. acid)	7.0	6.4	395	100	00	100	3.91	41.0
Lysine	7.0	6.4	415	100	10	90	3.78	41.0
Leucine	7.0	6.4	405	100	06	94	4.0	44.0
Glutamic acid	7.0	6.5	312	100	98	02	0	0
Aspartic acid	7.0	6.5	305	100	96	04	0.03	3.5
Phenylalamine	7.0	6.5	375	100	04	96	0.07	44.0
Valine	7.0	6.2	420	100	16	90	3.67	38.5
Proline	7.0	6.6	390	100	02	98	4.11	44.2
Alanine	7.0	6.5	390	100	32	68	2.77	23.9
Cysteine	7.0	6.1	400	100	48	52	2.08	28.0
Methionine	7.0	6.8	410	100	15	85	3.44	36.6
Threonic	7.0	6.8	400	100	50	50	2.49	22.4
Tyrosine	7.0	6.8	365	100	46	54	3.49	39.0
Tryptophan	7.0	6.3	355	100	60	40	2.31	29.5
Histidine	7.0	6.2	325	100	08	92	3.78	37.5
Glycine	7.0	6.5	355	100	05	95	3.44	41.5

Details of the experiment are same as Table 126 except the ODAP broth contained 0.1% of different amino acids and 0.5% glucose. (Stock, concentrated solution of each amino acid was prepared and autoclaved separately at 15 psi for 15 min).

amino acids (which are present in legumes) to the medium, Addition of lysine, tyrosine, methionine and leucine did not affect the breakdown of ODAP, but tryptophan, threonine, cysteine, alanine and tyrosine affected it to some extent. The most interesting observation made in this experiment was complete inhibition of ODAP degradation by addition of glutamic acid and aspartic acid. This can be attributed to the structural analogy of these amino acids with ODAP. Mehta et al. (1972) also showed that ODAP is a potent antagonist of L-glutamic acid transport in resting yeast cells.

Data presented in Table-134 show the effect of different antinutrients viz. phytate and trypsin inhibitor (present in seeds) on the degradation of neurotoxin. It was found that both phytate and trypsin inhibitor at higher concentration at 1.0% level decreased the utilization of ODAP and subsequently the production of ODAP hydrolysing enzymes. Although addition of these compounds at low concentrations did not affect the breakdown of ODAP.

In summary, Streptococcus sp. I grow well and produce ODAP hydrolysing enzymes in a basal salt medium containing 0.4% ODAP and 0.5% glucose when the initial pH of the medium adjusted to 7.0 and incubated at 30° for 24-36 h, with complete complete breakdown of ODAP. The vitamins, amino acids (except glutamic acid and aspartic acid) and sugars commonly present in L. sativus seeds seem to promote the growth of bacteria without affecting the rate of hydrolysis of ODAP. Since these compounds are generally present in legumes, it might be

TABLE-134 : Effect of other toxic/antinutritional factors present in L. sativus on growth, degradation of ODAP and production of ODAP hydrolysing enzymes.

Concentration (%)	Final pH of the medium	Turbidity (KR at 660 nm)	ODAP content (mg/flask)	Degradation of ODAP (%)	Enzyme activity (units/flask)	
					ODAP hydrolase	DAPA ammonia lyase
Control	6.2	380	03	97	4.4	40.5
Phytate - 0.10	5.0	380	08	92	4.4	42.0
0.20	5.2	340	10	90	4.0	40.2
0.50	5.4	320	12	88	3.6	34.2
1.00	5.9	310	52	48	2.3	19.5
Trypsin inhibitor 0.10	5.4	395	04	96	4.5	41.2
0.20	5.2	385	08	92	4.1	40.2
0.50	5.5	380	64	36	1.9	14.5
1.00	5.5	375	78	22	0.8	5.8

Details of the experiment are same as described under Table-126 , except ODAP broth contains 0.5% glucose and various concentrations of phytate/trypsin inhibitor.

justified in assuming that Streptococcus sp. I grow well during fermentation of L. sativus dhal.

Induction studies on ODAP hydrolysing enzymes :

Since the two bacterial isolates viz. Streptococcus sp. I and Bacillus sp. I showed the presence of ODAP hydrolysing enzymes studies were also carried out to find out whether the enzymes are inducible or constitutive. It was found that when bacteria were grown in basal medium containing glucose as sole 'C' source, ODAP hydrolysing enzymes were absent (Table-135), whereas presence of ODAP in the medium showed the presence of enzymes. These results suggested that ODAP hydrolysing enzymes are inducible in these bacteria. Fig.82 & 81 indicate that both the bacteria showed presence of ODAP hydrolase and DAPA-ammonia lyase when they were grown in ODAP broth. However, during their growth in DAPA broth only Bacillus sp. II showed the presence of both the enzymes. This experiment suggested that in Bacillus sp. II DAPA acts as an inducer of both ODAP hydrolase and DAPA-ammonia lyase, whereas in Streptococcus sp. I only ODAP acts as an inducer of both these enzymes.

Thus it can be concluded from these experiments that ODAP hydrolysing enzymes in bacteria are inducible and not consitutive.

TABLE-135 : Production of ODAP hydrolysing enzymes during growth of *Streptococcus sp.* I in presence of different 'C' sources.

	Specific activity of ODAP hydrolysing enzymes (units/mg protein)[a]	
	ODAP hydrolase	DAPA-ammonia lyase
ODAP broth (ODAP as sole 'C' and 'N' source)	0.415 ± 0.02	3.99 ± 0.19
DAPA broth (DAPA as sole 'C' and 'N' source)	0.008 ± 0.0	4.50 ± 0.28
Glucose broth (Glucose as 'C' source)	0	0

'a' - Values are mean of four different observations ± S.E.

Cells were grown in 100 ml basal medium containing 0.4% ODAP, DAPA or glucose for 24 h, at 30° on shaker (140 rpm). After the incubation, cell-free extract was prepared and it was assayed for the activity of ODAP hydrolase and DAPA-ammonia lyase, as described under "Materials and Methods".

FIG.8| : Production of ODAP hydrolysing enzymes when bacteria were grown in ODAP broth.

o—o -ODAP hydrolase, *--* DAPA-ammonia lyase.

