CHAPTER-II

MATERIALS AND METHODS

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The present studies are concerned with the isolation of oligosaccharides and neurotoxin (ODAP) hydrolysing bacteria from fermented foods and stored grains (commonly used in India) and detailed studies on these bacteria with special reference to the enzymes which hydrolyse raffinose family of oligosaccharides and neurotoxin (ODAP). The different aspects investigated are :

1) Isolation and identification of dominant bacteria present during fermentation of L. sativus dhal and bacteria of stored grains which can degrade antimutritional compounds and toxins such as flatulence producing oligosaccharides and neurotoxin (ODAP).

2) Studies on bacterial degradation of flatulence producing oligosaccharides with reference to -

   - standardization of thin layer chromatographic (TLC) and gas chromatographic (GC) methods for separation and estimation of oligosaccharides and their metabolites,

   - detection of enzymes of raffinose degradation in a strain of Bacillus Sp. I (isolated from stored soybean grains), optimization of growth conditions for this

bacteria for maximum production of enzymes, studies on regulation of production of raffinose hydrolysing enzymes in the same as well as purification and characterization of α-galactosidase and invertase from Bacillus Sp. I.

3) Studies on bacterial degradation of neurotoxin (ODAP) with reference to

- screening of bacterial isolates for their capacity to degrade ODAP and its metabolites,
- detection of enzymes involved in the degradation of ODAP in a strain of Streptococcus Sp. I (isolated from stored L. sativus grain) and kinetic studies of these enzymes in cell-free extract,
- optimization of growth conditions of Streptococcus Sp. I for maximum production of ODAP hydrolysing enzymes.

4) Screening the bacterial strains (which have capacity to breakdown flatulence producing oligosaccharides and neurotoxin) for the presence of plasmids and detailed studies on genetic basis of raffinose degradation in Bacillus Sp.I.

5) Production of antisera against α-galactosidase from Bacillus Sp. I.

Materials :

Lathyrus sativus seeds were obtained from the Agriculture Institute, Jambusar, Broach, Gujarat State, India, in bulk, dehusked and split in a local flour mill and used for the investigation. The dehusked pulse, henceforth referred to as 'dhal' were stored in polythene bags. The other grains for the investigation were purchased from local market and stored for 1-2 months in stoppered glass bottles.

The common chemicals used in the present studies were of analytical grade or research grade purity obtained from standard chemical companies such as Sarabhai Chemicals, Loba Chemicals, etc. The sources from which other chemicals were obtained are given in the Table-38a. The important reagents and standards used for various estimations are given in Table-38b.

For experiment of antibody production rabbits belonging to "Swiss Himalayan strain" maintained in the departmental animal house were used.

Preparation of a fermented product similar to 'khaman' from *L. sativus* dhal :

A fermented product similar to 'khaman' was prepared from *L. sativus* dhal according to traditional method. 500 g dhal were taken in a 2.0 litre beaker and washed twice with water. The washed dhal was steeped at 32° in 1000 ml water for 3 to 4 h. The soaked dhal was ground with appropriate amount of water for 1-2 min in a waring blender at high speed to get a coarse texture and thick batter. This preparation was then transferred to a 3.0 litre beaker and 25g of

TABLE-33a: List of Chemicals.

Name of the chemical	Source
Agar Agar	Difco Laboratories, Michigan, U.S.A.
Agarose	Sigma Chemicals, St. Louis, U.S.A.
Alkaline phosphatase	"
Acrylamide	E. Merck, Germany
Antibiotics (Kanamycin, tetracycline, ampicillin, novobiocin, chloramphenicol, rifampicin)	Sigma Chemicals, St. Louis, U.S.A.
2,5-bis (5'-tert-butyl Benz-oxarolyl-(2') thiophene (BBOT)	"
Blue dextran	"
Bovine serum albumin	"
N-N' methylenediacrylamide (Bis acrylamide)	Koch Light Lab. Ltd.,England.
Caesium chloride	BDH, Poole, England
Coommasie blue	BDH, Poole, England
Diamino propionic acid	Sigma Chemicals, St. Louis, U.S.A.
Dowex 50W (50 x 8 -400)	"
Dithiothreitol	"
DEAE Cellulose (DE-52)	Whatman Ltd., England
Ethidium bromide	Sigma Chemicals, St. Louis, USA.
E D T A	"
Freund's complete and incomplete adjuvant	"

TABLE-33a(Contd...)

Name of the chemical	Source
Glucose oxidase (A. niger)	Sigma Chemicals, St. Louis, U.S.A.
Hydroxylapetite	Bio-Rad Lab (Richmond, Calif.)
Hexamethyl disilazane (HMDS)	E. Merck, Dornstadt, Germany.
Lysozyme	Boehringer, West Germany
Lactate dehydrogenase (rabbit muscle)	Sigma Chemicals, St. Louis, U.S.A.
Mitomycin	
2-Mercaptoethanol	E. Merck, Schuchardt, W.Germany
MOPS (3-(N-Morpholino)-propane-sulfonic acid)	Sigma Chemicals, St.Louis, U.S.A.
Molecular weight markers for proteins	Sigma Chemicals, U.S.A. and Bio-Rad Laboratories.
Orthophthalaldehyde	Sigma Chemicals, St.Louis,USA
Phytic acid (sodium salt)	"
Pyridine	Fluka A.G. Chem.
Peptone	Difco Laboratories, Michigan, U.S.A.
p-Nitrophenyl α-D-galactoside	Sigma Chemicals,St.Louis, USA.
Peroxidase	"
p-Nitrophenyl α-D-fucoside	"
p-Nitrophenyl β-D-glucoside	"
QAE-Sephadex	Pharmacia Fine Chemicals, Sweden.
RNase	Sigma Chemicals, St.Louis, USA
Restriction endonucleases	Bethesda Research Lab, USA.
Radioactive chemicals (DL-leucine-1-C^{14}, Uridine-T(G), Oxalic acid $C-^{14}$)	Isotope Division, Bhabha Atomic Research Centre, Bombay, India.

TABLE-33a (Contd..)

Name of the chemicals	Source
Sodium Dodecyl sulfate	Sigma Chemicals, St.Louis, USA
Sugars (Stachyose, raffinose, melibiose, glucose, galactose, fructose, sucrose, maltose, cellobiose)	"
Silica Gel-G	E. Merck, W. Germany
Sephadex G-200	Pharmacia Fine Chemicals, Sweden
Sepharose 6B	"
Tetrazolium chloride	Sigma Chemicals, St.Louis, USA.
Trypsin	E. Merck, W. Germany
Trypsin inhibitor	Sigma Chemicals, St.Louis, USA.
Trifluoroacetic acid (TFA)	Fluka, A.G. Chem., Switzerland
N,N+,N',N'-tetramethylethylene diamine (TEMED)	Fluka, Switzerland
Triton X-100	British Drug House Ltd., USA
Yeast extract	Difco Laboratories, Michigan, U.S.A.

TABLE-33b : List of reagents and standards used for experiments.

Reagent	Method of preparation
Bovine Serum Albumin (Standard Stock Solution)	100 mg BSA dissolved in 0.1N NaOH and volume made to 50 ml to give a concentration of 2 mg/ml.
Bovine Serum Albumin (Working standard solution)	The BSA stock solution was diluted 1:10 to give a concentration of 200 µg/ml.
Bromocresol green reagent	A 0.1% spray solution was prepared by dissolving 100 mg of bromocresol green in 100 ml of 50% ethanol solution and pH adjusted to 7.2.
Bradford's reagent for protein estimation	100 mg of coomassie blue was dissolved in 50 ml 90% ethenol and then 100 ml 85% orthophosphoric acid was added and volume was made upto 200 ml with glass distilled water. The reagent was stored at 5°C. The reagent was diluted by adding 4 volumes of distilled water before use.
Destaining reagent for PAGE	400 ml of methanol was mixed with 70 ml glacial acetic acid and volume made upto 1000 ml with distilled water.
Kovac's reagent	10g of p-dimethylaminobenzaldehyde was dissolved in 150 ml of amyl alcohol and to this 50 ml of concentrated HCl was added slowly and mixed. It was stored at 4°C.

TABLE-33b (Contd..)

Reagent	Method of preparation
Methyl red solution	0.1g of methyl red dissolved in 300 ml of 95% alcohol and volume made upto 1 litre with distilled water.
Nessler's reagent	70g potassium iodide and 100g of mercuric iodide dissolved in 400 ml water, and then mixed with 500 ml of 20% aqueous potassium hydroxide and volume made upto 1 litre. The solution allowed to settle and clear supernatant decanted and preserved in a brown bottle.
Ninhydrin-acetone reagent (for detection of amino acids).	0.4% ninhydrin was prepared by dissolving 400 mg of ninhydrin in 100 ml of acetone.
Nitrite detection reagent for nitrate reduction test.	(A) Sulfanilic acid solution : 0.8g of sulfanilic acid dissolved in 100 ml of 5N acetic acid. (B) α-naphthylamine solution : 0.5g α-naphthylamine dissolved in 100 ml of 5N acetic acid. One ml each of the solution A and B are added to test solution.
Omera's reagent	To 100 ml of 40% aqueous solution of sodium hydroxide 0.5g of creatine was added just before use.
OPT reagent	To 100 mg of O-phthalaldehyde was added 0.2 ml of 2-mercapto ethanol and 1 ml of ethyl alcohol in a 100 ml measuring flask and then volume made to 100 ml with 0.5M, potassium tetraborate buffer, pH 9.9.

TABLE-33b (Contd...)

Reagent	Method of preparation
Potassium tetraborate buffer, 0.5M, pH 9.9	76 g of potassium tetraborate and 15 g of boric acid dissolved in 200 ml of distilled water and pH adjusted to 9.9 with 4N NaOH and then volume made to 500 ml with distilled water.
p-Nitrophenol standard	7 mg of p-nitrophenol were dissolved in 50 ml of water.
p-CMB, 0.005M	36 mg of p-CMB dissolved in 0.2N NaOH and pH adjusted to 8.0 with 0.2N HCl and made to 20 ml with distilled water.
p-dimethl aminobenzaldehyde (p-DAB reagent) or Ehrlich's reagent.	0.8 g of p-dimethyl aminobenzaldehyde dissolved in 30 ml of distilled ethanol and 30 ml of conc. HCl.
Staining reagent for PAGE	1.25 g coomassie blue was dissolved in 400 ml methanol. To this solution 70 ml glacial acetic acid was added and volume made upto 1000 ml with distilled water.
Schiff's reagent for glycoprotein staining	It was prepared by dissolving 2.5g of basic fuchsin in 500 ml of water, then adding 5g of sodium metabisulphite and 50 ml of 1N HCl. The solution was stirred for 6 hrs at 30°C and decolorised with about 8g of activated charcoal.
Sample buffer for SDS-PAGE	0.39g of NaH_2PO_4, 1.02g of Na_2HPO_4 and 1g SDS were dissolved in 100 ml distilled water. To this 1 ml 2-mercaptoethanol and and 0.015g bromophenol blue were added and mixed.

TABLE-33b (Contd....)

Reagent	Method of preparation
Spray reagent for detection of sugars on TLC	20 mg of naphthoresorcinol was dissolved in 10 ml distilled alcohol and 0.2 ml of concentrated sulfuric acid was added to this.
Tris-glucose oxidase (TGO) reagent	20 mg of glucose oxidase (Aspergillus niger - 25,000 units/gm solid) were dissolved in 80 ml of 0.5M Tris-HCl buffer (pH 7.0). To this 0.5 ml peroxidase (horse-raddish having 40 units/mg solid, 1 mg/ml in distilled water) and 2.0 ml O-dianisidine di-HCl (10 mg/ml in distilled water) were added and volume made upto 100 ml with buffer.
Scintillation fluid	400 mg of BBOT (2,5-bis-(5'-tert-butylbenzoxazonyl-(2') thiophene) was dissolved in 750 ml of toluene and to this 250 ml of Triton X-100 was added.

salt was added and mixed. This was covered with glass plate and allowed to ferment for 12-14 h at 32°. At the end of incubation period, the batter was poured in a steamer and steamed for 10 minutes. This was then cooled and cut into pieces and seasoned with mustard seeds cracked in oil.