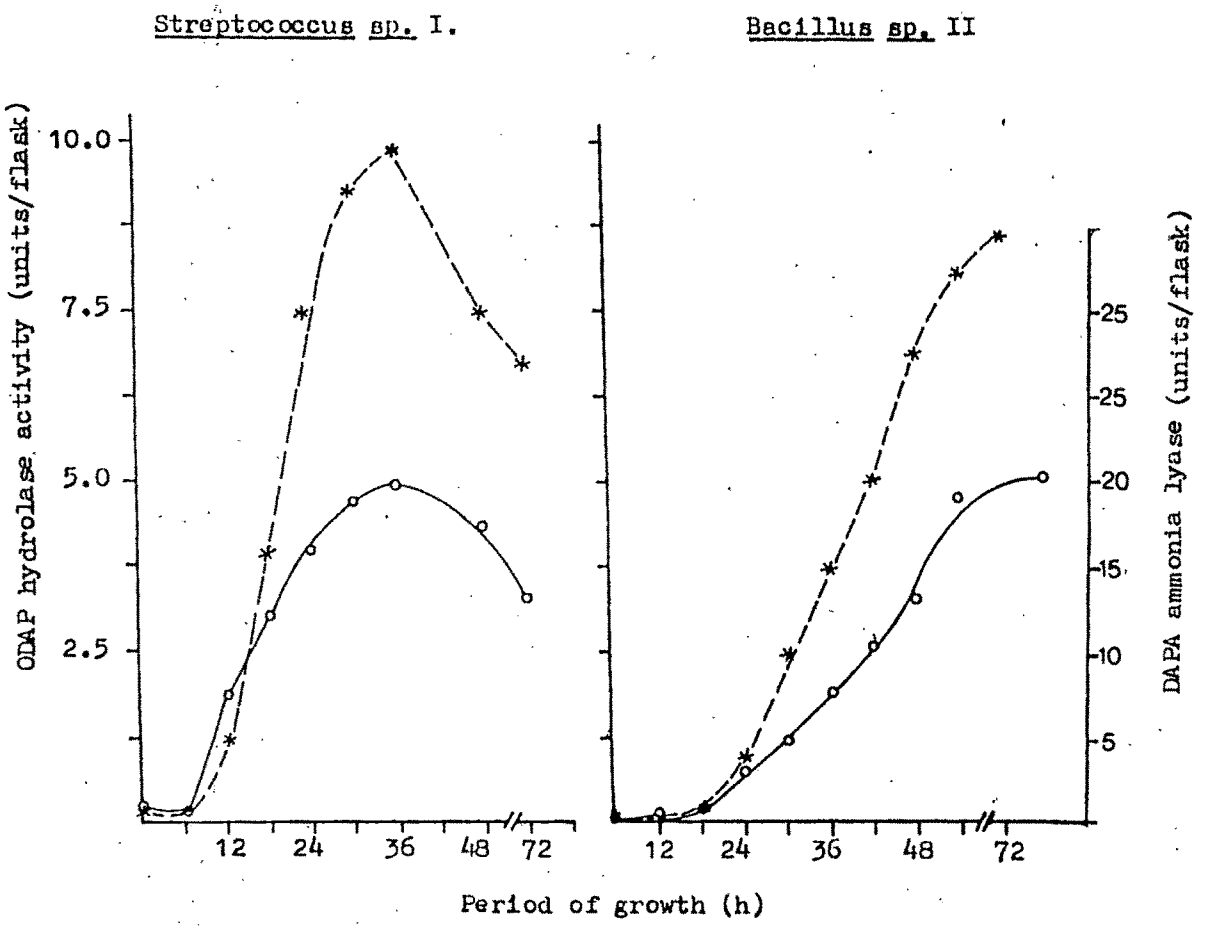
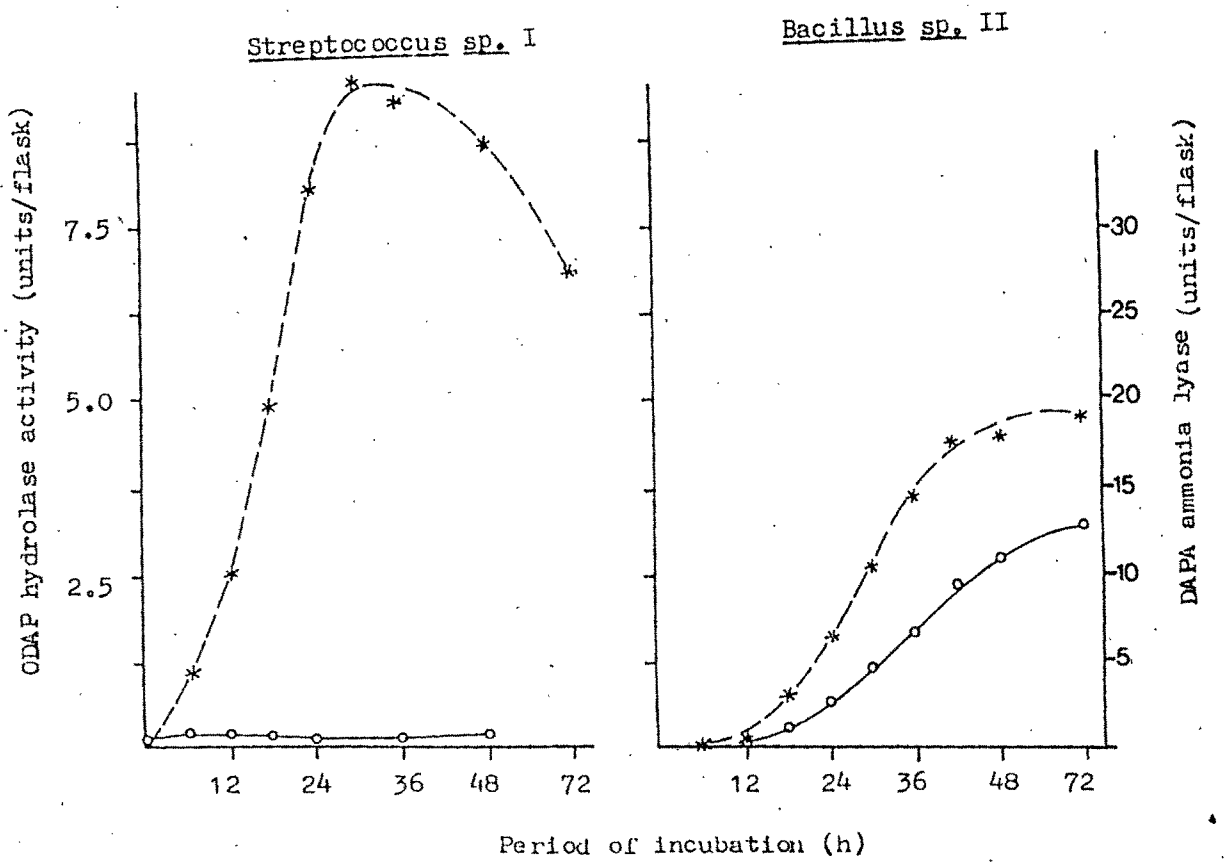


FIG.82: Production of ODAP hydrolysing enzymes during growth of bacteria in DAPA broth.

o—o ODAP hydrolase, *—* DAPA-ammonia lyase.



Section - V

Studies on genetic basis of degradation of antinutritional factors and toxins in bacteria with special reference to raffinose degradation.

As discussed earlier, recombinant DNA technology can be a useful tool if one wants to prepare a novel strain having capacity to degrade the toxins and antinutritional compounds present in commonly consumed cereal-legume based foods. Since different bacterial strains isolated from fermented foods and stored grains exhibited the capacity to degrade antinutritional factors like hemagglutinin, phytate, flatulence producing oligosaccharides and neurotoxin (ODAP), there is a potential to prepare a nowel strain which can grow in a fermented batter and breakdown these compounds.

The basic stratagies for the selection of new and improved strains of industrially important micro-organisms are screening strains and selection techniques, selection after mutation or recombination (by sexual hybridization or protoplast fusion in eukaryotes and gene cloning in both prokaryotes as well as eukaryotes) and gene amplification (Johnston,1985). A major step in the construction of bacterial strains capable of degrading novel compounds is the recognition that a complete set of a genes allowing the degradation of a compound is borne on some transmissible plasmids or on main chromosome

(Reineke and Knackmuss, 1979; Chatterjee et al, 1981). Plasmids are known to have the capacity to express enzymes which participate in a number of peripheral metabolic pathways (Magalhaes and Veras, 1977). As discussed earlier, plasmids also serve as experimental tools in recombinant DNA technology. Effective genetic improvements of the strains can be made by studying the biochemistry and genetics of degradation of various compounds (Chakrabarty, 1983).

As mentioned earlier, different bacteria are known to have certain degradative plasmids, it was of interest to study whether the cultures isolated during the present investigation contain plasmids, because the genes coding for the enzymes responsible for the breakdown of food toxins could be present either on chromosomes or on plasmids. Therefore, the studies were carried out on screening of bacterial isolates for the presence of plasmids and the genetic basis of neurotoxin and raffinose degradation in the same.

Screening of bacterial strains for the presence of plasmids :

Bacillus sp. I which can degrade raffinose, Bacillus Sp. II and Streptococcus sp. I which can degrade neurotoxin, and L. mesenteroides which is a dominant bacteria for the fermentation of L. sativus dhal were screened for the presence of plasmid. For this experiment E. Coli strain harbouring plasmid pBR-322 was used as a positive control and wild type E. Coli strain C-600 was used as a negative control.

The isolation of plasmid DNA from these strains was carried out according to the procedures described in 'Materials and Methods'. Plasmid DNA preparations obtained from all the above strains were loaded on agarose gel and electrophoresis was carried out. Figs. 83 & 84 show the electrophoretic run of the DNA isolates obtained from various bacterial strains. As can be seen in Figures all the bacterial strains screened during the present investigation showed the presence of at least one plasmid DNA band along with the contamination of a band of chromosomal DNA. Thus Bacillus sp.I, Bacillus sp. II, Streptococcus sp. I and L. mesenteroides showed presence of plasmid DNA in them. Bacteria belonging to these genera are reported to have plasmids. Mainly degradative plasmids are detected in the genus 'Pseudomonas'.

Earlier investigations have shown that many plasmids carry degradative genes, which code for the utilization of xenobiotic compounds (Chakrabarty, 1980; 1982). As discussed earlier, there are number of degradative plasmids detected so far for the degradation of various hydrocarbons and pesticides. Chakrabarty (1983) successfully transferred various plasmids like Oct^+, XYL^+ and NAH^+ to a single strain of bacteria, thus constructing a novel superbug which can degrade wide range of hydrocarbons. Degradative genes for the number of hydrocarbons are known to be borne on plasmids and construction of multi copy plasmid strains capable of enhanced growth on crude oil has been reported (Friello et al, 1976; Shapiro et al, 1984). The use of micro-organisms in the

The enzyme ODAP hydrolase has not been reported so far in the literature. In the present investigation, for the first time the presence of this enzyme from prokaryotes is reported. Table-119 summarizes the optimum conditions for the activity of ODAP degrading enzymes from Streptococcus sp.I from which it can be seen that the rate of second enzyme (DAPA-ammonia lyase) reaction is much faster than that of the first enzyme (ODAP hydrolase). The amount of crude enzyme protein required for the hydrolysis of DAPA was also much less than that for ODAP hydrolysis. These studies indicate that DAPA ammonia lyase is more active than ODAP hydrolase during conversion of ODAP into final products pyruvate and ammonia in Streptococcus sp. I.