Measurement of increase in batter volume during fermentation :

As the typical texture of Indian fermented food is due to leavening effect of fermentation because of production of CO_2, increase in volume was used as a measure of fermentation. The height of the column of batter was noted initially and after incubation with a plastic scale and the increase in height was taken to indicate increase in volume.

pH measurement :

A 10 percent suspension of the batter was prepared using deionized water and its pH was measured in a Beckman pH meter.

Sensory evaluation :

A sensory evaluation was carried out on a fermented product prepared from L. sativus dhal by a panel of 10 judges. A traditional fermented product 'khaman' obtained from popular shop was used as the standard for comparison.

Microbiological analyses of the L. sativus soak water and the batter :

To form a complete picture of the micro-organisms involved in the fermentation, the fermented L. sativus batter was analysed, as described below, according to the method of Mukherjee et al. (1965). Unfermented and fermented samples of batter in triplicate were removed with sterile spatula. 10.0g of sample was weighed and diluted 10 fold with sterile normal saline. (Mixing of the sample was carried out using acid-washed sand as dispersing agent). Further dilutions of this were made viz. 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. 0.1 to 1.0 ml of serially diluted sample was used to inoculate into APT agar and potato dextrose agar containing 30 µg/ml streptomycin sulphate, (Composition of the media is given in Tables-34 & 35), using spread plate and pour plate technique. These agar plates were then incubated at 30° for 48 h. The total colonies were counted using colony counter after the incubation. Representative colonies of the various isolates were maintained on slants containing APT agar or GYE agar or lactic agar (composition of media are described in Tables-34, 36 & 37).

The L. sativus dhal soak water was also sampled at 0 h and 4 h intervals for microbiological analyses as described above.

TABLE-34 : Composition of APT Agar.
(Evans and Niven, 1959).

Ingredients	Amount (g/1000 ml of distilled water)
Peptone	10.0
Yeast extract	7.5
Sodium chloride	5.0
Potassium phosphate (monobasic)	5.0
Sodium citrate	5.0
Dextrose	10.0
Polysorbate-80	0.20
$MgSO_4.7H_2O$	0.80
$MnCl_2.4H_2O$	0.14
$FeSO_4$	0.04
Na_2CO_3	1.25
Agar agar	20.0
Final pH 6.7	

Sterilized at 15 lb psi for 15 min.

TABLE-35 : Composition of potato dextrose agar.

Ingredients	g/litre of distilled water
Potatos, Infusion from	200
Dextrose	20
Agar	20
Streptomycin	35 mg/litre

200 g of peeled and cut potatoes were boiled in water for one hour, filtered and the filtrate was made upto 1000 ml. Glucose and agar were added to the filtrate, dissolved and autoclaved at 121°C for 20 minutes.

To the autoclaved medium, 5.0 ml Streptomycin solution sterilized by filtration was added to give a final concentration 35 mg/litre.

TABLE-36 : Composition of GYE agar. (Collins, 1967).

Ingredients	g/1000 ml distilled water
Tryptone	5.0
Yeast extract	2.5
Dextrose	5.0
Agar	20.0
pH	7.0

TABLE-37 : Composition of the medium for maintenance of lactic cultures.

(Mital et al, 1973)

Ingredients	Amount (g/1000 ml distilled water)
Tryptone	2.5
Yeast extract	0.5
Gelatin	2.5
Glucose	2.5
NaCl	4.0
Sodium acetate	1.5
Ascorbic acid	0.5
Agar	15.0

Isolation of bacteria from stored grains :

Bacteria which can degrade raffinose family of oligosaccharides and neurotoxin were isolated from stored grains. The grains used for the study were rice, wheat, bajra, bengalgram, soybean, kesari dhal (L. sativus) and blackgram and greengram (Appendix-I).

10.0 g of each grain were washed twice with 50 ml sterile distilled water. The grains were then soaked in sterile distilled water for 2-3 h at 30° with constant shaking. The supernatant was used for the isolation of bacteria. For isolation, 1-2 loopful of supernatant was streaked in triplicates on neurotoxin agar and raffinose agar plates. After streaking, the plates were incubated at 37°C for 48-72 h and at the end of incubation period, colonies were picked up and transferred to 20 ml sterile basal medium containing neurotoxin as 'C' and 'N' source or raffinose as 'C' source. (Composition of the media are given in Tables-38 & 39) and incubated at 30° for 48 h on shaker (140 rpm). The bacteria were again subcultured in the same media for six times and after the sixth subculture, culture was plated out on agar plates of appropriate isolation medium again to get isolated colonies. These colonies were then transferred on ODAP agar/ raffinose agar slants and preserved at 4°C.

TABLE-38 : Composition of raffinose broth.

Ingredients	Amount (g/1000 ml distilled water)
$(NH_4)_2 SO_4$	2.0
K_2HPO_4	7.0
KH_2PO_4	3.0
$MgSO_4.7H_2O$	0.2
$CaCl_2$	0.02
Micronutrient solution*	0.5 ml
Yeast extract	0.1
Raffinose	5.0
pH	7.0

10 X solutions of $MgSO_4$, $CaCl_2$ and yeast extract were prepared separately and sterilized at 15 lb for 15 min. 20% stock solution of raffinose was prepared and sterilised separately at 15 lb for 15 min.

K_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$ were mixed and autoclaved together. Final medium was prepared by adding appropriate concentrations of stock solutions after autoclaving in sterile conditions.

Raffinose agar was prepared by adding 20g agar agar to 1000 ml of raffinose broth.

TABLE-38 (Contd....)

* Micronutrient solution was prepared by dissolving 11.0g $ZnSO_4.7H_2O$, 5.0g $MnSO_4.H_2O$, 0.05 g $Co(NO_3)_2$, 0.05 g H_3BO_3, 2.0 g $Na_2MoO_4.2H_2O$ and 0.007 g $CuSO_4.5H_2O$ in 1000 ml of distilled water.

TABLE-39 : Composition of ODAP broth.

ODAP broth is a basal salt medium containing ODAP as sole 'C' and 'N' source, whose composition is given below :

Ingredients	Amount (g/1000 ml of distilled water)
K_2HPO_4	7.0
KH_2PO_4	3.0
$MgSO_4$	0.2
$CaCl_2$	0.02
Micronutrient solution	0.5 ml
Yeast extract	0.1
ODAP	4.0
pH	7.0

The method of medium preparation and composition of micronutrient solution are same as described under Table-38.

ODAP agar was prepared by adding 20g agar agar to 1000 ml of ODAP broth.

Identification of bacteria :

In order to identify the bacteria isolated, the tests described in the Manual of Microbiological Methods (Society of American Bacteriologists, 1957), Cruickshank et al.(1965), Norris and Ribbons (1971), Sharpe et al. (1966) and Skerman (1967) were carried out and bacteria were identified according to Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). Various media used for testing cultures are given in Tables-40 to 59.

The relative population of each species of bacteria during fermentation of L. sativus dhal was determined according to the methods of Pederson and Albury (1950; 1954); Mukherjee et al. (1965) and Davidson and Gronin (1973).

TABLE-40 : Composition of MRS agar.

(DeMan Rogosa and Sharpe, 1960).

Ingredients	g/1000 ml of distilled water
Tryptone	10.0
Lab lemco (oxoid)	10.0
Yeast extract	5.0
Tween-80	1.0 ml
$K_2H\ PO_4$	2.0
Tri-ammonium citrate	2.0
Dextrose	20.0
Sodium acetate	5.0
$MgSO_4.7H_2O$	0.2
$MnSO_4.4H_2O$	0.05
$FeSO_4$	0.034
Agar	20.0
pH 6.0 - 6.5	

Sterilized at 15 lb. psi for 15 min.

TABLE-41 : Differential medium for lactic Streptococci

(Mullan and Walker, 1979)

Ingredients	g/1000 ml of distilled water
Sodium Carboxymethyl cellulose	10.0
Yeast extract	5.0
Peptone	5.0
Lab lemco (oxoid)	5.0
Lactose	1.5
Calcium citrate	10.0
Arginine hydrochloride	1.0
Bromocresol purple	2.0 ml. (0.1%)
Agar	20.0
pH 7.0	

This medium differentiates following 3 types of Streptococci.

S. lactis - purple smear - no clearing.

S. cremoris - yellow smear - no clearing.

S. diacetylactis - purple smear extensive clearing.

TABLE-42 : Composition of Sodium Azide agar.

(Difco Manual, 1971)

Ingredients	g/1000 ml of distilled water
Beef extract	4.5
Tryptone	15.0
Dextrose	7.5
Sodium chloride	7.5
Sodium azide	0.2
Agar	20.0
pH	7.2

Medium was sterilized at 15 lb. psi for 15 min.
and sterile glucose solution was added separately.

TABLE-43 : Composition of the medium for Indole production.
(MacKie and McCartney, 1956).

Ingredients	Amount (g/1000 ml of distilled water)
Peptone	20.0
Sodium chloride	5.0

The pH of the medium wasadjusted to 7.4 and the medium autoclaved at 121° for 15 min.

Test for Indole production :

The medium was inoculated with the culture and incubated at 30° for 48 hrs. After incubation 0.5 ml of xylene was added. The tube was vigorously shaken, allowed to stand and xylene layer separated out. Indole, if present, would be extracted in xylene layer. To this, 0.5 ml of Kovac's reagent is added. Red colour in the xylene layer indicated the presence of indole.

TABLE-44 : Composition of medium for carbohydrate fermentation.

(Fred and Waksman, 1928)

	Amount (g/1000 ml of distilled water)
Peptone	2.0
Sodium chloride	5.0
Potassium phosphate (monobasic)	0.3
Bromocresol purple	0.005

The initial pH of the medium was adjusted to 7.1.

5 ml peptone medium was taken in a tube to which Durham's fermentation tube was inserted and stoppered with cotton wool. It was sterilised in the autoclave at 121° for 15 min. (15 psi).

Sugars to be tested were separately prepared at a concentration of 20% in distilled water and sterilised in the autoclave at 121° for 15 min. 0.25 ml of sugar was added to each tube.

Fermentation test : A loopful of the liquid culture (young culture) was inoculated into each tube containing the medium. Before inoculation the tubes were inspected to confirm the absence of gas bubbles from the Durham's tubes. The tubes are incubated at 37° for 2 days. The presence or absence of an acid by colour change and gas formation in the Dorham's tube were noted.

TABLE-45 : Composition of medium for aesculin hydrolysis.

(Collins and Taylor, 1967).

To 100 ml of peptone water or melted peptone water agar, 0.1 g of aesculin and 0.05 g of ferric citrate were added. The medium was then distributed into tubes (5.0 ml) and autoclaved at 10 lb for 10 min.

Ingredients

Peptone water	-	100 ml
Aesculin	-	0.1 g
Ferric citrate	-	0.05g
Agar	-	2.0 g

Medium was distributed into tubes (5.0 ml) after melting the agar, and sterilized at 10 lb for 10 min.

Test :

Stabs were inoculated with the culture and incubated for 2-7 days at 32°. Blackening of the medium was considered as a positive test.

TABLE-46 : Composition of the medium for citrate utilisation test.

(Koser, 1923)

Ingredients	Amount (g/1000 ml of distilled water)
NaCl	5.0
$MgSo_4 \cdot 7H_2O$	0.2
KH_2PO_4	1.0
Sodium citrate	25.0

The pH of the medium was adjusted to 6.8 and the medium was sterilised by autoclaving at 121° for 15 min.

Procedure for citrate utilisation test :

The tubes were inoculated with the culture and incubated at 37° for 96 hrs. The presence of turbidity was used as an index of growth and citrate utilisation.

TABLE-47 : Tests for catalase and oxidase production.

Catalase test

One ml of hydrogen peroxide solution is poured over a 24 hr. nutrient agar slope culture of the test organism and the tube is held in a slanting position. The production of gas bubbles from the surface of the culture indicates a positive reaction.

Oxidase test

A freshly prepared 1% solution of tetra-methyl-p-phenylene-diamine dihydrochloride is poured onto a 24 hr. nutrient agar slope (or APT agar) culture of the test organism so as to cover the surface and is then decanted. The colonies of oxidase-positive organisms rapidly develop a purple color.

TABLE-48 : Composition of medium for litmus milk test.

Preparation of Litmus milk

To 100 ml of skim-milk 1.5 ml of litmus solution (4% ethanolic solution) was added to give pale muave color and then distributed in test tubes (5 ml each) plugged with cotton, sterilized by autoclaving at 110°C for 30 min on each of the three successive days.

Test : The cultures of the test organisms was inoculated into sterile litmus milk and incubated for 48 hr at 37°C. The changes brought about by the cultures in Litmus milk regarding acid production (Litmus change to pink), precipitation of casein and gas production (stormy clot), proteolytic changes (litmus change to purple) were observed at the end of incubation period and reported in results.

TABLE-49 : Composition of the medium for nitrate reduction test.

(Fred and Waksman, 1928)

Ingredients	Amount (g/1000 ml of distilled water)
$K_2H\ PO_4$	0.5
$CaCl_2.2H_2O$	0.5
$MgSO_4.7H_2O$	0.2
Glucose	10.0
KNO_3	1.0

Sterilised at 15 psi for 15 min.

Procedure for nitrate reduction test :

The culture was inoculated in 5.0 ml of the above medium and incubated at 30°C for 48 hrs. After the incubation, 1.0 ml of sulfanilic acid reagent and 1.0 ml of -napthylamine reagent are added. Production of red colour indicated the reduction of nitrates.

TABLE-50 : Composition of Gelatin medium.

(Mackie and McCartney, 1956)

Ingredients	g/1000 ml of distilled water
Peptone	10
Meat extract	10
Sodium chloride	5
Gelatin	15

The medium was prepared and kept at 4° overnight. Next day the gelatin was dissolved by heating at 45°. The pH adjusted to 8.4 and steamed for 10 min. It was cooled quickly to 45° and 10g of egg albumin dissolved in 50 ml water was slowly added. It was steamed for 30 min, filtered and the pH of the filtrate adjusted to 7.6. It was sterilised by holding in the autoclave, and then steam at (100°) for 10 min, followed by keeping at 115° for 10 min. It was removed from the autoclave as soon as possible and cooled to 25°.

Procedure

The solid media was streaked with the organism to be identified.

TABLE-51 : Composition of the medium for methyl red test and Voges-Proskauer test.

(Machie and McCartney, 1956)

Ingredients	Amount (g/1000 ml of distilled water)
Peptone	5.0
KH_2PO_4	5.0

The pH of the medium was adjusted to 7.6 and autoclaved at 121° for 15 min. A 10% glucose was separately sterilised by autoclaving at 121° for 15 min. The sugar solution was added to the medium to give a concentration of 0.5%.

Procedure :

The medium was incoculated with a loopful of the liquid culture (nutrient broth culture) and incubated at 37° for 48 hrs. Methyl red reagent was added to one tube, mixed and observed. Bright red colour indicated a positive test. To another tube, 0.5 ml of O' Meara's reagent was added and incubated at 37° for 4 hrs. It was aerated by shaking at intervals. A positive reaction was denoted by the development of an pink colour usually in 2-5 minutes.

TABLE-52 : Composition of the medium for H_2S production.

(Untermohlon and Georgi, 1940).

Ingredients	Amount (g/1000 ml distilled water)
Tryptone	10.0
Sodium chloride	5.0
Agar-agar	5.0
2,3,5-triphenyl-tetra zolium chloride	0.05
Ferrous ammonium sulfate	0.2

The ingredients were completely mixed with water and boiled till completely dissolved. The medium was distributed in tubes and autoclaved at 121° for 15 min.

Procedure for the H_2S production test :

Culture was inoculated into the experimental and control tubes by stabbing. A red line along inoculum indicated nonmotile H_2S negative. Black line along inoculum indicated nonmotile H_2S negative. Diffused black colour through out the medium indicated motile H_2S positive.

TABLE-53 : Composition of the medium for phenylalanine deaminase test.

(Untermohlen and Georgi, 1940)

Ingredients	Amount (g/1000 ml distilled water)
Yeast extract	3.0
DL-phenylalanine	2.0
Na_2HPO_4	1.0
NaCl	5.0
Agar agar	20.0

The pH of the medium was adjusted to 7.4 and sterilized by autoclaving at 121° for 15 min.

Procedure for phenylalanine deaminase test

The medium was incubated with the culture incubated for 24 h at 37°. A few drops of 10% solution of ferric chloride was allowed to run down over the growth of the slant. Development of green colour indicated positive test.

TABLE-54 : Composition of the medium for urease test.

(Mckie and McCartney, 1956)

Ingredients	Amount (g/1000 ml of distilled water)
Peptone	1.0
Sodium chloride	5.0
Potassium dihydrogen phosphate	2.0
Phenol red (1g in 500 ml of aqueous solution)	6.0 ml

The pH of the medium was adjusted to 6.8 and sterilized by autoclaving at 121° for 15 min. After cooling to 50°, sterile solution of glucose was added to give a final concentration of 0.1% and 100 ml of sterile 20% solution of urea was added.

Procedure for urease test

Culture was inoculated into the medium and incubated at 37° for 24 h. Urease positive culture produced a purple pink colour.

TABLE-55 : Composition of medium for arginine hydrolysis.

Ingredients	Amounts (g/1000 ml of distilled water)
Tryptone	5.0
Yeast extract	5.0
K_2HPO_4	2.0
L-arginine monchydro-chloride	3.0
Dextrose	0.5

For arginine breakdown of lactobecilli MRS broth was used in which the ammonia citrate was replaced by 0.3% arginine-HCl.

Test for NH_3 production from arginine hydrolysis :

Culture was inoculated into arginine broth for 24-48 h and added few drops Nessler's reagent. Brown colour indicated ammonia production due to arginine hydrolysis.

TABLE-56 : Composition of the medium for Malonate utilization test.

(Ewing, 1962)

Ingredients	g/1000 ml distilled water
Yeast extract	1.0
Ammonium sulphate	2.0
K_2HPO_4	0.6
KH_2PO_4	0.4
NaCl	2.0
Sodium Malonate	3.0
Bromothymol blue	0.035

pH 7.4

Ingredients were dissolved in water and distributed 5 ml each in test tubes plugged with cotton and sterilized 15 lb psi for 15 min.

TABLE-57 : Composition of thioglycollate medium

(Difco Manual, 1971)

Ingredients	Amount (g/1000 ml distilled water)
Tryptone	15.0
Yeast extract	5.0
Dextrose	5.5
Sodium thioglycollate	0.5
Sodium chloride	2.5
L-cystine hydrochloride	0.5
Methylene blue	0.002

Medium was autoclaved at 15 lb psi for 15 min.

TABLE-58 : Composition of Gibson's semi-solid medium

- a medium for the detection of carbon dioxide production from glucose.

(Gibson and Abd-el-Malek, 1945)

Yeast extract	-	2.5 g
D-glucose	-	50.0 g
Tomato juice (pH 6.5)	-	100 ml
Reconstituted skim milk	-	800 ml
Nutrient agar	-	200 ml

Tomato juice was mixed with the reconstituted skim milk and added the yeast extract and glucose and heated in a steamer. While still hot, added the molten nutrient agar and mixed well. pH was adjusted to 6.5 if necessary and the medium was distributed in test tubes to a depth of 5-6 cm, sterilized by steaming at 30 min, on each of three successive days.

TABLE-59 : Starch agar medium composition.
(Harrigan and Mc Cance, 1966)

Ingredients	g/1000 ml
Soluble starch	2.0
Nutrient agar	1000 ml

Dissolved the starch in the molten nutrient agar. Sterilized by autoclaving at 121°C for 15 min, and poured into petridishes aseptically.

TABLE-60 : Composition of LB (Luria-Bertani) medium.
(Maniatis et al, 1982).

Ingredients	Amount (g/litre of distilled water)
Peptone	10.0
Yeast extract	5.0
Sodium chloride	10.0

pH was adjusted to 7.5 with 0.1N NaOH and autoclaved at 15 lb (PSI) for 15 minutes.

TABLE-61 : Composition of DAPA broth.

DAPA broth is a basal salt medium containing DAPA (diaminopropionic acid) as a sole 'C' and 'N' source, whose composition is given below :

Ingredients	Amount (g/litre of distilled water)
K_2HPO_4	7.0
KH_2PO_4	3.0
$MgSO_4$	0.2
$CaCl_2$	0.02
Micronutrient solution	0.5 ml
Yeast extract	0.1
DAPA (Diaminopropionic acid)	4.0
pH - 7.0	

The method of medium preparation and composition of micronutrient solution are same as described under Table-38.

DAPA agar was prepared by adding 20g agar agar to 1000 ml of DAPA broth.

TABLE-62 : Composition of oxalate broth.

(Chandra and Shetna, 1975)

Ingredients	g/1000 ml of distilled water
$(NH_4)_2SO_4$	0.5
$MgSO_4.7H_2O$	0.2
K_2HPO_4	4.4
KH_2PO_4	3.4
$FeCl_3$	0.015
Mineral stock solution*	1.0 ml

Five grams per litre of potassium oxalate and 10 mg/ml of yeast extract was added to the above medium. As an indicator of the pH, 5 ml of 0.4% aqueous solution of phenol red per litre of the medium was used.

* Mineral stock solution contained $ZnSO_4.7H_2O$, 11.0g; $MnSO_4.H_2O$, 5.0g; $CoSO_4$, 0.05g, H_3BO_3, 0.05; $Na_2MoO_4.2H_2O$, 2.0g; $CuSO_4.5H_2O$, 0.5g per litre of distilled water.

Experimental fermentation of L. sativus flour with pure cultures :

For experimental fermentation, about 200g of L. sativus dhal flour was sterilized at 120° for 15 min. in 500 ml conical flask. This autoclaved flour was cooled and then transferred to 1 litre sterile beaker, and slurry was made by addition of sterile distilled water. (All preparations were carried out in a sterile inoculation chamber previously exposed to U.V. light). The autoclaved batter was inoculated with test cultures under sterile condition. Consistency of batter was adjusted by adding sterile distilled water, after adding 2.0% common salt. The batter was then allowed to ferment at 32° for 14 h. The change in pH and increase in volume were measured.

The inoculum of the bacteria to be used for above experiment was adjusted so that the resulting batter would resemble the naturally unfermented batter with regard to approximate initial total bacterial count. The inoculum was adjusted for bacterial count using turbidity measurement in colorimeter at 660 nm. A fresh culture (24 h old) of the organism to be inoculated was prepared on agar slants and transferred with the sterile wireloop to 10 ml sterile normal saline, and mixed well. 5.0 ml of this was taken and the amount of dilution required to match the inoculated batter

with natural unfermented batter regarding microbial population was calculated and the inoculum diluted accordingly. Appropriate amount of this diluted inoculum was added to the sterilised batter.

Preparation of neurotoxin from L. sativus dhal :

The neurotoxin was isolated from L. sativus by the method of Rao et al. (1964). The decorticated seeds of Lathyrus sativus (kesari dhal) were taken, washed with water and air dried and powdered using an electric grinder, 400 g of the flour was extracted with 2 litres of distilled water at 60 to 65° for 6 hrs with occasional stirring. The slurry was centrifuged at 8000 xg for 10 min and the supernatant (S_1)was collected. The residue was re-extracted with 1 litre of water and kept at 60 to 65° for 6 hrs, centrifuged at 8000 xg for 10 min and the supernatant (S_2) obtained was added to the first supernatant (S_1). To the combined extracts, 6 litres of 95% ethanol were added and the final concentration of alcohol was made to 70% using distilled water and allowed to precipitate at -4° for 12 h. The precipitated protein was centrifuged at 8000 xg for 10 min. The supernatant was collected and the volume reduced to 100 ml in water bath at 60° and was then treated with 100 ml of diethyl ether in a separate funnel, shaken well and the aqueous layer was separated to remove organic acids. The aqueous layer was again taken in a separating funnel and 100 ml of chloroform was added and

shaken to remove the lipids. The aqueous lipid free phase was removed and passed through Dowex 50x 8-H^+ (200-400 mesh size) column (25 cm x 1.8 cm) and eluted with water. 10 ml fractions of the eluent were collected. Those fractions which gave colour with ninhydrin reagent were tested for colour formation with OPT reagent with and without hydrolyses with 1N NaOH. The fractions which gave positive test with ninhydrin and with OPT after hydrolysis were collected. The volume was reduced to 20 ml by freeze-drying in lyophilizer for further purification steps. When this neurotoxin was tested for the presence of sugars, using Dubcı's method (Dubois et al, 1956) it showed the presence of sugars. Therefore the sample was further purified by rechromatography on second Dowex-50 x 8-H^+ column as described above. The purified sample was then freeze dried in lyophilizer.