Cofactor requirement and inhibitor studies of ODAP hydrolysing enzymes of Streptococcus sp. I :

During preliminary experiments, it was found that the activity of ODAP hydrolysing enzymes is lost during dialysis. From the literature studies, it was observed that in general lyases including 'DAPA-ammonia lyase' require pyridoxal phosphate (PLP) as a cofactor. Hence the present studies were carried out to check the requirement of PLP by ODAP hydrolysing enzymes of Streptococcus sp. I. Since carbonyl binding reagents and sulphydryl binding agents are known to inhibit 'hydrolases' and 'lyases' from various sources, attempts were also made to see the effect of these compounds on the activity of ODAP hydrolysing enzymes.

Data presented in Table-120 show that dialysis of crude enzyme preparation against 50 mM potassium phosphate buffer pH 8.0, resulted in the complete loss of 'ODAP hydrolase' and 'DAPA ammonia lyase' activity. The inactivation due to dialysis was partially reversed by the addition of pyridoxal phosphate (1 mM) and mercaptoethanol (1 mM) thus suggesting the requirement of both PLP and sulphydryl groups to restore the full activity of both the enzymes. Table-121 shows that EDTA does not have any effect on these enzymes indicating that the divalent cations are not required for their activity.

Data given in Tables-121 & 122 indicate that ODAP hydrolase and DAPA ammonia lyase were inhibited by both sulfhydryl binding agents and agents which bind carbonyl groups at 0.1 to 1.0 mM concentrations. However, the loss of activity due to agents binding carbonyl and sulfhydryl groups was reversed by the addition of excess of pyridoxal phosphate (1 mM) and dithiothreitol (1-10 mM) respectively.

Table-123 summarizes the effect of certain known organic and inorganic inhibitors on activities of ODAP hydrolase and DAPA-ammonia lyase. ODAP hydrolase activity was inhibited by glutamate and aspartate at 100 mM concentration to the extent of 89% and 75% respectively. Inhibition of DAPA ammonia lyase by these compounds was much less compared to ODAP hydrolase. The inhibition by these compounds can be

TABLE-120 : Effect of dialysis and the pyridoxal requirement on ODAP hydrolysing enzym of Streptococcus sp. I.

Dialysis system	Enzymes activities			
	Only enzyme		Enzyme + pyridoxal phosp + mercaptoethanol	
	ODAP hydrolase	DAPA-NH_3 lyase	ODAP hydrolase	DAPA-NH lyase
1. Undialyzed	0.193	0.602	0.228	0.852
2. Dialysed - 6 h.	0.094	0.271	0.219	0.822
3. Dialysed - 12 h.	0 (0)	0 (0)	0.137 (60)	0.614 (72)

Crude enzyme preparation (50 mg) was subjected to dialysis at 4°C against 2 litre 50 mM sodium phosphate buffer, pH 8.0. The enzyme activities were measured in terms of μmoles of pyruvate liberated by the colorimetric procedure described under 'Materials and Methods', with PLP and without PLP addition.

Values in the parentheses are per cent of control (undialyzed) values.

TABLE-121 : Effect of compounds which bind carbonyl groups or metal ions on ODAP hydrolysing enzymes by Streptococcus sp. I.

Inhibitor	Inhibitor concentrations (mM)	Pyridoxal phosphate (mM)	Enzyme units (µmoles of pyruvate liberated)		Inhibition (%)	
			ODAP hydrolase	DAPA Ammonia lyase	ODAP hydrolase	DAPA ammonia lyase
	0	0.0	0.181	0.725	0	0
1. Control	0	0.1	0.200	0.804	0	0
	0	1.0	0.220	0.861	0	0
	0.1	0.0	0.181	0.725	0	0
2. EDTA	1.0	0.0	0.181	0.725	0	0
	5.0	0.0	0.180	0.721	0	0
	0.1	0.0	0.036	0.058	80	92
3. Hydroxylamine	0.1	0.1	0.045	0.021	75	70
	0.1	1.0	0.145	0.616	20	15
	0.1	0.0	0.069	0.011	62	85
4. Semicarbazide	1.0	0.0	0	0	100	100
	1.0	1.0	0.144	0.65	20	10
	0.1	0.0	0.100	0.35	45	52
5. Sodium cyanide	0.1	1.0	0.179	0.721	0	0
	1.0	0.0	0.027	0.074	85	90
	1.0	1.0	0.180	0.717	0	0

TABLE-121 (Contd.....)

The enzymes were incubated with the inhibitor in the presence of buffer for 10 minutes before assay. In studies with pyrid phosphate, the assay system were incubated first with the inhibitor and then with pyridoxal phosphate. Pyruvate formed was estimated colorimetrically as described under "Materials a Methods".

TABLE-122 : Effect of sulphydryl binding reagents on catalytic activity of ODAP hydrolysin enzymes of Streptococcus sp. I.

	Inhibitor (mM)	Dithio-threitol (mM)	µmoles of pyruvate formed			
			ODAP hydro-lase	% inhibition	DAPA-ammonia lyase	% inhibitio
Control	0	0.0	0.180	0	0.725	0
	0	0.1	0.182	0	0.725	0
	0	1.0	0.182	0	0.785	0
	0	10.0	0.211	0	0.825	0
p-chloromercuri-benzoate	0.1	0.0	0.050	72	0.072	90
	0.1	1.0	0.126	30	0.435	40
	1.0	0.0	0.0	100	0.0	100
	1.0	10.0	0.162	10	0.706	5
N-Ethyl Maleimide	0.1	0.0	0.104	42	0.088	88
	0.1	1.0	0.144	20	0.54	25
	1.0	0.0	0.0	100	0.0	100
	1.0	10.0	0.177	8	0.715	0

Crude enzyme preparations were incubated with the inhibitors in the presence of pyridoxyl-phosphate for 10 min at 37°C before addition of substrate. In reversal studies dithiothre was added after incubation with the inhibitor, reincubated for another 10 min at 37°C and then the reaction was carried out by the addition of substrate. Pyruvate formed was estim colorimetrically.

TABLE-123 : Effect of some organic and inorganic compounds on catalytic activity of ODAP hydrolysing enzymes of Streptococcus sp. I.

		Activity of ODAP hydrolase	% Residual activity	Activity of DAPA ammonia lyase	% Residua activity
Glutamate	(0.0 mM)	0.180	100	0.726	100
"	(1.0 mM)	0.178	99	0.721	99
"	(10 mM)	0.171	95	0.701	97
"	(25 mM)	0.126	70	0.581	80
"	(50 mM)	0.094	53	0.461	64
"	(75 mM)	0.057	32	0.392	54
"	(100 mM)	0.019	11	0.099	14
Aspartate	(1.0 mM)	0.180	100	0.725	100
"	(10 mM)	0.179	99	0.720	99
"	(100 mM)	0.045	25	0.295	41
Urea	(1.0 mM)	0.180	100	0.726	100
"	(10.0 mM)	0.178	99	0.725	100
"	(100 mM)	0.176	98	0.712	98

TABLE-123 (Contd.....)

	Activity of ODAP hydrolase	% Residual activity	Activity of DAPA ammonia lyase	% Residual activity
Guanidine-HCl (10.0 mM)	0.178	99	0.722	99
" (100 mM)	0.180	100	0.706	97
$MnSO_4$ (1 mM)	0.178	99	0.727	100
NH_4NO_3 (1 mM)	0.294	163	1.36	188
$Li(OH)_2$ (1 mM)	0.107	59	0.579	80
$MgSo_4$ (1 mM)	0.180	100	0.737	102
$ZnSO_4$ (1 mM)	0.176	98	0.707	97
$MgCl_2$ (1 mM)	0.012	07	0.04	06
$Ag\ NO_3$ (1 mM)	0	0	0	0
Raffinose (50 mM)	0.184	102	0.745	103
Trypsin inhibitor (50 mM)	0.185	103	0.736	101
Phytate (50 mM)	0.178	99	0.766	106

All the compounds were added in the assay ~~mmm~~ systems during the enzyme reaction, except urea and guanidine-HCl which were added before assay to the enzyme.

attributed to the structural analogy of glutamate and aspartate with ODAP. Urea and guanidine-HCl did not bring about denaturation of the enzymes indicating that the enzymes do not exist in multimeric structures. Among the inorganic salts studied, Hg^{2+}, Ag^{+} and Li^{2+} inhibited both the enzymes, whereas NH_4^{+} acted as an activator of both the enzymes. The antinutritional compounds present in L. sativus, viz. trypsin inhibitor, raffinose and phytate did not affect the activity of any of the enzymes.

Since ODAP hydrolase from bacteria is reported for the first time during present investigation, comparison of the characteristics of this enzyme is made with other hydrolases (from various sources) which can act on 'C-N' bonds of nonprotein compounds as can be seen from the Table-124.