Chemical synthesis of neurotoxin (Ramchand et al, 1983) :

1.4 g of diaminopropionic acid monohydrochloride was dissolved in 20 ml of distilled water and to this 10 ml of distilled methanol was added. 2.0g of dimethyl oxalate was dissolved in 10 ml of methanol and added slowly (during the course of 1 hr) from a burette with constant stirring using a magnetic stirrer and the stirring was continued for another 3 h. The pH of the solution was adjusted to 9.5 with a saturated solution of $Li(OH)_2$ in methanol. The solution was

diluted to 100 ml with warm water (80°) and the pH was lowered to 3.0 with 1N HCl. Excess dimethyl oxalate, which precipitated out as lithium oxalate was removed by filtration. The filtrate (100 ml) was then charged to a column (25 x 1.8 cm) containing previously activated Dowex 50 x $8H^+$ (200-400 mesh) and eluted with distilled water. The fractions containing ODAP were collected and the volume was reduced to 50 ml. To this 1.75g of $NaHCO_3$ was added to convert ODAP into disodium salt, and 1 litre of acetone was added and kept at -4° for 12 hrs. The precipitated ODAP was filtered through Whatman No.1 filter paper and dried.

Synthesis of Dimethyl Oxalate :

Dimethyl oxalate required for the chemical synthesis of ODAP was synthesized according to the method of Dey (1972). Oxalic acid was dehydrated at 110° for 1 hr in an oven. 100g of anhydrous oxalic acid and 200 ml of methanol were taken in a 500 ml round bottom flask and a water condensor was attached to it. The mixture was refluxed at 80° for 4 hrs. After the refluxing was over, the water condensor was removed and a distillation condenser was attached and dimethyl oxalate was distilled out at 168°. It was recrystallised using methanol using sand bath.

Synthesis of labelled dimethyl oxalate :

Labelled dimethyl oxalate was synthesized by refluxing (^{14}C) oxalic acid + cold oxalic acid with distilled methanol using a semi-micro refluxing set. Oxalic acid and methanol were taken in a 50 ml round bottom flask fitted with a condenser and the solution was refluxed for 4 hrs in a water bath at 80°. After this the flask was fitted with an air condensor and transferred to a sand bath heated at 200°. First the excess methanol was distilled off at 65° and then the dimethyl oxalate at 168°. The dimethyl oxalate was recrystallised using distilled methanol and used for the synthesis of labelled ODAP.

Chemical synthesis of oxalyl labelled ODAP was carried out using a method of Ramchand et al. (1983) as described earlier except labelled dimethyl oxalate was used instead of cold dimethyl oxalate.

The characteristics and purity of neurotoxin (ODAP) preparations were checked by IR Spectrum analysis as well as thin layer chromatography.

I.R. Spectrum of ODAP preparations :

The purity of the synthesized compounds was confirmed by obtaining IR spectrum using IR spectrophotometer with

automatic recorder. The frequency range used was 800 to 4000 cm^{-1}. A thin film of the compound was made with KBr pellets and used for IR studies.

Thin layer chromatography for separation of neurotoxin (ODAP), diaminopropionic acid (DAPA) and oxalic acid :

To check the purity of the neurotoxin prepared in the laboratory as well as for the degradation studies, a thin layer chromatgraphy (Mehta et al, 1972) was used to separate ODAP, DAPA and oxalic acid.

20 cm x 20 cm size glass plates were washed with distilled water, dried and coated with silica gel H to a thickness of 0.25 mm. The plates were activated at 110° for 1 hr before use. Samples containing ODAP, diaminopropionic acid (DAPA) and oxalate were spotted in duplicate with the help of micropipette, standard diaminopropionic acid and oxalic acid were also spotted. The plates were developed using the solvent system 95% ethanol + water (63 : 37 v/v) for 2.5-3 h at 30°. Colour was developed by spraying 0.4% ninhydrin in acetone in the case of ODAP and diaminopropionic acid and by spraying bromocresol green reagent in the case of oxalic acid. For this, half the TLC plate was sprayed with ninhydrin reagent and another half with bromocresol green by keeping the partition in the middle of the plate.

Thin layer chromatography (TLC) and gas chromatography (GC) methods to separate and estimate oligosaccharides and their breakdown products :

(A) Extraction of sugars from L. sativus samples

All the samples to be analysed were dried at 70° in a hot air oven to a constant weight and ground in an electric grinder to get fine powder.

Sugars (oligosaccharides and simple sugars) were extracted using method of Cerning and Guilbot (1973). Five grams of the dried sample was mascerated in 70% aqueous ethanol (250 ml) and the mixture boiled by keeping in hot water bath at 70° for 5 minutes, and then kept on a shaker for 2 h at 140 rpm. This solution was then filtered using Whatman filter paper No.3 and the residue was washed several times with approximately 150 ml of 70% alcohol till the last extract showed the absence of sugars by not giving colour with phenol sulphuric acid reagent. The ethanol extracts were combined together and concentrated to 50 ml by evaporating in a water bath at 70°. This extract was then treated with double the volume of chloroform and shaken in a separating funnel. The layer containing sugars (by testing with phenol-sulphuric acid reagent) then removed and

centrifuged at 3000 rpm for 30 minutes to remove denatured proteins. The supernatant was then used for estimating mono-, di- and oligosaccharide components.

In some of the experiments, sugars were extracted by the method of Goel and Verma (1981) as described below :-

5 gm of dried sample was defatted by grinding with chloroform + methanol (2:1) mixture. Lipids were then removed by filtration through Whatman filter paper No.1 and the sample was dried in air. This defatted powder was then transferred to 250 ml conical flask and added 50 ml 70% aqueous ethanol and shaken on rotary shaker for 10-12 h. The suspension was then centrifuged at 3000 rpm for 30 minutes to remove denatured proteins. The supernatant was made to final volume 50 ml with 70% alcohol and used for estimating sugars.

Analysis of sugars :

Since there was no GC available during the early stages of investigation, the TLC method was standardized. The method of Stefanis and Ponte (1968) was modified in the present investigation as follows :-

(a) Preparation of plates

30g of silica gel 'G' was mixed with 60 ml of 0.05 M sodium phosphate buffer, pH 7.0. The suspension

was applied on 20 x 20 cm glass plates (0.5 mm thickness). These plates were dried in air and activated at 110° for 45 minutes. 20-200 ug of each standard sugar prepared in 70% alcohol as well as samples 50-100 ul were spotted on plates. These sugars were then detected after the run, by spraying the plates with naphthoresorcinol reagent. For development of coloured spots plates were heated immediately for 10 min at 110° in a hot air oven.

(b) Solvent system :

The solvent system used was a mixture of chloroform : acetic acid : water (6 : 10 : 1). The plates were run at 30° thrice for 2-3 h, each time.

Quantitative estimation of sugars :

Since it was not possible to separate galactose, fructose and glucose by the TLC method during the present investigation indirect estimation of these sugars was done. From the TLC plates, a mixture comprising of all the three sugars was eluted with 1.0 ml distilled water and estimated by the method of Dubois et al. (1956) which will give the values for all the three sugars. From the portion of the eluted sample, glucose was estimated by glucose oxidase method (Dahlquist and Nordstrom, 1966) and fructose by Roe's method (Roe, 1934). The corresponding values for these two sugars (glucose and fructose) were determined referring to standard graphs in Dubois' method. These values

were subtracted from the value obtained for the mixture of all the three sugars by Dubois' method. From this value galactose was calculated again by referring to standard graph for galactose by the method of Dubois et al. (1956).

Well separated sugars on TLC plate, viz. sucrose, raffinose, stachyose and verbascose were estimated by the method of Roe (1934), after extracting from gel. Melibiose was estimated by the method of Dubois et al. (1956).

Estimation of all the sugars by the method of Dubois et al. (1956) :

Suitable aliquotes of the sample were made to 1.0 ml with distilled water and to this 1.0 ml of 5% aqueous phenol was added, and mixed properly. 5 ml of sulphuric acid was added from a fast blowing pipette to each tube in order to produce good mixing and even heating. The contents were mixed thoroughly on a vortex mixer. After 30 min the colour was measured at 490 nm against a blank containing distilled water and gel.

Estimation of fructose, sucrose, raffinose, stachyose and verbascose by the method of Roe (1934) :

Suitable aliquotes of the eluted sample were made to 2.0 ml with 70% alcohol, to this 2.0 ml of 0.1% alcoholic

resorcinol was added and mixed properly, 6 ml of concentrated HCl was added to this and heated at 80° for 8 min. The tubes were cooled and read at 420 nm against a reagent blank.

Glucose oxidase method of estimation of glucose :

The samples were made to 1.0 ml with distilled water and 1.0 ml of TGO (Tris-glucose oxidase) reagent was added. Tubes were then incubated at 37° for 60 min. Reaction was terminated with 1.0 ml of 66% conc. H_2SO_4. The colour was read at 540 nm against a blank containing silica gel and distilled water.

Reference curves were prepared for the sugars by spotting known quantity (25-200 µg) of them on TLC plates.

GC method for analysis of sugars :

Since gas liquid chromatograph was available, the method of Perl *et al*. (1984) was modified and standardized to separate and estimate oligosaccharides and their metabolites.

Apparatus

The gas chromatograph used was Hewlett Packard model 5840 A equipped with flame ionization detector. The column used was a 50 cm x 3 mm I.D. stainless steel

packed with 10% UCW-98 on chromosorb 80-100, WAW DMCS (Hewlett Packard, Avondale, USA). The peak area determination was made with HP 5840 A computing integrator attached to the gas chromatograph. The peaks were normalized for detector response and amount of sugar calculated with respect to internal standard.

Preparation of TMS (Trimethylsilyl) derivatives :

The standard solutions of different sugars as well as the extracts of the samples containing 0.001 to 0.01 g of different sugars were evaporated to dryness under a stream of nitrogen gas at 50-60°C or by lyophilisation. To the dehydrated residue 500 µl of pyridine was added. This was then trimethyl silylated with a mixture of 900 µl of hexamethyl disilazane and 100 µl trifluoroacetic acid for 60 min at 70-72°C. Two microlitres of this solution were injected into the gas chromatograph. To all the samples a known amount of internal standard namely phenyl β-D-glucoside was added.

Seperation of trimethylsilyl (TMS) derivatives :

The temperatures of injection port and detector were 300° and 380° respectively. The oven was programmed from an initial temperature of 150° for one minute to a final temperature of 380° at the rate of 10° per min. Twenty three minutes were required to elute the TMS derivatives. The nitrogen flow rate was 30 cm^3 per min.

Methods for processing L. sativus seeds or dhal :

The traditional methods used for processing legumes in India were tried with a view to see the effect of these processings on oligosaccharides. The different methods tried are described below :-

1) Soaking and autoclaving/boiling :

L. sativus dhal was soaked in 4 volumes of water for 6 h at 25° and then cooked by autoclaving at 15 psi for 20 min in double the amount of water.

The soaked samples were also cooked by boiling in four volumes of water for 30 min.

2) Roasting and parching :

For roasting, the dhal was kept in a frying pan on the fire for 15-20 min with occasional stirring.

For parching, the whole seeds were soaked for 2 h in water. The soaked seeds were then puffed in a hot sand medium by rapid mixing.

3) Germination :

The seeds were surface sterilized with 1% sodium hypochlorite solution, washed thoroughly with distilled water and allowed to germinate on a filter paper at 28°C. They were analysed at 12,24,48 and 72 h of germination.

4) Fermentation :

Natural fermentation of the dhal was carried out as described earlier.

Estimation of ODAP :

ODAP was estimated by the method of Rao (1978b). In this method of estimation, neurotoxin is hydrolysed to diaminopropionic acid which gives yellow colour with ortho-phthalaldehyde.

For estimation of neurotoxin from L. sativus dhal samples during fermentation, 1.0 g of dried sample was weighed and to this 10 ml of distilled water was added, shaken for 15 min. The suspension was then centrifuged at 3000 xg for 15 min in a clinical centrifuge. The supernatant was used for estimating neurotoxin content.

Aliquots of the sample were taken in duplicate tubes and the volume was made to 1.0 ml with distilled water and mixed. To each tube 0.5 ml of 10% TCA was added and centrifuged at 3000 xg for 15 min. The supernatant was collected and to this 0.5 ml of 4.5N NaOH was added. One tube was kept in a water bath at 80° for 30 min for hydrolysing ODAP to DAPA whereas the other was kept at 25°. After the hydrolysis was over the tubes were cooled to room temperature. 1 ml of OPT reagent was added to each tube. The intensity of the colour produced was measured after 10 min at 420 nm in Beckman spectrophotometer against a blank, which contained 1 ml of distilled water, 0.5 ml 10% TCA, 0.5 ml 4.5N NaOH

and 1 ml OPT reagent. The difference in reading after and before hydrolysis will give the value for ODAP content.

A standard graph was prepared as follows. Aliquots containing different concentrations (4-20 µg) of ODAP were taken in test tubes. The volume in each tube was made to 1 ml with distilled water and they were treated as described in the previous paragraph. A standard graph was drawn using the values obtained.

Estimation of DAPA (Diaminopropionic acid) :

DAPA was estimated using the method of Rao (1978b) for ODAP estimation as described above except for the hydrolysis step which was ommitted.

Estimation of Pyruvic Acid :

Pyruvic acid liberated by action of ODAP hydrolysing enzymes was estimated by LDH (Lactate dehydrogenase) method of Gloster and Harris (1962). After termination of the enzyme reaction, during the assay the contents of the tube were centrifuged at 3000 rpm for 10 minutes. The supernatant was used for estimation of pyruvate. To the suitable aliquots of supernatant containing pyruvate 1.0 ml of 1.1M phosphate buffer (pH 6.9), 0.05 ml NADH (5 mg/ml) were added and volume made upto 2.5 ml with distilled water. The absorbance was read at 340 nm in a Beckman spectrophotometer (0 minute reading).

The reaction was started by the addition of 0.05 ml of LDH (50 units) and the decrease in absorbance was measured after 1 min. From the difference in initial and final readings the pyruvic acid was calculated using the standard graph. (Range of the method for pyruvic acid was 2-20 µg).

Pyruvate was also estimated using a colorimetric method (Friedemann and Haugen, 1943) during the inhibitor studies of ODAP hydrolysing enzymes.

Screening of bacterial isolates for their capacity to utilize neurotoxin (ODAP) and oligosaccharides :

The basal salt medium was used for screening the bacterial isolates for their capacity to utilize neurotoxin (ODAP) and oligosaccharides as sole 'C' and 'N' or only 'C' source. Since raffinose is cheap and readily available it was used as a representative of raffinose family of oligosaccharides for studies on bacterial degradation. Other sugars belonging to raffinose family of oligosaccharides viz. stachyose, verbascose were not readily available.

In order to find out the capacity of the bacteria isolated from various sources, to utilize flatulence producing oligosaccharides or neurotoxin (ODAP) as sole carbon or carbon and nitrogen source in vitro, the following experiments were conducted :

The utilization of raffinose or neurotoxin during growth of the test organisms was investigated by estimation of raffinose or neurotoxin in the culture filtrate and cell free extract before and after incubation period.

25 ml of the basal salt medium were dispensed in 150 ml conical flask and sterilized by autoclaving at 121° for 20 min. Raffinose (20%) and ODAP (10%) were sterilized separately by autoclaving (autoclaving did not destroy any of these two compounds) and then the appropriate amount of these solutions were added to the sterile basal salt medium to get the final required concentrations. Basal medium containing raffinose was termed as raffinose broth (composition of the medium is given in Table-38 and basal medium containing neurotoxin was termed as ODAP broth (composition of the medium is given in Table-39). The inoculum was prepared by growing the isolates in luria broth (composition is given in Table-60) for 24 h at room temperature. 0.5 ml of this inoculum (approximately 5-50 x 10^6 cells) were added to the 25 ml basal medium containing raffinose or ODAP and incubated at 30° for 24 to 48 h on rotary shaker (150 rpm). The flasks containing only basal salt medium with inoculum and flask with basal medium containing 'C' or 'C' and 'N' source without inoculum served as controls. After the incubation period, growth of bacteria in terms of turbidity was measured at 660 nm in Klett Summerson colorimeter, and final pH was measured using Beckman pH meter. The culture was then centrifuged at 8000 xg for 20 min at 4° and the supernatant was removed (SN_1). For preparation of

cell free extract, pellet was suspended in 10 ml distilled water and disrupted by sonication using Branson sonifier B-12 20 KHz for 10 min. This cell homogenate was then treated with chloroform:methanol mixture (2:1) to remove lipids. This fat free supernatant (SN_2) and culture filtrate (SN_1) were lyophilized to powder forms and used for the quantitative analysis of raffinose or neurotoxin. This solution was centrifuged at 20,000 xg for 30 min and the supernatant (SN_2) was removed. SN_1 and SN_2 (culture filtrate and cell free extract) were used for the quantitative analysis of neurotoxin or raffinose.

Neurotoxin (ODAP) was estimated by the method of Rao (1978b) as described earlier. Raffinose was estimated using the GC method described previously.

Raffinose degrading enzyme system of Bacillus Sp. I

As described earlier, α-galactosidase and invertase are the two enzymes involved in the degradation of flatulence producing oligosaccharides into their monosaccharide end products. Hence these two enzymes were investigated from Bacillus Sp. I (isolated from stored soybean grains) which could give maximum growth in raffinose broth.

Cells were grown in 100 ml raffinose broth for 12 h, 24 h and 48 h at 30° on shaker at 140 rpm. After the incubation period was over, the medium was centrifuged at 8000 xg for 10 min at 0-4°. The supernatant was used for

extracellular enzyme activity and cells were suspended in 0.05M sodium phosphate buffer, pH 7.0. The suspension was surrounded by ice-salt mixture and sonicated in an ultrasonic disintegrator (Branson B-12 sonifier) at 20 KHz. by giving 20 pulses of one minute each. This cell homogenate was then centrifuged at 20,000 xg for 30 min at 0-4° and supernatant viz. cell-free extract was used for intracellular enzyme activity.

α-Galactosidase activity was assayed using para-nitrophenyl α-D-galactoside (α-PNPG) as a substrate in all the studies. Sucrose hydrolase (invertase) was assayed by the method of Gascon and Lampen (1968) using sucrose as a substrate. Details of the assay system for both the enzymes are given in Table-63.

α-Galactosidase activity against melibiose was determined by measuring the liberated glucose. The reaction mixture consisted of 10 µmoles of melibiose, 150 umoles of sodium phosphate buffer, pH 7.0 and 0.1-0.2 ml appropriately diluted enzyme solution in 1.0 ml assay system. The reaction was carried out at 37°C for 30 min and stopped by the addition of 0.5 ml 0.25N Ba $(OH)_2$ and 0.5 ml of 0.25N $ZnSo_4$. The assay tubes were then centrifuged at 5000 rpm for 15 min in a clinical centrifuge and liberated glucose was estimated from the supernatant by using glucose oxidase method. Enzyme units are defined as the amount of enzyme which liberates 1.0 µmoles of glucose per min. under assay conditions.

TABLE-63 : Details of the assay system for ∝ -galactosidase and invertase.

	∝ -Galactosidase	Invertase
Basis of the method used	Schmid and Smitt (1976)	Gascon and Lampen (1968)
Substrate	p-nitrophenyl- ∝ -D-galactoside (∝ -PNPG) 1.0 µmole.	Sucrose 100 µmoles.
Buffer	Sodium phosphate buffer, pH 7.0, 50 µmoles	Sodium phosphate buffer, pH 7.0, 50 µmoles.
Enzyme extract	50 µg protein (crude enzyme)	300 µg protein (crude enzyme)
Final volume (ml)	1.0 ml	2.0 ml
Temperature and period of incubation	37°, 10 min.	37°, 30 min.
Start of reaction	Enzyme added	Enzyme added.
Termination of reaction	1.0 ml of 0.5M Na_2CO_3 added and centrifuged if required.	0.5 ml of 0.25N $Ba(OH)_2$ and 0.5 ml of 0.25N $ZnSO_4$ added and then centrifuged to remove precipitated proteins.
Treatment of blank	0.5M Na_2CO_3 added before incubation or boiled enzyme added.	Reaction was terminated before enzyme addition or boiled enzyme extracts were added.
Parameter measured	Liberated paranitrophenol estimated by reading absorption at 420 nm.	Liberated glucose estimated by glucose oxidase method of Dahlquist and Nordstrome (1966)

TABLE-63 (Contd...)

	∝-Galactosidase	Invertase
Enzyme unit	Amount of enzyme required to liberate one micromole (µmole) of PNP per minute under assay conditions, unless and until specified.	Amount of enzyme required to liberate one micromole (µmole) of glucose under assay conditions.
Specific activity	Enzyme units per mg protein.	Enzyme units per mg protein.

The activity of enzyme against raffinose and stachyose was measured by estimating the amount of galactose released according to the method of Somogyi (1952). Enzyme units are defined as the amount of enzyme which liberates 1.0 µmoles of galactose per minute under assay conditions.

Protein determination :

Protein was measured spectrophotometrically according to the procedure of Lowry et al. (1951). During the later stages of enzyme purification, when protein concentration was low or interfering substances were present Bradford's method (1976) was used for the estimation of proteins.

Studies on regulation of -galactosidase production of Bacillus Sp. I :

An attempt was made to study the regulation of raffinose metabolism in Bacillus Sp.I. For these studies production of enzymes ∝-galactosidase and invertase were studied using various sugars. The evidence for induction of ∝-galactosidase at transcriptional level was provided using rifampicin as an inhibitor of transcription.

Enzyme assays during induction :

During this experiment growth and production of enzymes were studied using various sugars as 'C' source. Stock solution (20%) of each sugar was sterilized separately by autoclaving at 15 lb. for 10 min and appropriate amount (the required concentration) of each sugar stock solution was added in a sterile 250 ml Erlenmeyer flask. Basal-salt medium was prepared separately in a bulk and to this was added 0.5% inoculum (12 h old culture grown in luria broth). 40 ml of this inoculated basal medium was distributed in 250 ml conical flasks containing different sugars and shaken well. (Controls were removed immediately from this flask (containing 0.5% sugar) for 0 h readings). These flasks were then incubated on shaker (240 rpm) at 32°. During the growth at different time intervals, 3.0 ml aliquts were removed for assay of -galactosidase, invertase and for measurement of turbidity. Growth in terms of turbidity was measured at 660 nm using spectrophotometer. For α-galactosidase assay, 1.0 ml cell suspension was toluenized for disruption of cells for 10 min at room temperature and added 1 µmole of α-pNPG prepared in sodium phosphate buffer, pH 7.0 to give final concentration of buffer to 100 µmoles. The assay mixture (1.1 ml) was incubated at 37°C for 10 min. Reaction was stopped by the addition of 1.0 ml of 1M Na_2CO_3 solution and then tubes were centrifuged at 5000 rpm for 10 min. The supernatant was read at 420 nm for measurement of liberated paranitrophenol. Controls included reaction mixtures to which boiled extracts (culture) had been added.

Sucrose hydrolase (invertase) activity during growth was assayed according to Gascon and Lampen (1968) by measuring liberated glucose from sucrose. Cells to be assayed for this enzyme (1.0 ml) were washed thrice with 0.05 M sodium phosphate buffer, pH 7.0 using micro-centrifuge and suspended in 1.0 ml of the same buffer. The cells were then toluneized for 15 min at room temperature. The enzyme reaction was started by the addition of 10 µmoles of substrate (0.5 ml of 0.02M sucrose) and 100 µmoles of buffer and it was allowed to react for 30 min at 37°. Reaction was terminated by the addition of 0.5 ml of 0.25 N $Ba(OH)_2$ and 0.5 ml of 0.25N $ZnSo_4$ solution. The precipitates were centrifuged at 5000 rpm for 15 min and the supernatant was estimated for glucose liberated by the method of glucose oxidase.

Inhibitor studies :

In order to study the effect of rifampicin on induction of α-galactosidase, first the concentration of rifampicin was standardized for inhibition of only mRNA synthesis and not protein synthesis in Bacillus Sp. I during the growth in raffinose broth. Synthesis of RNA (mRNA) was monitored by incorporation of (^3H) uridine, whereas synthesis of proteins was monitored by the incorporation of (^{14}C) leucine.