The present studies on DAPA-ammonia lyase from Streptococcus sp. I catalysed α,β-elimination reaction similar to the reactions catalyzed by other PLP requiring enzymes viz. typtophanase (E.C. 4.1.99.1), tyrosine phenol-lyase (E.C.4.1.99.2) threonine dehydratase (E.C.4.4.1.1), cystathione β-synthase (E.C.4.2.1.22) reported by Davis and Metzler (1972) and DAPA-ammonia lyase (E.C.4.3.1.___) reported by Vijayalakshmi et al. (1975). Currently the enzyme nomenclature lists more than 90 pyridoxal phosphate x requiring enzymes, these are mainly classes, E.C. 2-, 4- and 5- with quite a few in the

other classes of this system. In all living beings, pyridoxal phosphate proteins catalyze key step and many specialized reactions in the assimilation and metabolic transformations of nitrogen and sulfur containing compounds. These include a broad variety of elimination exchange and condensation reactions, particularly at the α, β and γ -carbon atoms in amino acids and in other 'NH_2' containing compounds (Braunstein and Goryachenkova, 1984).

In the present investigation DAPA-ammonia lyase from Streptococcus sp. I showed pH optima at pH 8.0 (in 50 mM potassium phosphate buffer) which is comparable with DAPA-ammonia lyase from Pseudomonas sp. (Vijayalakshmi et al,1975), threonine dehydratase from S. typhimurium, aspartate ammonia lyase from Bacteroides cadavaries (William and Hartigue,1967) and E. Coli (Rudolph, 1971). Tris-HCl buffer at pH 8.0 was found to decrease the activity of DAPA-ammonia lyase from Streptococcus sp. I in the present study, which is similar to the observation made by Greenberg and Matsao (1958) and Vijayalakshmi et al. (1975) during their studies on homoserine cystathione γ -lyase and DAPA-ammonia lyase respectively.

NH_4^+ activates whereas Li^+ inhibits the DAPA-ammonia lyase from Streptococcus sp. I in the present studies. Strong activation by NH_4^+ and K^+ is observed with tyrosine phenol lyase (Toraya et al, 1976), tryptophan synthase (Miles, 1970), threonine dehydrates from mammalian

tissues, plants and microbes (Davies and Metzler, 1972) and all bacterial tryptophanase (Snell, 1974). Braunstein and Goryachenkova (1984) have reported that in α,β-specific lyases, the mode and strength of coenzyme binding is modified by monovalent cations.

DAPA-ammonia lyase from Streptococcus sp. I resembles the other ammonia lyases in being dependent on pyridoxal-phosphate and thiol groups. The enzyme is also inhibited by agents which bind carbonyl and sulphydryl groups, however, inhibition can be reversed by pyridoxal phosphate and dithiothreitol respectively, as discussed earlier. The inactivation of the enzyme caused by dialysis during present studies could be due to the removal of certain ions or PLP. Since EDTA did not have any effect on the activity of the enzyme, the possibility of the loss in activity due to loss of divalent cations can be ruled out. This inactivation caused by dialysis was reversed by PLP and 2-mercaptoethanol, therefore, it was concluded that DAPA-ammonia lyase from Streptococcus sp. I requires thiol group for binding to PLP. In the binding site of PLP enzymes carbonyl group binding reagents with highly reactive amine groups, e.g. free and alkyl or acyl-substituted hydroxylamines displace C^4 aldehyde group of the coenzyme and bind to it in the form of fairly stable PLP aldoximes or hydrazones inhibiting the activity of the enzymes (Braunstein and Goryachenkova, 1984). Similar observations were made by Vijayalakshmi et al.(1975) in other ammonia lyases, who also found noncompetitive reversible binding of these agents.

During the present investigation, the specificity of the enzyme towards various substrates was not studied, since the protein was not purified. The rat liver cystathione lyase has a broader specificity acting not only on L-diaminopropionic acid, but also on the isomers of homoserine, allocystathione, cysteine and serine (Mushawar and Koeppe, 1973). Tryptophanase, the most extensively studied α, β -ammonia lyase has also broad specificity (Newton et al, 1965; Morino and Snell, 1967) and furthermore the reaction is reversible and in fact it has been used to prepare tryptophan analogues (Watanabe, and Snell, 1972).

The properties of PLP dependent lyases from different sources are compared in Table-125.

Optimization of growth conditions for the breakdown of ODAP and for the production of ODAP hydrolysing enzymes by Streptococcus sp. I :

During the present experiment, studies were carried out to find out the optimum cultural conditions for growth and production of ODAP hydrolysing enzymes of Streptococcus sp. I.

For these studies, bacteria (maintained on ODAP agar medium) were subcultured and 24 h old culture (50 x 10^6 cells) were inoculated into 25 ml of basal medium containing 0.4% ODAP in 100 ml conical flasks, incubated at room temperature (30°). Control flasks were incubated at 0-2°. Details regarding

natural decomposition of oil spills as well as residues in oil tankers and storage drums has been reported (Gutnick and Rosenberg, 1977; Atlas, 1981). Many of the genes involved in the biodegradation of chlorinated compounds are found to be clustered and borne on plasmids (Weightman et al, 1984; Ghosal et al, 1985) as shown in Table-136.

The presence of plasmids in Streptococcus sp. is also reported, which are as common in this genus as they are in gram negative enteric bacteria (Clewell, 1981). As described in Table-137 in the case of S. faecalis and S. lactis, it is not uncommon to find strains containing five or more plasmids, whereas plasmids seem to be less frequent in isolates of S. mutans and S. pneumoniae. Plasmids present in streptococcal strains determine variety of different functions including conjugal activity, drug resistance, hemolysis, protease production, bacteriocin production, U.V. resistance and utilization of lactose, sucrose and citrate. In the case of lactic streptococci, which are very important in dairy fermentations, the ability to coagulate milk is very much dependent on the ability of the organism to metabolize lactose and also to breakdown casein which are also properties governed by plasmid DNA as shown in Table-137. Recently, it has been suggested that plasmid DNA is associated with the ability of some strains of S. lactis to ferment sucrose, produce nisin and grow in the presence of nisin, by data on curing studies, 'rec'-independent transfer and bilateral incompatibility with known plasmids.

TABLE-136 : Plasmids encoding degradation of some halogenated compounds.

	Molecular size (Mdal)	Halogenated substrate	Reference
pUO1	43	Haloacetate	Kawasaki et al. (1981)
pAC21	65	p-Chlorobiphenyl	Kamp and Chakraborty (1979)
pKF1	53	p-Chlorobiphenyl	Furukawa and Chakraborty (19
No designation	35,50	p-Chlorobiphenyl	Sayler et al. (1984)
pJP2	36	2, 4-D	Don and Pemberton (1981)
pJP4	52	2,4-D,3-Chlorobenzoate	"
pWR1	72	3-Chlorobenzoate	Reineke and Knackmass (1979)
pAC25	76	3-Chlorobenzoate	Chaterjee and Chakraborty (1
pAC31	72	3,5-Dichlorobenzoate	"

TABLE-137 : List of some streptococcal plasmids. (Cf. Clewell, 1981).

Species	Plasmid	Mol. wt. (x 10^6)	Related phenotypes	Transmissibility
S. faecalis	p AM 1	6.0	Tc^r	-
	p AM 1	35	Hly-Bac, UV^r, PR	+
	p PD 1	35	Bac, UV^r, PR	+
	p IP685	20	Tc^r	+
	p IP683	44	Cm^r, Gm^r, Km^r	+
S. agalactiae	PIP 639	18	Em^r	+
	pAC1	17	Em^r	+
	pIM 3001	30	Lac, Prt, Asa^r, Asi^r, Cr^r	ND
	p SK04	40	Lac, Prt	ND
	pDR 1	32	Lac	ND
	pDR 2	29	Suc	ND
S. eremons	pIM3601	36	Lac	ND
S. diacetylactis	pGK4102	41	Lac, Prt	ND
S. Lactis	-	30	Suc, Nip^+, Nis^r	+
S. acidilacti	p SR210	-	Em^r, Cm^r	+

(Clewell, 1981; Stele and McKay,1986; Gonzalez and Kunka,1983) contd.

TABLE-137 (Contd.....)

Abbreviations :

Tc	=	Tetracycline,
Em	=	Erythromycin,
Sm	=	Streptomycin
Km	=	Kanamycin
Gm	=	Gentamycin
Cm	=	Chloramphenicol
Cr	=	Chromate
Hly-Bac	=	Hemolysin Bacteriocin
Bac	=	Bacteriocin
PR	=	Pheromone Response
Lac	=	Lactose utilization
Prt	=	Protease production,
Suc	=	Sucrose utilization
Nip^+	=	Nisin production
Nis^r	=	Nisin resistance.

However, physical evidence linking the 'Suc$^+$', 'Nip$^+$, 'Nis$^+$' markers to a distinct plasmid was not obtained (Steele and McKay , 1986). Occurrence of plasmid DNA in Leuconostoc spp. associated with vancomycin resistance (Orberg and Sandine, 1984) and lactose fermentation (O'Sullivan and Daly, 1982) have been reported. Evidence that plasmid DNA is involved in carbohydrate fermentation and other phenotypic characters expressed by various lactic streptococci is well documented.