Cells were grown in basal medium containing 0.5% raffinose at 37° on a rotary shaker (250 rev/min) till log period ($A_{660} \simeq 0.8$). The culture was then centrifuged in sterile cups at 8000 xg for 10 min and suspended in 2.0 ml normal saline. These cells were used to inoculate 100 ml raffinose broth in 250 ml Erlenmeyer flasks to which 50 µci of (^{14}C) leucine (sp. activity = 52.4 mCi/mmole) and 100 µci (^{3}H) uridine (sp. activity = 2.7 mCi/mmole) were added previously. The culture was then distributed in sterile culture tubes containing different amount of rifampicin (10-200 µg/ml) and allowed to grow at 32° in shaker water-bath. From these tubes at different time intervals, 0.5 ml of sample was removed and to this was added 0.5 ml of cold 10% TCA and kept for 1 hr at -4°, the precipitate was collected on Whatman filter paper and washed twice with 10 ml of cold TCA. The filters were then dried and placed in scintillation vials with scintillation fluid (composition is given in Table-33). Radioactivity was measured using LKB Rack Beta Liquid Scintillation Counter.

For studying the effect of rifampicin during induction, the cells were allowed to grow in 200 ml raffinose broth for the induction of α-galactosidase. During the induction, at different time intervals 3.0 ml culture was withdrawn for assay of enzyme and measurement of growth. At the same time 25 ml culture was removed for the addition of rifampicin in 100 ml conical flask to which was added 50 µg/ml rifampicin. Cultures containing rifampicin were

also allowed to grow further and at every 30 min time intervals, 3.0 ml culture was removed for growth and enzyme assays. Measurement of growth and assay of enzymes viz. ∝-galactosidase and invertase were carried out as described earlier.

Purification of ∝-galactosidase and invertase :

Cell free extracts of Bacillus Sp. I (isolated from stored soybean grains) having inducible ∝-galactosidase served as the source of both the enzymes.

1) Preparation of cell free extract

The bacteria were grown in 200 ml raffinose broth in 500 ml conical flasks with aeration at room temperature for overnight (total 5 litre of medium was used). The cells were harvested at early stationary phase of growth by centrifugation at 8000 xg for 15 min at 0-4°. 35 g of cells (wet weight) were washed with four volumes of 50 mM sodium phosphate buffer, pH 7.0 containing 1 mM mercaptoethanol. After recentrifugation, cells were frozen at -20°. All the steps of purification were carried out at 5-10° unless and until specified. Preliminary investigations showed that both the enzymes required -SH group for their activity, hence 1 mM mercaptoethanol was added in all the buffer solutions used during purification steps.

At the time of purification the cells were thawed and resuspended in 4 volumes of 50 mM sodium phosphate buffer pH 7.0 and then disintegrated by using Branson B-12 Sonifier at 20 KH_z by giving 20 pulses of one minute each in cold condition. (The glass container containing sample was surrounded by ice-salt mixture during ultrasonication). The sonicated homogenate was made upto volume 115 ml and then centrifuged at 20,000 xg for 60 min at 0-5°. Since both the enzymes were cytoplasmic, supernatant was used for purification of enzymes and the pellet was discarded.

The complete lysis of the cells during sonication were checked by reading change in absorbance of the homogenate at 260 nm and 280 nm in a Beckman spectrophotometer. To the supernatant (115 ml) 5% streptomycin sulfate solution was added (final concentration to 0.7%) for removal of nucleic acids. This solution was kept on ice for 1 h in stirring condition, centrifuged at 15,000 xg for 30 min. Precipitated nucleic acids were discarded and the supernatant (125 ml) was submitted to fractionation by ammonium sulfate precipitation. Removal of nucleic acids was checked by reading the supernatant at 260 and 280 nm, before and after precipitation.

To the supernatant (125 ml) solid ammonium sulfate was added with continuous stirring to give 0-50% saturation. After the addition of ammonium sulfate, the solution was kept at 1-2° for 2 h, and centrifuged at 15,000 xg for

30 min at 0-4°. The precipitates were dissolved in 10 ml of 50 mM sodium phosphate buffer, pH 7.0. Since the dissolved precipitate did not show the activity of ∝-galactosidase and invertase the supernatant was used for further precipitation. It was brought to 50-70% saturation with solid ammonium sulfate addition and kept for precipitation for 6 hr at 2-4°. The suspension was centrifuged and the precipitates were dissolved in 5.0 ml of 50 mM sodium phosphate buffer, pH 7.0. These precipitates showed 90% of the total activity for both the enzymes, hence the supernatant was discarded. 50-70% fraction containing maximum activities of both the enzymes was dialyzed against 2 litres of 50 mM sodium phosphate buffer, pH 7.0 for 12 h at 4° with frequent changes of buffer to remove the salt.

Fractionation by gel filtration on Sephadex G-200 :

The dialysed extract was applied to a Sephadex G-200 column of bed dimensions 66 x 2.5 cm, previously equilibrated with the same 50 mM sodium phosphate buffer, pH 7.0. Proteins were eluted with the same buffer at a flow rate of 8 ml/h. Fractions (4 ml each) containing ∝-galactosidase and those which contained invertase were pooled separately. Both the enzymes were concentrated by dialysing against 50% polyethylene glycol in buffer to 2.0 ml.

Fractionation by ion-exchange chromatography :

Both the enzymes were further purified by ion exchange chromatography using DEAE cellulose hydroxyl-apatite and DAE sephadex columns. All the 3 resins were previously equilibrated in 0.1M sodium phosphate buffer, pH 7.0 and then columns were packed at 4-8°. All the three columns were washed with 4-5 column volumes of 50 mM sodium phosphate buffer, pH 7.0.

Concentrated enzymes were applied separately to DEAE cellulose columns (Whatman DE-52) (25 x 2.5 cm) and columns were flushed with 200 ml of 0.05M sodium phosphate buffer, pH 7.0. Since both the enzymes were adsorbed on the columns, they were eluted with a linear gradient of 0.1 to 1 M NaCl. 5 ml each fractions were collected at the rate of 50 ml/h. The active fractions were pooled and dialysed against 2 lit. buffer for 6 hr at 4°. For dialysis 50 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM mercaptoethanol was used.

The 20 ml extracts containing 198 units of α-galactosidase and 110 units of invertase were adsorbed on hydroxyl apatite columns (25 x 1.8 cm). Proteins were eluted by batch elution using 40 ml each 0.1 to 0.6 M sodium phosphate buffer, pH 7.0. 4.0 ml fractions were collected at a flow rate of 30 ml/h and active fractions

were pooled, dialyzed against buffer. These pooled fractions were then loaded on second DEAE cellulose column (25 x 1.8 cm) for concentrating the enzymes. First, columns were washed with 100 ml 0.1M NaCl solution (prepared in buffer) and then enzymes were eluted by washing the columns with 100 ml of 1M NaCl. The active fractions of both the enzymes were then dialysed against 2 lit of buffer for 6 h at 4°. ∝-galactosidase was further purified by using QAE sephadex. Dialyzed fraction was loaded on QAE Sephadex column (20 x 2.5 cm), and the column was flushed with 200 ml of buffer. The column was eluted with a linear gradient of 0.1 to 0.6 M NaCl at a flow rate of 60 ml/h by collecting 4 ml fractions. Active fractions were pooled and dialysed.

Both the purified enzymes were dialysed against 50% glycerol prepared in 50 mM sodium phosphate buffer, pH 7.0 containing 1 mM mercaptoethanol for overnight at 4°. for reduction of the volume of enzyme as well as for better stability.

Polyacrylamide disc gel electrophoresis :

Polyacrylamide disc gel electrophoresis (PAGE) was carried out to check the purity of ∝-galactosidase and invertase. Simple PAGE and SDS-PAGE were carried out according to Weber et al. (1972), using 7.5% gels. For simple PAGE purified enzymes, 20-100 µg proteins were loaded on gels directly. For SDS-PAGE, the enzymes were

denatured by incubation for 1 hr at 30° in 0.01M phosphate buffer, pH 7.0,containing 0.1% of 2-mercaptoethanol and 6M urea. Samples were also denatured by boiling with equal volume of 0.01M phosphate buffer containing 0.1% mercapto-ethanol and SDS for 3 minutes. Electrophoresis was performed at 5 MA/tube for 5-6 h. After electrophoresis, the gels were stained for 4-5 h with staining reagent, and then destained with destaining reagent for 24 h with frequent changes of the destaining reagent. (Composition of staining reagent and destaining reagent are given in Table-33).

The activity band of α-galactosidase was detected on gel by running the gel in simple PAGE at 8° and then staining with 0.005M α-PNPG. The gels were also stained with periodic acid-fuchsin reagent (Zacharius et al, 1969) for detecting the presence or absence of glycoprotein nature of enzymes.

Molecular weight determination of purified enzymes :

The molecular weights of both the enzymes were determined by analytical gel filtration using the method of Andrews (1964) as well as SDS-PAGE method. Molecular weight by gel-filtration was determined using Sepharose-6B and Sephadex G-200 columns.

Sepharose-6B (pharmacia) column (1.5 x 48 cm) equilibrated with 0.05M sodium phosphate buffer, pH 7.0 was

used for determination of molecular weight by gel filtration. The column was calibrated with known molecular weight makers viz. bovine serum albumin (68,000), ovalbumin (45,000), cytochrome-C from horse heart (13,000), alcohol dehydrogenase from yeast (1,50,000), ferritin (4,00,000) and blue dextran (20,00,000). Elution of the proteins was carried out with the equilibration buffer at a flow rate of 20 ml/h and 2.0 ml fractions were collected. Blue dextran was detected spectrophotometrically by measuring absorption at 660 nm; BSA, ferritin, ovalbumin, alcohol dehydrogenase, [illegible] at 280 nm and cytochrome-C at 412 nm. Alkaline phosphatase was detected by assay for its enzyme activity.

20 ug of purified α-galactosidase and 100 µg of purified invertase were loaded to the calibrated column. Elution was carried out with equilibration buffer at a rate of 20 ml/h and 2 ml fractions collected. The void volume of the column (Vo) was measured with respect to elution volume of Blue dextran. The elution volume (Ve) of proteins of known molecular weights were found out and the ratio (Ve/Vo) was plotted against the log molecular weight of corresponding protein. From the standard graph, the molecular weights of samples were calculated.

Sephadex G-200 column (2.3 x 70 cm) was equilibrated with 0.05M sodium phosphate buffer, pH 7.0, and calibrated

with the known molecular weight markers as described above. Purified enzymes were also loaded onto the column. All the proteins were eluted with the equilibration buffer at a flow rate of 8 ml/h (4.0 ml fractions each). Molecular weights of the two enzymes viz. α-galactosidase and invertase were determined by using calibration curve as described above.

For determination of molecular weights of enzymes by SDS-PAGE, known markers were used as molecular weight standards. 10-100 µg samples as well as protein markers were loaded onto gels in the tubes after mixing with the loading buffer and electrophoresis was carried out as described above.

ODAP hydrolysing enzyme system of bacteria :

Preparation of cell-free extract from ODAP hydrolysing bacteria

50×10^6 cells were inoculated in 100 ml ODAP broth, containing 0.4% ODAP and incubated at 30° for 24 h. After the growth, the cells were harvested by centrifuging at 8000 xg for 10 min at 4°C. The supernatant viz. culture filtrate was brought to pH 7.0 using 0.05N HCl and used to check the enzyme activity. For preparation of cell free extract, the cells were washed with 50 mM potassium phosphate buffer, pH 8.0, recentrifuged and suspended in a 10 ml buffer in glass beaker surrounded by ice-salt mixture, and sonicated

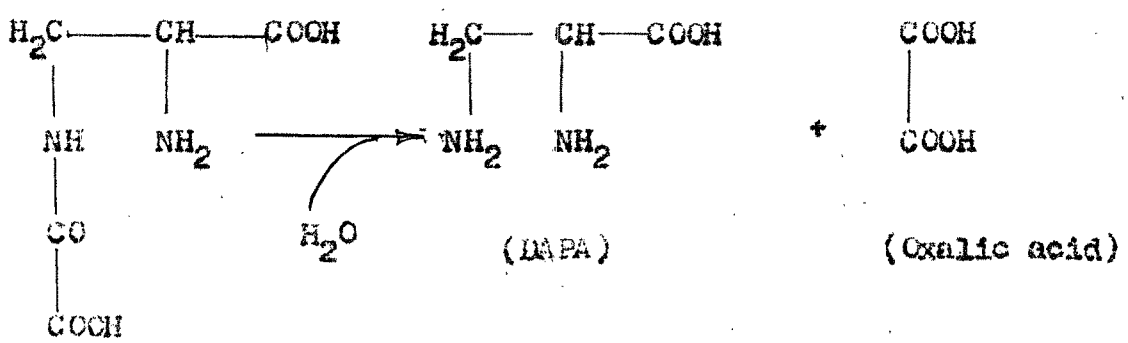
in Branson sonifier at 20 KH_z by giving 20 pulses of one minute each in cold condition. This homogenate was used as an enzyme source.