Extrachromosomal DNA amongst the genus Bacillus, was first demonstrated in B. megaterium, and since then many reports have been made of plasmids in Bacillus sp. e.g. B. subtilis and B. pumilus (Carlton and Smith, 1974; Tonaka and Koshikawa, 1977; Bernhard et al, 1978; Rostas et al,1980; Uozumi et al, 1980). However, the majority of these plasmids lack readily identifiable markers except the ones which are described in Table-13B.

In the present investigation the most interesting results were obtained with Bacillus sp. I capable of degrading raffinose family of oligosaccharide which showed the presence of five plasmid DNA bands (Fig.83). As discussed earlier (Section-I),the genetic basis of raffinose degradation is very well studied in E. Coli. In enterobacteria, isolated from man and domestic animals, raffinose degrading plasmids were detected (Smith and Parsell, 1975; Magalhes and Veras, 1977). Orskov and Orskov (1975) reported that six of eleven strains of E. Coli that transmitted ability to produce hydrogen sulfide

TABLE-138 : Bacillus plasmids developed as cloning vehicles.

Plasmid	Source	Mol. weight (x 10^{-6})	Copy number	Restriction sites	Marker	Reference
p LS 28	B.subtilis (natto)	4.1	5	Eco RI, Bam HI, Hind III	-	Tanaka and Koshikawa (1977).
p BS 1	B. subtilis	5.5	6	Eco-R1, Bam H1, Sal-I, Pst I, Hind III	-	Bernhard et al. (1978)
p BC 16	B. cereus	3.0	20	Eco-RI, Bam HI	Tc^R	Bernhard et al. (1978)
p AB 124	B. stearothermophilus.	2.9	-	EGo-RI, Hind-III, Single sites for BstEII, Cau I, Hpa I, Xba I.	Tc^R	Bingham et al. (1979)

also transmitted ability to ferment raffinose. Later on it was reported by Burkardt et al. (1978) that the tetracydine resistance (Tc) and raffinose-hydrogen sulfide (Raf-HyS) characters of E. Coli D 1021 are located on two compatible, conjugative fi⁻ plasmids namely pRSD$_1$ and pRSD$_2$, respectively. 'pRSD$_1$', showed a buoyant density of 1.716 gms/cm^3 (56% GC) and the 'CC' form had sedimentation coefficient of 35 s whereas pRSD$_2$ had buoyant density 1.718 gms/cm^3 (53% GC) and the sedimentation coefficient of 'CC' form was 58s. During experimental growth of E. Coli one copy of pRSD2 per chromosome was found indicating stringent control of plasmid replication (Burkardt et al, 1978). Schmitt et al. (1977) have reported that raffinose plasmids enable strains of E. Coli to use a trisaccharide raffinose as sole carbon source by the action of three inducible plasmid coded functions, namely a transport system ('raf' permease), ∝ -galactosidase and invertase whose structural genes form an operon. Evidence for plasmid linked ∝ -galactosidase and sucrose hydrolase activity in Pediococcus pentosaceus has also been shown recently by curing methods (Gonzalez and Kunka, 1986).

Thus there is possibility that the genes responsible for the degradation of antinutritional factors and toxins may also reside on plasmids. For further studies, Bacillus sp. I capable of degrading raffinose and Streptococcus sp. I capable of degrading neurotoxin were used to investigate whether the raffinose and neurotoxin degradation are controlled by degradation plasmids present in these bacteria.

Studies on genetic basis of ODAP degradation in Streptococcus sp. I :

As described earlier, Streptococcus sp. I which is capable of degrading ODAP showed presence of plasmid DNA. Therefore, this strain was further used for studying the genetic basis of ODAP degradation. Curing studies were carried out to locate the genes coding for the degradative enzymes. For this, tubes of Luria broth containing various concentrations of curing agents (acridine orange, acriflavine, mitomycin or novobiocin) were inoculated with 10^4 to 10^5 cells/ml. These were incubated on shaker for 24 h at 30° and the minimum inhibitory concentration was determined for each curing agent (Table-139). Set of tubes containing different concentrations of acriflavine was also incubated at 40°. Aliquotes were taken from the tubes showing minimum growth and then diluted with normal saline, plated to give 30-300 colonies (appropriately on luria agar plates containing 1% glucose). The colonies obtained were replica plated onto different media to select the cured colonies. The media used for selection (replica plating) were Basal media + agar (control), Basal media + glucose, Basal media + ODAP (neurotoxin) and Basal media + DAPA.

As can be seen from the Table-140, some of the colonies did not grow on ODAP and DAPA agar, eventhough they did show growth on glucose agar. Further when these colonies were individually inoculated in liquid media containing ODAP or DAPA as sole 'C' and 'N' source, they did not show any growth when

TABLE-139 : Minimum inhibitory concentration of different curing agents tried for Streptococcus sp. I.

Curing agent	Minimum inhibitory concentration (MIC) (µg/ml)
Acridine orange	500
Acriflavine	500
Novobiocin	250
Mitomycin-C	10

TABLE-140 : Frequency of loss of neurotoxin degrading ability of Streptococcus sp. I by different curing agents.

Curing agent	ODAP$^+$ clones	ODAP$^-$ clones	Total colonies tried	Loss of ODAP$^+$ (curing %)
Acriflavine (30°)	162	08	170	4.7
Acriflavine (42°)	110	10	120	8.3
Novobiocin (30°)	196	04	200	2.0
Mitomycin (30°)	232	08	240	3.3

checked after 48 h. Thus these colonies might have lost the capacity to degrade ODAP and DAPA which could be due to two reasons :

1) The chromosomal DNA might be affected,
2) The resident plasmids might code for the degradative enzymes.

To confirm this, the colonies which turned ODAP⁻ were analysed for the plasmid DNA by small scale alkali lysis method. When electrophoresis of DNA preparation was carried out with E. Coli harbouring pBR-322 and wild type untreated Streptococcus sp. I as controls, it was found that Streptococcus sp. I had lost the plasmids after treatment with curing agents (Fig.84). These experiments provided a preliminary evidence for plasmid linked ODAP utilisation in Streptococcus sp. I.

Thus in the present investigation, it was found that the bacterial strains capable of degrading neurotoxin do have plasmids. Curing studies with different agents on Streptococcus sp. I provided preliminary evidence for the presence of plasmid coded degradative enzymes for neurotoxin degradation. However, further studies are necessary to confirm the linkage of this phenotype (ODAP degradation) with plasmid DNA by transformation or conjugation studies. Thus, if neurotoxin utilization is a plasmid coded phenotype, it will be advantageous for further genetic manipulations.

Studies on genetic basis of raffinose degradation in Bacillus sp. I

As discussed earlier, Bacillus sp. I capable of hydrolysing raffinose showed the presence of plasmid DNA bands with "SDS lysis" method. Experiments were conducted to determine the role of these resident plasmids to code for detectable phenotype such as 'raf' and 'mel' utilization. Various methods of curing plasmid DNA, like treatment with mutagenic agents, SDS and elevated temperature were used ("Materials and Methods") and the minimum inhibitory concentration (MIC) of the chemicals used for curing plasmids of Bacillus sp. I are shown in Table-141. When bacteria were grown at MIC of these chemicals, the derivatives free of plasmids were not obtained. However, the bacteria treated with acriflavine and acridine orange at 6 µg/ml and 10 µg/ml concentration respectively and incubated at 35°. 42° and 45° (according to the method describes by Gonzalez and Kunka, 1986) resulted in the detection of 'Raf⁻', aggregates as shown in Table-142. A drop in curing frequency was observed at highest temperature (45°) due to a drop in cell viability at this temperature as shown in Table-143.

Analysis of plasmid DNA from these isolates after curing studies showed that the cured derivatives had lost the plasmids suggesting that utilization of raffinose in Bacillus sp. I might be plasmid coded (Fig. 83). Therefore it was of

TABLE-141 : Minimum inhibitory concentration (MIC) of different curing agents tried for the growth of Bacillus sp. I.

Curing agent	MIC (µg/ml)
Acridine orange	6.0
Acriflavine	10.0
Novobiocin	0.6
Mitomycin-C	1.0
Sodium dodecyl - sulfate	40
Ethidium bromide	0.2

TABLE-142 : Frequency of loss of raffinose degrading ability of Bacillus sp. I by treatment of different curing agents.

Curing agent	Raf^+ colonies	Raf^- colonies	Total colonies tried	Loss of Raf^+, Mel^+ (curing %)
Novobiocin	410	0	410	0
Mitomycin	300	0	300	0
SDS	500	0	500	0
Acriflavine (30°)	448	2.0	450	0.44
Acridine orange (30°)	375	0	375	0
Acriflavine (37°)[a]	445	5.0	450	1.1
Acriflavin (40°)	341	9.0	350	2.57
Acriflavin (42°)	377	33.0	410	8.0
Acriflavine (45°)	192	8	200	4.0

(a) Bacteria were grown in Luria broth containing 10 µg/ml acriflavin at 37° for 24 hr.

TABLE-143 : Viability of cells (Bacillus sp. I) at elevated temperature during their growth with acriflavine.

	No. of cells/ml or CFU/ml of medium
Control*	5×10^9
Acriflavine (30°)	5×10^7
Acriflavine (37°)	4.5×10^7
Acriflavine (40°)	1.5×10^7
Acriflavine (42°)	5.0×10^6
Acriflavine (45°)	2.5×10^5

Bacteria were grown in luria broth containing 10 µg/ml acriflavine at different temperatures for 24 h and the survival of cells was investigated by using spread plate method as described under 'Materials and Methods'.

* For control, the culture was grown in luria broth at 30° without acriflavine.

interest to determine whether quantitative differences in enzyme activity in parental and cured derivatives were correlated with the presence or absence of plasmid DNA. The parental and cured strains were assayed for α-galactosidase with permeabilized cells as well as from cell free extracts as described under 'Materials and Methods'. The specific activities of inducible α-galactosidase of parental strains were 0.428 and 0.495 in raffinose and melibiose broth respectively, whereas the cured strains were unable to utilize these compounds as growth substrates (Table-144). When the parental strain was grown in luria broth, it showed α-galactosidase activity 0.165, whereas cured strain did not show enzyme activity.

Cornelis et al. (1978) have suggested toluene resistance as an useful marker for defining strains which harbour a raffinose plasmid and for distinguishing them from 'Raf$^-$' strains. In the present studies also a permeabilized cell assay for α-galactosidase activity of Bacillus sp. I showed the plasmid associated enzyme activity in parental strains to be toluene resistant. Characterization of α-galactosidase activity associated with raffinose plasmid in E. Coli has also shown that in contrast to the chromosomal α-galactosidase (Mel α-gal) specified by the 'mel' system of E. coli (Burstein and Kepes, 1971; Schmitt, 1968) 'raf' coded α-galactosidase (Raf α-gal) which is plasmid encoded can be assayed in toluene treated cells (Schmid and Schmitt, 1976).

TABLE-144 : Alpha-galactosidase activity of crude extracts from cured and parental strains of *Bacillus* sp. I grown in different substrates.

Strain	Specific activity of enzyme in extracts of cells grown in :				
	luria broth	Glucose broth	Sucrose broth	Melibiose broth	F
Parental (Wild type strain)	0.165	0.0	0.0	0.495	
Cured strain (Plasmid free strain)	Trace	0	0	ND	

ND = Not detected

The selective advantage or role of raffinose plasmid in Bacillus sp. is not studied during the present investigation. However, this strain resides on the surface of stored grains and is associated with legumes where raffinose and sucrose are the most common sugars found in them. It is therefore possible that plasmid encoded raffinose utilization may give these isolates a selective advantage in being able to ferment sugars which are commonly found in legumes.

Knowledge of plasmid coded enzyme which govern the fermentation of carbohydrates in bacteria is of importance to the food fermentation industry. In product development, it may be necessary to promote or prevent the utilization of certain naturally occurring or added carbohydrates in fermentation systems. By addition or deletion of certain naturally occurring plasmids in micro-organisms, its fermentation pattern may be tailored either to ferment or not to ferment a given carbohydrate. One such example is a recently issued patent (Gonzalez, U.S. Patent, 1985) in which naturally occurring 'raffinose' and 'sucrose' plasmids were eliminated so that P. pentosaceus could be used to ferment meats and vegetables in the presence of added sucrose without fermenting sucrose.

To determine if the transfer of the "Raf$^+$" phenotype was possible by transformation and if this mode of transfer will result in a detectable "Raf$^+$" phenotype, transformation

of cured Bacillus sp. I and E. Coli with "Raf$^+$" plasmid were attempted using $CaCl_2$ method. These experiments did not give "Raf$^+$" transformants which might be due to inefficiency of the method used in the case of Bacillus sp. and inability of "Raf$^+$" plasmid to multiply in a new host viz. E. Coli. Since the attempts of transformation of cured Bacillus strain and E. Coli were unsuccessful, it was thought of transferring this foreign DNA using another plasmid vector pBR-322 using a recombinant DNA technique. For this the plasmid DNA from Bacillus sp. I and pBR-322 plasmid DNA from E. Coli were isolated from a large bulk of cultures and were purified using caesium chloride density gradient centrifugation (details of the methods for isolation and purification of plasmid DNA are described under "Materials and Methods"). The purity of the purified plasmid DNA preparations was checked by agarose gel electrophoresis. The restriction analysis of plasmid DNA from Bacillus sp. I and pBR-322 plasmid (which was used as a vector) were carried out using restriction enzymes 'Pst-I', 'Eco-RI' and Bam-H_1. Fig.85 shows the number of fragments generated after the digestion of DNA with restriction endonucleases.

A gene bank of Bacillus sp. I plasmid DNA was constructed by ligating the Eco-RI and pst-I fragment of plasmid DNA with pBR-322 fragments cut with the same enzymes using T4 DNA ligase (Fig.86). E. Coli was transformed with the resulting recombinant plasmids followed by selection on LB agar plates containing either ampicillin or tetracycline using a principle

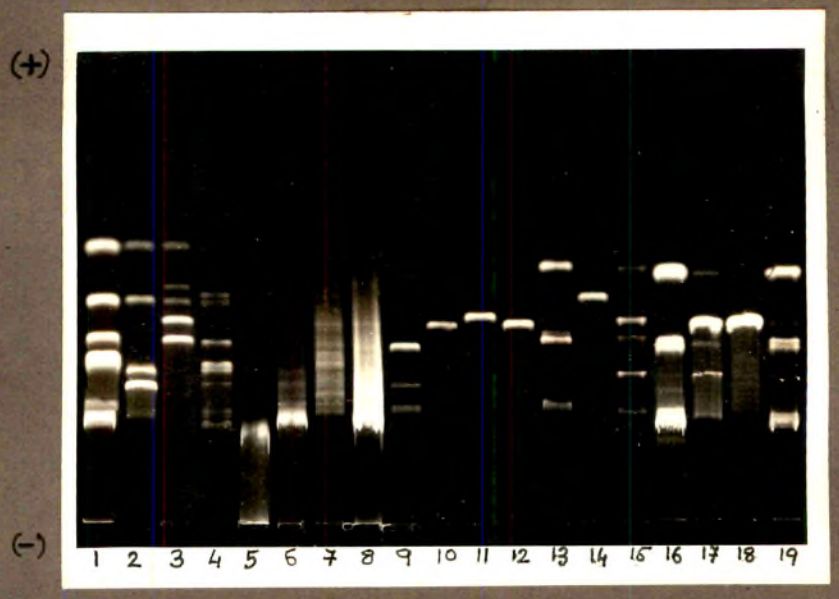


FIG.85 : Agarose gel electrophoretic pattern of DNA preparations digested with restriction enzymes:

Lane-1 : Purified plasmid DNA from raffinose degrading strain Bacillus sp. I ('Raf' plasmid),
Lane-2 : 'Raf' plasmid DNA digested with Bam-H_1,
Lane-3 : 'Raf' plasmid DNA digested with Pst-I,
Lane-4 : 'Raf' plasmid DNA digested with Eco-R1,
Lane-5 : Chromosomal DNA preparation from raffinose degrading Bacillus sp. I,
Lane-6-8: Chromosomal DNA from Bacillus sp.I digested with Bam-H_1, Pst-I and Eco-R1 respectively.
Lane 9-15: Other standard DNAs,
Lane-16 : Purified pBR-322 DNA,
Lane-17 : Purified pBR-322 DNA digested with Bam-H1,
Lane-18 : Purified pBR-322 DNA digested with Pst-I,
Lane-19 : Purified pBR-322 DNA digested with Eco-R1.

of "Insertional inactivation". When these transformants (viz. amp^S tet^R in pst-I cut fragments) were then replica plated on raffinose agar plates, it was possible to get 3 transformants out of 700 which had 'raf' gene inserted into them. A recombinant plasmid obtained from clones was found to contain an additional fragment as shown in Fig.86 when digested with pst-I. When E. Coli strain (C-600) contained only pBR-322, no α-galactosidase activity was detected in cell free extracts or by plating on minimal medium containing raffinose as 'C' source. In contrast to this, E. Coli strain harbouring a recombinant plasmid could grow on minimal medium containing raffinose and the cell-free extract possessed α-galactosidase activity. This data support the condusion that in Bacillus sp. I Raf^+ phenotype is plasmid coded. However, the expression of α-galactosidase gene cloned from Bacillus sp. I into E. Coli is not studied here.