For the preliminary investigations on the presence of ODAP hydrolysing enzymes in bacteria (which could degrade ODAP), the enzyme activities were measured in terms of disappearance of the substrate viz. ODAP. The assay system consisted of 20 µmoles of ODAP, 50 umoles of potassium phosphate buffer, pH 8.0, 0.5 to 1.0 ml of crude enzyme preparation (either cell free extract or culture filtrate) and water to a final volume of 2.0 ml. After incubation for 1 h, 0.5 ml of 10% TCA was added to inhibit the reaction and centrifuged for 20 min in a clinical centrifuge. The supernatant was used for the estimation of ODAP by the method of Rao (1978b). Blank was same as that of experimental except that TCA was added before the incubation. The enzyme units were defined as µmoles of ODAP disappeared per minute under assay conditions.

Measurement of activities of ODAP hydrolase and DAPA-ammonia lyase of Streptococcus Sp. I :

When crude extract of enzyme preparation from Streptococcus Sp. I (cell free extract) was incubated with ODAP as a substrate, diaminopropionic acid (DAPA) was detected during the reaction. During preliminary studies on the growth of Streptococcus Sp. I, DAPA was detected as an

intermediate during the breakdown of ODAP. So it was assumed that ODAP gets degraded enzymatically first into DAPA and oxalic acid. This can be a 'hydrolase' reaction as follows :



(ODAP)

Diaminopropionic acid liberated by enzymatic hydrolysis of ODAP can be further cleaved into pyruvic acid and ammonia. This assumption was made on the basis of DAPA-ammonia lyase reported from Pseudomonas Sp. (Vijayalakshmi et al, 1975). Therefore, considering all the above stated facts, the first enzyme of ODAP breakdown pathway is reported here as ODAP hydrolase whose units are defined as micromoles of pyruvate liberated per minute per assay system when the extract was incubated with ODAP as a substrate. ODAP hydrolase was also assayed for oxalate liberated as a product, using oxalyl labelled ODAP as a substrate. Second enzyme of the pathway, DAPA ammonia lyase was assayed for pyruvate as a product using DAPA as a substrate. The enzymatic reactions are as follows :-

Diaminopropionic acid (DAPA) + H_2O ⟶ Pyruvate + $2NH_3$ (1)

Pyruvate + NADH + H^+ ⟷ Lactate + NAD^+ (2)

Lactate dehydrogenase

Separation of oxalyl labelled ODAP and C^{14}-oxalic acid by solvent extraction method :

The method of measuring activity of ODAP hydrolase is standardized for the first time using labelled ODAP. The crude cell-free extract was incubated with C-^{14} oxalyl labelled ODAP as a substrate and the enzyme activity was measured in terms of labelled oxalate liberated. The assay system consisted of 20 umoles of labelled ODAP, 50 umoles of potassium phosphate buffer, pH 8.0, 0.1 to 1.0 ml of enzyme (crude cell free extract) and water to a final volume of 2.0 ml. After the incubation for 1 h at 37°C, 1 ml of 4.5N NaOH was added to inhibit the enzyme reaction. The pH of the assay mixture was then made to 1.0 by addition of 0.5 ml of 12 N HCl. After the enzyme reaction was terminated, it was necessary to separate labelled oxalate (product) from labelled ODAP. So a simple solvent extraction method was standardized for this purpose. The basic principle involved is extraction of organic acid (oxalic acid) in solvent ether at pH 1.0. When the method

When the method was standardized with known amount of labelled ODAP and labelled oxalic acid + cold oxalic acid, it was found that the extraction of oxalic acid was highest at pH 1.0, when carrier 100 mg of oxalic acid was present along with labelled oxalic acid. Accordingly after the termination of enzyme reaction, to each tube 100 mg cold oxalic acid was added before the extraction with ether. To each tube 2.0 ml of solvent ether was added, components were mixed by vortexing and allowed to stand at room temperature for 5 minutes for separation of two layers. The upper ether layer containing labelled oxalic acid (product formed from labelled ODAP), was pipetted out in a scintillation vial with a pasteur pipette. This procedure was repeated 5-7 times for complete extraction of oxalic acid. The ether collected in a vial was then evaporated at 50° in water bath to dryness. Residue of oxalic acid was dissolved in 0.5 ml distilled water and 10 ml of scintillation fluid was added. The extracted labelled oxalate was measured by counting the radioactivity in Beckman liquid scintillation counter (LS-7000). The assay systems for the measurement of activity of ODAP hydrolysing enzymes viz. ODAP hydrolase and DAPA ammonia lyase are given in Table-64.

TABLE-64 : Details of the assay system for ODAP hydrolysing enzymes.

	ODAP hydrolase	DAPA-ammonia lyase
Basis of the method used	—	Vijayalakshmi et al. (1975).
Substrate	ODAP, 20 µmoles.	DAPA, 20 µmoles.
Buffer	Potassium phosphate buffer, pH 8.0, 100 µmoles.	Potassium phosphate buffer, pH 8.0, 100 µmoles.
Cofactor added	0.1 µmole of pyridoxal phosphate.	0.1 µmoles of pyridoxal phosphate.
Enzyme concentration	500 µg protein (crude enzyme)	200 µg protein (crude enzyme)
Final volume	2.0 ml	2.0 ml.
Temperature and period of incubation	37°, 45 min.	37°, 10 min.
Start of reaction	Enzyme added.	Enzyme added.
Termination of reaction	0.5 ml of 10% TCA added	0.5 ml of 10% TCA added.
Treatment of blank	TCA added before incubation.	TCA added before incubation.
Parameters measured	Liberated pyruvate estimated by LDH method (Gloster and Haris, 1962), or left over ODAP estimated by the method of Rao (1978).	Liberated pyruvate estimated by LDH method.
Enzyme unit	The amount of enzyme required to liberate 1 µmole of pyruvate per minute under assay conditions or the amount of enzyme required to breakdown 1 µmole of ODAP under assay conditions.	The amount of enzyme required to liberate 1 µmole of pyruvate per minute under assay conditions

TABLE-65 : List of reagents used for experiments on "Genetic basis of degradation of neurotoxin and raffinose family of oligosaccharides in bacteria".

All glasswares and plasticwares used for these experiments were sterilized by autoclaving. Whenever possible, the reagents listed below were also sterilized by autoclaving. All antibiotics solutions were sterilized by filtration.

Reagent	Method of preparation
Acridine orange/ acriflavine solution	Stock solutions were prepared in distilled water at concentration of 5 mg/ml.
Ampicillin solution	Stock solution of sodium salt of ampicillin was prepared in distilled water at concentration of 25 mg/ml.
Chloramphenicol solution	Stock solution was prepared in 100% ethanol at concentration of 34 mg/ml.
Chloroform:Isoamyl alcohol (24:1) mixture	A mixture of chloroform and Isoamyl alcohol (24:1 v/v) was used to remove proteins from DNA preparations.
EDTA, 0.2M, pH 8.0	74.4 g of disodium EDTA $2H_2O$ was dissolved in 800 ml of distilled water using magnetic stirrer, then pH was adjusted to 8.0 with 1 N NaOH and the volume was made upto 1 litre distilled water. It was sterilized by autoclaving at 15 psi for 15 min before use.

TABLE-65 (Contd....)

Reagent	Method of preparation
0.25M EDTA - 50 mM Tris (pH 8.0)	50 ml of 0.5M EDTA (pH 8.0) was mixed with 50 ml of 100 mM Tris-HCl buffer (pH 8.0).
Ethidium bromide solution (10 mg/ml)	1 g of ethidium bromide was added in 100 ml of distilled water and stirred using magnetic stirrer for several hours to ensure complete dissolution. The container was wrapped in aluminium foil and stored at 4°C.
Gel loading buffer for agarose gel electrophoresis	0.25 g of bromophenol blue and 40 g of sucrose dissolved in 100 ml of dissolved water and stored at 4°C.
Ligase buffer, 10X, for T_4 DNA ligase	0.5 ml of 2M Tris (pH 7.4), 0.2 ml of 1M $MgCl_2$, 0.4 ml of 1M DTT, 0.2 ml of 100 mM ATP (pH 7.0 with NaOH) and 100 ug BSA were mixed together and volume made upto 2.0 ml with distilled water.
Mitomycin-C solution	1.0 µg/ml of solution was prepared in 10% dimethyl sulfoxide solution.
Novobiocin solution	Stock solution was prepared in distilled water at concentration of 10 mg/ml.
NaCl solution, 5M	292.2g of NaCl was dissolved in 800 ml of distilled water. The volume was adjusted to 1 litre and sterilized by autoclaving at 15 psi for 15 min.

TABLE-65 (Contd..)

Reagent	Method of preparation
Phenol saturated with buffer	Crystalline phenol was redistilled at 160° to remove contaminants and stored below 0°. Whenever needed, it was melted at 68°. The melted phenol was extracted several times with an equal volume of buffer (1.0 M Tris, pH 8.0) followed by 0.1M Tris, pH 8.0, until the pH of the aqueous phase was more than 7.6. The phenol solution saturated with buffer stored at 4°.
Pronase solution	Dissolved pronase at a concentration of 20 mg/ml in distilled water and stored as stock solution at -20°. Working standard was made by diluting stock solution in 10 mM Tris HCl (pH 7.8) buffer at a concentration of 1 mg/ml before use.
Restriction enzyme buffer (medium), 10X	This medium salt buffer was prepared by mixing 100 mM Tris (pH 7.4), 100 mM $MgCl_2$, 10 mM DTT and 500 mM NaCl.
Restriction enzyme buffer (high), 10X	High salt buffer was prepared by mixing 100 mM Tris (pH 7.4), 100 mM $MgCl_2$, 10 mM DTT and 1M NaCl.
RNase solution	Dissolved pancreatic RNase (RNase A) at a concentration of 10 mg/ml in 10 mM Tris HCl (pH 7.5) and 15 mM NaCl. Heated to 100°C for 15 minutes (to make it free from DNase) and

TABLE-65 (Contd...)

Reagent	Method of preparation
	allowed to cool slowly to room temperature. Dispensed into aliquots and stored at -20°.
Spheroplast medium	10% sucrose solution was made in 50 mM Tris (pH 8.0).
STET buffer	A litre of solution was made containing 0.1M NaCl; 10 mM Tris (pH 8.0); 0.1 mM EDTA and 0.5% Triton X-100.
SDS, 10% solution	100g of SDS was dissolved in 900 ml of distilled water and heated at 68° to enhance dissolution. The pH of the solution was adjusted to 7.2 with conc. HCl and then volume made upto 1 litre.
Bacterial strains used for cloning experiments	1) *E. coli* strain C-600, harbouring pBR-322. 2) Wild type *E. coli* - C-600. F^-, thi^{-1}, thr^-, Leu B6, lae Y_1, ton A21, Sup E44 , Rk^-, Mk^-. (The strains were obtained from Biochemistry Division, BARC, Bombay).

Preparation of plasmid DNA from bacterial isolates as well as E. Coli strains :

It was necessary to screen all the major bacterial isolates for the presence of plasmid DNA for further genetic experiments. Leuconostic mesenteroides, Bacillus Sp. I, Bacillus Sp. II and Streptococcus sp.1 were used for screening of plasmids. Standard E. Coli K-12 strain containing a plasmid pBR-322 was also used for comparison and further restriction analysis.