Cloning of 'raf' operon that mediates catabolism of raffinose was also carried out by Konishi et al. (1985) from E. Coli D-1138 into E. Coli C600 using a vector pBR-322. Their experiments gave an evidence that genes for raffinose utilization constitute an operon; the gene order is the regulator, α-galactosidase, the permease and invertase. However, the location and number of the promotor of this operon are still not known. Kosugi et al. (1986) constructed a cointegrate plasmid in vivo between a plasmid that mediates raffinose catabolism in E. Coli and IncP-1 plasmid

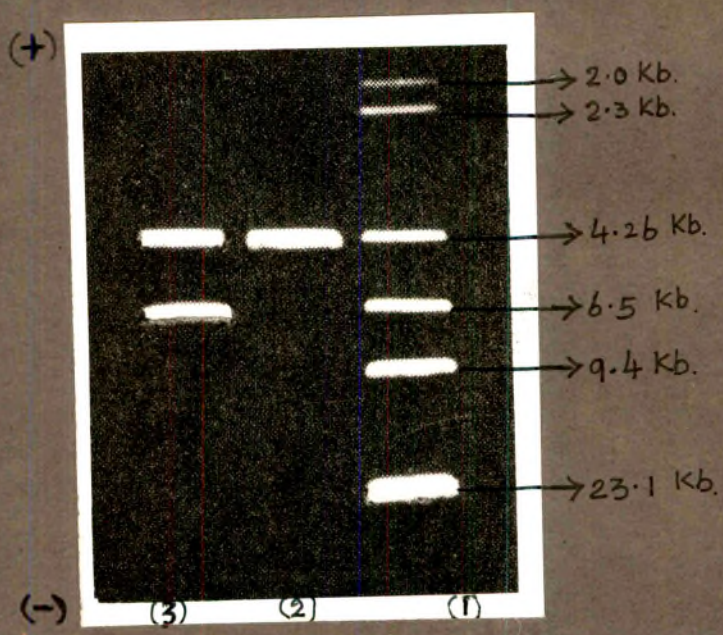


FIG.86 : Agarose gel electrophoretic pattern of Pst-I restriction digestion of pBR-322 and recombinant (cloned) pBR-322;

Lane-1 : Bacteriophage λ-DNA digested with Hind-III, used as a reference standard,

Lane-2 : pBR-322 DNA digested with Pst-I,

Lane-3 : Cloned pBR-322 DNA digested with Pst-I.

R-68-45 from Pseudomonas sp.. This cointegrate plasmid was then introduced into Zymomonas mobilis to improve the strain. But still there are no data available on the size of the genes which constitute a 'raf' operon in E. Coli or other bacteria. Whereas there are reports on the size of genes coding for melibiose utilization in E. Coli. Melibiose utilization in E. Coli is determined on the melibiose locus 'mel' which is located at 93 min on the genetic map (Bachmann, 1983). The 'mel' operon is induced by several α-galactosides (Prestidge and Pardee, 1965; Schmitt, 1968) and its expression is controlled by cAMP-CRP regulatory circuit (Okada et al, 1981). 'Mel' locus is believed to form an operon consisting of at least two structural genes 'mel-A' and 'mel-B' coding for α-galactosidase and mel-permease respectively (Hanatani et al, 1984). 'Mel-AB' region has been cloned into another E. Coli strain using a vector pBR-322 by Hanatani et al (1984). Using genetic complementation tests and recombination analysis of recominant plasmids and 'mel-A$^-$', and 'mel-B$^-$' mutants enabled them to determine the physical location of the promotor, 'mel-A' and 'mel-B' genes on the DNA segment, viz. size of 'mel-AB' region is about 3000 base pairs. Complete nucleotide sequence of the 'mel-B' gene coding for the melibiose carrier in E. Coli was determined by Yazyu et al. (1984). The melibiose carrier is predicted to consist of 469 amino acid residues (70% nonpolar amino acids) resulting in a protein with a molecular weight of 52,029. Recently, 'mel-A' gene of E. Coli is also cloned and sequenced, which showed that

it codes for 450 amino acids long protein, with a molecular weight of 50.6 kilodaltons (Liljestrom and Liljestrom, 1987).

In conclusion, the present experiments reported on Bacillus sp. I suggest that Raf$^+$ phenotype is plasmid coded in this bacteria. The involvement of plasmid DNA is suggested by the curing data and transformation of plasmid genes using recombinant DNA technique.

Studies on immunological properties of α-galactosidase of Bacillus sp. I :

As discussed earlier (Section-III), "raffinose induced" α-galactosidase of Bacillus sp. I was purified to homogeneity having molecular weight of 1,90,000 and tetrameric structure. "Melibiose induced" α-galactosidase from Bacillus sp. I was also purified to homogeneity by other workers in the laboratory. Therefore comparative studies between 'raf' induced and 'mel' induced α-galactosidases from Bacillus sp. I were carried out. Studies carried out on the purified enzymes showed that these two ensymes have similar kinetic properties, electrophoretic mobility (Fig.87) and molecular weights (Table-145). As discussed earlier, curing studies also proved the plasmid linked raffinose and melibiose degradation in Bacillus sp. I unlike that of E. coli system (Schmid and Schmitt, 1976). Therefore the studies were also carried out to detect whether 'mel' induced α-galactosidase and 'raf' induced α-galactosidase are immunologically related or not. For this investigation antisera were raised against both the purified enzymes in rabbits as described under "Materials and Methods".

Sera obtained from the rabbits after injection of α-galactosidase were tested for the presence of antibodies against them by immunodiffusion techniques, it was found that the sera obtained after 28 days, 35 days and 42 days showed clear precipitation lines in the gels, indicating the presence of antibodies. For further studies 'anti-raf' and 'anti-mel' sera of 35 days (having high antibody titer) were use

TABLE-145 : Comparison of melibiose induced and raffinose induced ∝-galactosidase of Bacillus sp. I.

Properties	Melibiose induced ∝-galactosidase	Raffinose induced ∝-galactosidase
(A) Kinetic properties		
1. Optimum pH	7.0	7.0
2. Optimum temperature (°C)	37	37
3. Km for ∝-PNPG (mM)	0.626	0.50
4. Km for melibiose (mM)	6.0	8.0
5. Km for raffinose (mM)	40	36
6. Km for stachyose (mM)	83	66
7. Inhibitors	Ag^{+}, Hg^{2+}, Cu^{2+}, p-CMB, Iodoacetate, Tris, urea.	Ag^{+}, Hg^{2+}, Cu^{2+}, p-CMB, Tris, Iodoacetate, urea.
8. Competetive inhibition by	galactose, melibiose, raffinose.	galactose, melibiose, raffinose
9. Cofactor requirement	none	none
(B) Molecular properties		
1. Molecular weight	1,82,000 (Tetramer)	1,90,000 (Tetramer)
2. Electrophoretic mobility in activity staining.	Both are migrating at the same distance on the gel.	
(C) Other properties		
Toluene sensitivity	not sensitive	Not sensitive

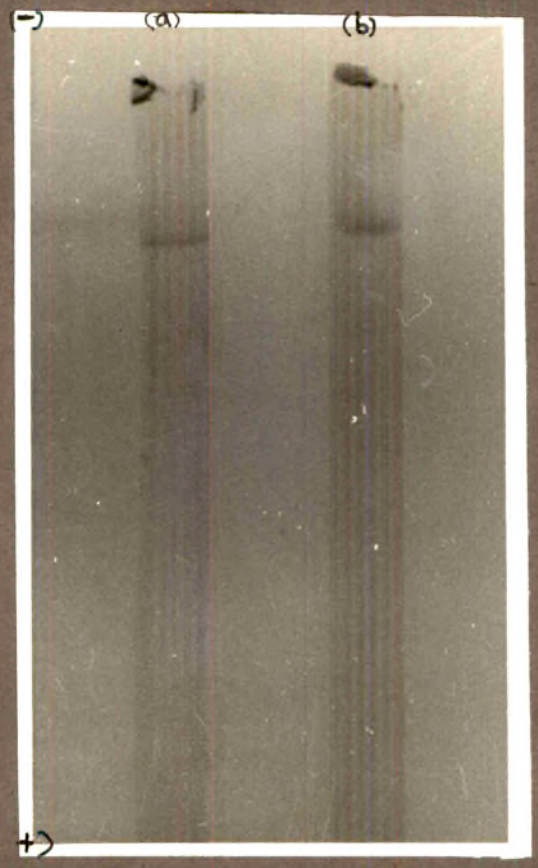


FIG.87 : Activity staining of raffinose induced and melibiose ∝-galactosidase of Bacillus sp. I, after polyacrylamide disc gel electrophoresis on 7.5% acrylamide gels:

(a) Raffinose induced ∝-galactosidase,
(b) Melibiose induced ∝-galactosidase.

For studying the immunological relationship between 'mel' induced and 'raf' induced α-galactosidase, cross-reactivity and immunotitration were carried out using 'anti-mel-α-gal' and 'anti-raf-α-gal' sera. The result of double-immunodiffusion are given in Fig.8B from which it can be seen that the precipitin arc formed between the antibody and test antigens fused indicating that the antibody is precipitating identical epitopes in each antigen. This does not mean that both the enzymes are necessarily identical, but they are identical as far as the antibody can distinguish the difference. Thus this experiment confirmed a common antigenic determinants of the two enzymes because of the fusion of precipitin lines.

Rabbit antisera raised against the two enzymes were titrated against the enzymes by assaying changes in the activity in the supernatants as described under 'Materials and Methods'. Data given in Fig.8C show that in both the cases immunoprecipitation occurred although the reaction was relatively poor in 'anti-raf-α-gal' sera. The immunoprecipitation curve using 'anti-mel-α-gal' sera indicate homology amongst both the enzymes. Since there is no difference in the slopes of the curves with both the enzymes, it can be concluded that 'mel' induced and 'raf' induced α-galactosidases in Bacillus sp.I are structurally closely related. Thus results obtained by immunotitration curve confirmed the results of double immunodiffusion method.

Therefore from all above mentioned studies, it can be concluded that in the present investigation, in Bacillus sp. I

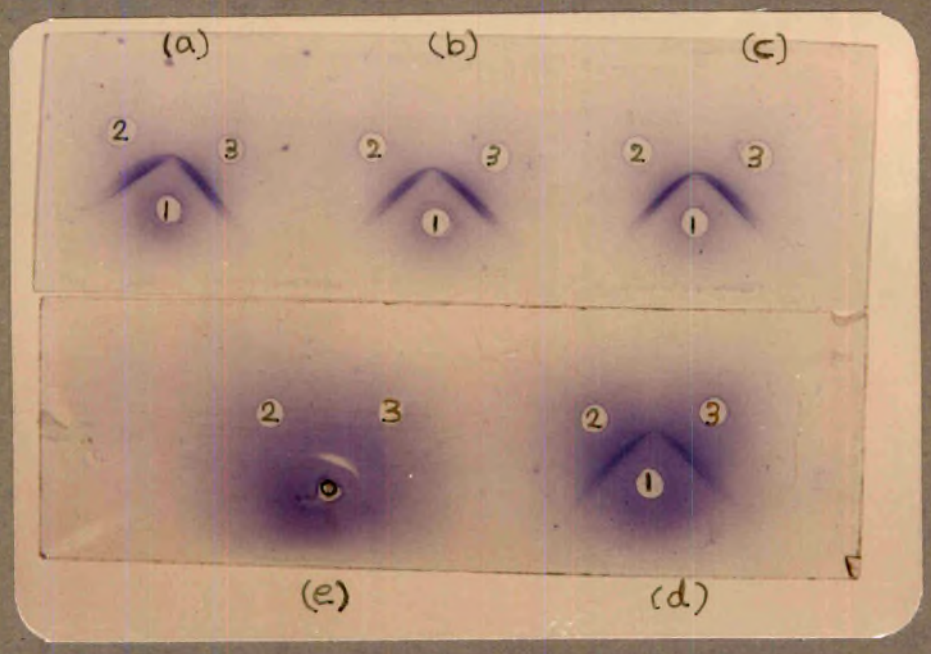


FIG.88 : Double immunodiffusion of raffinose induced and melibiose ∝-galactosidase from Bacillus sp. I:

Antiserum raised against pure raffinose induced ∝-galactosidase of Bacillus sp. I (anti-raf -∝-gal) was applied to the centre wells viz. well No.1 (in a,b,c,d), Raffinose induced ∝-galactosidase was applied to the well No.2, and melibiose induced ∝-galactosidase was applied to the well No.3,(in a,b,c,d) Control serum was applied to the central well in 'e'.

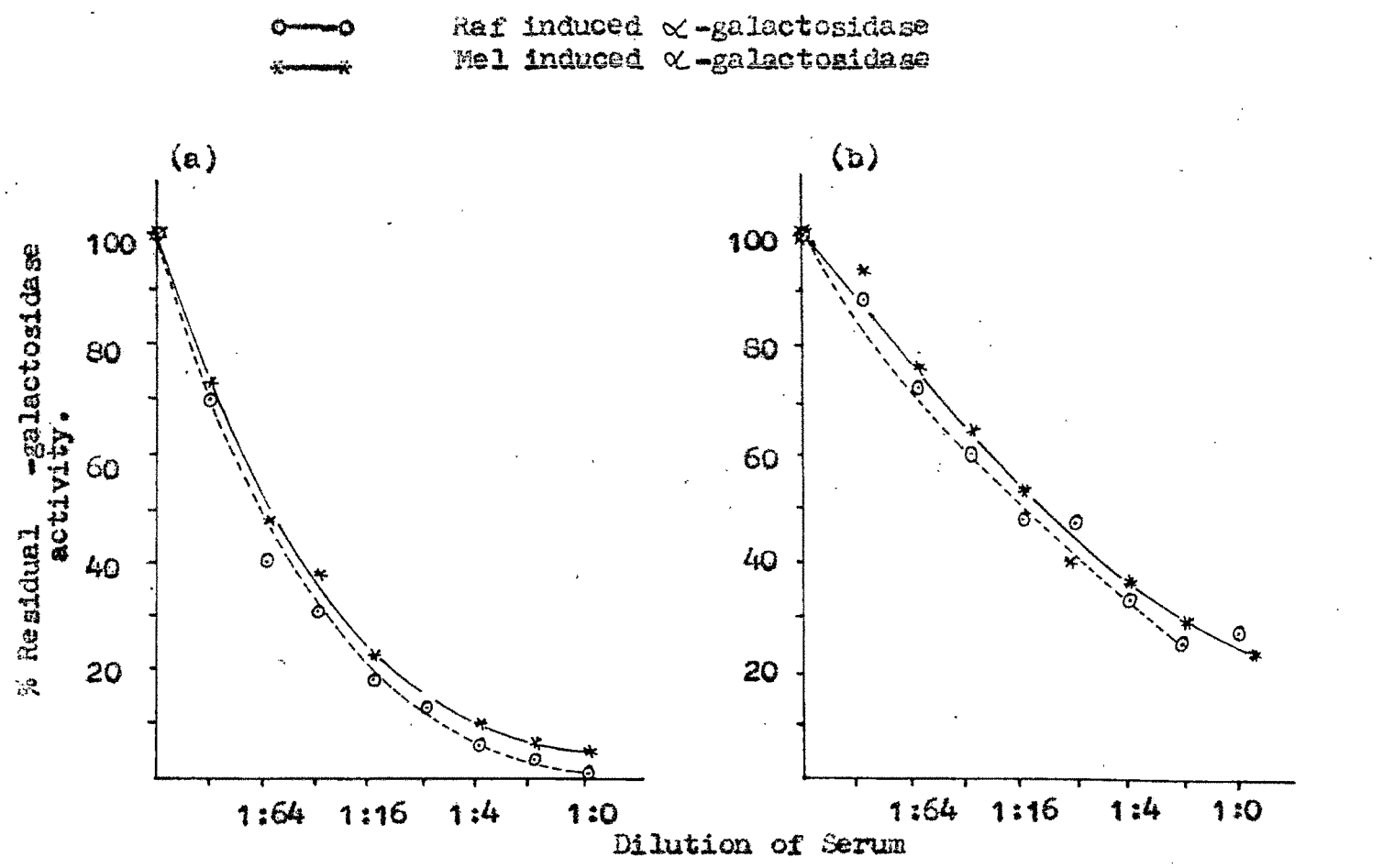


FIG.89 : Immunoprecipitation curves with melibiose induced and raffinose induced -galactosidase using: (a) anti-mel-∝-gal serum and (b) anti∝-gal serum

)LE-146 : Comparison of α-galactosidases coded by 'Raf' plasmid D1021 and x by E. Coli K12, respectively.

Criteria	α-Galactosidase specified by plasmid D1021	α-Galactosidase specified by the 'Mel' operon of E. Coli K12.
ntact enzyme : Molecular weight	3,29,000 ± 4000	2,00,000
ofactor requirement	None	NAD^+
on requirement	None	Mn^{+2}
H optimum	7.2	7.5
m for α-PNPG	0.15 mM	3.0 mM
m for melibiose	3.2 mM	10.0 mM
oluene sensitive	No	Yes

References : Schmitt and Rotman (1966);
Burstein and Kepes (1971);
Schmid and Schmitt (1976).

'raf' coded and 'mel' coded enzyme proteins might be the same proteins or they must be having homology in their structural and functional properties. It can be also postulated that 'raf' and 'mel' genes are components of the same operon in Bacillus sp. I. Thus raffinose and melibiose utilization is different in Bacillus species than that of E. coli, because in E. coli 'raf' coded and 'mel' coded α-galactosidases have different molecular and immunological properties (Schmid and Schmitt, 1976) as shown in Table-146.

Immunochemical relationships were established among 'Raf' plasmids of 39 independent isolates from man and domestic animals (from three continents) by using antiserum against α-galactosidase. Immunodiffusion revealed three serological subclasses of α-galactosidase, which were correlated with the biological and geographical origin of the host strains. It is concluded that the 'raf' determinants of all 'Raf' plasmids tested have evolved from a common ancestor (Schmid et al, 1979).