Different methods have been used to isolate plasmid DNA. All of them involve three basic steps ; growth of bacteria and amplification of plasmids, harvesting and lysis of the bacteria and purification of the plasmid DNA. These purification procedures exploit in one way or another the two major differences between the chromosomal DNA and plasmid DNA :

1) The chromosomal DNA is much larger than plasmid DNA.
2) The bulk of chromosomal DNA from the cell is obtained as broken, linear molecules, whereas plasmid DNA is extracted in a covalently closed, circular form. Most of the purification protocols therefore involve a differential precipitation step in which long strands of chromosomal DNA, entangled in the remnants of lysed cells, are preferentially removed.

Each of the complementary strands of plasmid DNA is covalently closed circle, therefore, the strands cannot be separated by conditions such as heating or exposure to mild alkali (upto pH 12.5), which break most of the hydrogen bands in DNA. Closed circular molecules regain their native configuretion when cooled or returned to neutral pH, whereas chromosomal DNA generally remains in the denatured state.

Growth of E. Coli containing pBR-322 and amplification of the plasmid :

Amplification in rich medium

Plasmid pBR-322 undergoes relaxed mode of replication in E. Coli K-12 harbouring them, hence the amplification can be carried out if chloramphenicol is added during the log phase. For amplification of these plasmids following protocol was followed (Maniatis et al. 1982).

1) 10 ml of LB medium containing appropriate concentration of ampicillin (25 µg/ml) and tetracycline (12 µg/ml) was inoculated with a single colony of E. Coli K-12 harbouring pBR-322. This was then incubated at 37° overnight with vigorous shaking.

2) Next day morning, 25 ml of LB medium in 100 ml flask containing antibiotics was inoculated with 0.1 ml of overnight culture and incubated at 37° until the culture

reached late log phase ($O.D._{660nm}$ ≃ 0.6) with vigorous shaking.

3) 25 ml of the late log culture was inoculated into 500 ml of LB medium prewarmed at 37° with the appropriate antibiotic in 2 litre flask. The flask was then incubated for 2.5 to 3.0 h, with vigorous shaking. To this flask 2.5 ml of a solution of chloramphenicol (34 mg/ml in ethanol) when the $O.D._{660nm}$ became approximately 0.4-0.5.

4) The cells were incubated at 37° with vigorous shaking for further 12-16 h.

Amplification in minimal medium :

1) A single colony grown in the presence of appropriate antibiotics, was inoculated into 10 ml of LB medium containing antibiotics and grown overnight at 37° by vigorous shaking.

2) 2.5 ml of overnight culture was inoculated into a 2 litre flask containing 500 ml minimal medium (M_9) with requirements of E. Coli C-600 strain (composition of the medium is shown in Table-66 and 67) containing 1.0% glucose and appropriate antibiotics. This was incubated at 37° with vigorous shaking until the culture reached an O.D. at 660 nm of 0.4-0.5.

TABLE-56 : Composition of Mq (minimal) medium.
(Maniatis et al, 1982)

Ingredients	Amount (g/1000 ml distilled water)
Na_2HPO_4	6.0
KH_2PO_4	3.0
NaCl	0.5
NH_4 Cl	1.0
pH - 7.0	

Ingredients mentioned above were mixed together autoclaved and cooled. To this medium, following solutions (separately sterilized) were added :

1M $MgSO_4$	-	2.0 ml
1M $CaCl_2$	-	0.1 ml

TABLE-67 : Composition of medium for the cultivation of E. coli $K_{12} - C_{600}$.

Ingredients	Amount
Mg medium	1000 ml
Glucose	5.0 g
Threonine	50.0 mg
Leucine	50.0 mg
Thiamine	5.0 mg

Stock solutions of glucose, threonine, leucine and thiamine were prepared, sterilized separately and then added to Mg medium to give appropriate final concentrations.

Note:- For selection of 'Raf^+' transformants during the cloning experiment, 0.5% raffinose was used as carbon source instead of glucose.

Appropriate type of antibiotic viz. ampicillin (25 µg/ml medium) or tetracycline (12 µg/ml medium) were added to the medium whenever required.

3) 2.5 ml of solution of chloramphenical (34 mg/ml in ethanol) was added and incubation was continued for further 12-16 hours.

Large scale isolation of plasmid DNA by SDS-lysis method :

(Maniatis et al, 1982)

Plasmid DNA preparation from Bacillus Sp. I, Bacillus Sp. II and E. Coli C-600 containing pBR-322 were carried out using the method described as follows :

a) Growth of bacteria and harvesting

1) Bacillus Sp.I capable of degrading raffinose and Bacillus Sp. IIcapable of degrading neurotoxin were grown in 500 ml each of raffinose broth and ODAP broth respectively till late log period (as mentioned earlier) for preparation of plasmid DNA. E. Coli K_{12} (C-600) harbouring pBR-322 was grown in rich medium as well as minimal medium as mentioned in amplification protocol.

2) All the bacterial cells were harvested by centrifugation at 8000 xg for 10 min at 4°. The supernatant was discarded.

3) The cells (each collected separately) were washed in 100 ml ice-cold spheroplast medium. (Composition given in Table. 65).

b) Lysis :

1) The washed cells were resuspended in 10 ml ice-cold spheroplast medium (in a transperant centrifuge cup of 100 ml capacity).

2) 2.5 ml of lysozyme (freshly made 10 mg/ml in 10 mM Tris, 1 mM EDTA) and 2.5 ml of 0.2M EDTA pH 8.0 were added to each tube. The tubes were shaken gently and incubated on ice for 15 min and at room temperature for 15-20 min.

3) 4.0 ml of 10% SDS was added to each tube and the solution were shaken immediately to disperse the SDS evenly through suspension, care was taken not to shear liberated DNA. Complete lysis of the cells were checked by seeing the clearing of the suspension.

4) 4.0 ml of 5M NaCl was added to each tube and mixed immediately by inverting the tubes gently. The tubes were kept immediately on ice for overnight incubation.

c) Purification of plasmid DNA

Removal of proteins from DNA preparation can be carried out by extracting aqueous solution of DNA with phenol and/or chloroform. The standard way is to extract once with phenol, once with a 24:1 mixture of chloroform and isoamyl alcohol. This procedure takes advantage of the fact that deproteinization is more

efficient, when two different organic solvents are used, instead of one. Furthermore, although phenol denatures proteins efficiently, it does not completely inhibit RNase activity and it is a solvent for RNA molecules that contain long tracts of poly C:A (Brawerman et al, 1972). Both of these problems can be circumvented by using chloroform in addition to phenol. Also chloroform removes any lingering traces of phenol from the DNA preparation.

1) The supernatant obtained after salt precipitation was mixed with an equal volume of phenol saturated with TE buffer in polypropylene tube with a cap.

2) The contents of the tube were mixed until an emulsion formed.

3) The sample was centrifuged for 10 min at 5000 xg and aqueous phase was transferred to a glass centrifuge tube with pasteur pipette.

4) An equal volume of chloroform : isoamyl alcohol mixture (24:1) was added to the aqueous phase and steps (2), (3) and (4) were repeated.

d) Concentration of DNA

1) The volume of aqueous phase obtained after the second centrifugation in step C-(4) was measured.

2) Two volumes of ice-cold distilled ethanol (exactly) was added to the solution, mixed well and chilled.

3) The sample was stored, overnight at -20° to allow DNA to precipitate. Next day, it was centrifuged at 0-4° for 30 min at 25,000 xg.

4) The supernatant was discarded and the tubes were kept in a vacuum dessicator for drying precipitates of DNA.

5) The DNA pellet was dissolved in the desired volume of TE (Tris 10 mM, EDTA 1 mM) buffer.

e) Treatment with RNase

1) The volume of the DNA solution in TE was measured and to that DNase free RNase (Table-65) was added to a final concentration of 10 µg/ml. Tubes were incubated at 37° for 1 h.

2) Suspension was treated with phenol and chloroform:iso-amyl alcohol mixture (24:1) as described in Step (c) for removal of RNase and other proteins.

3) To the aqueous phase was added double the volume of distilled alcohol and kept for DNA precipitation at -20° for 24 h.

4) Next day DNA precipitates were dissolved in desired amount of TE.

Isolation of plasmid DNA from Streptococcus Sp. I and Leuconostoc species by alkaline lysis method :

(Birnvoin and Doly, 1979)

1) The bacterial pellet from the 500 ml culture was resuspended in 16 ml of 50 mM Tris and 20 mM EDTA. 1 ml of freshly made lysozyme (10 mg/ml) was carefully added and incubated at 30° for 20 min followed by 2 ml of pronase (5 mg/ml) and the sample was kept at 37° for 1 hour.

2) 2 ml of 10% SDS was now added and the suspension kept at 37° for one hour.

3) 0.5 ml of 3M NaOH was added. The solution was mixed well and kept at room temperature for 15 minutes (pH of solution was made to 12.5).

4) Now, 1.5 ml of 2M Tris (pH 7.0) was added to bring the pH of the solution to 8.0. It was allowed to stand for 15 mins at room temperature.

5) 5.2 ml of 5M NaCl (final concentration = 1 M) was added and the solution was kept overnight at 4°C.

6) Next day the sample was centrifuged to remove high molecular weight DNA and bacterial debris at 12,000 xg for 30 min.

7) The supernatant was treated with 30% PEG (in distilled water) so as to give a final concentration of 10% PEG.

8) The solution was allowed to stand for 8 hours at 4°.

9) It was then spinned down at 20,000 xg for 15 minutes, which yields DNA pellet.

10) The DNA pellet was dissolved in about 200 µl 50 mM Tris ~20 mM EDTA.

Rapid isolation of plasmid DNA from drug treated colonies:

(Roberts et al, 1986)

1) 2 ml volume of LB medium was inoculated with each single colony and grown to late log phase i.e. for 18 hours.

2) A 1.5 ml of this culture was transferred to an Eppendorff tube, and the cells were harvested by centrifugation.

3) The pelleted cells were resuspended by vortexing in 400 µl of Tris-EDTA-Sucrose buffer (50 mM Tris, 1 mM EDTA, 6.7% Sucrose) pH 8.0 and suspension warmed at 37°.

4) 100 µl of lysozyme solution (20 mg/ml) in 25 mM Tris, pH 8.0 was added.

5) This was mixed by inversion and incubated at 37° for 5 to 10 mins.

6) 100 µl of 0.25M EDTA - 50 mM Tris (pH 8.0) and 60 µl of 20% SDS (in 50 mM Tris, 20 mM EDTA) (pH 8.0) were added and mixed by inversion and the mixture was incubated at 37° for 10-15 mins.

7) 28 µl of fresh 3.0M NaOH was added and again mixed by inversion.

8) Mixture was allowed to stand at room temperature for 10 mins.

9) Then 10 µl of 2.0 M Tris hydrochloride (pH 7.0) was added and mixed by inversion and this was allowed to stand at room temperature for 3 mins.

10) 75 µl volume of 5.0M NaCl was added and mixed by inversion. Mixture allowed to stand at 4° for 90 mins or more.

11) This mixture was centrifuged for 5 mins and the pellet which contained the bulk chromosome DNA was discarded.

12) Supernatant was treated with 2 µl of pancreatic RNase (10 mg/ml, boiled for 2 mins, before use) and allowed to stand at room temperature for 15 mins or more.

13) To the RNase treated solution was added 600 µl of phenol saturated with 3% NaCl and 100 µl of chloroform-isoamyl alcohol (24:1). This was mixed thoroughly by vortexing and centrifuged for 2 min.

14) The upper aqueous phase was removed and precipitated with double volume of distilled alcohol, incubated at -20° for 10 mins and centrifuged at 4° for 5 mins.

15) The supernatant was discarded and the tube was inverted to allow the DNA pellet to drain. The pellet was dried under vacuum for 15 mins to remove alcohol and then taken up in 20 µl of TE buffer for agarose gel electrophoresis.

Purification of DNA by centrifugation to equilibrium in caesium-chloride-ethidium bromide gradients :

(Maniatis et al, 1982)

1) The volume of DNA solution was measured and for every millilitre, 1 g of caesium chloride (solid) was added and gently mixed till all the salt dissolved.

2) 0.8 ml of ethidium bromide solution (10 mg/ml in H_2O) was added for every 10 ml of caesium chloride solution and mixed well, with final density being 1.55 g/ml (n = 1.3860) and concentration of ethidium bromide being approximately 600 µg/ml.

3) The caesium chloride solution was transferred to a tube suitable for centrifugation. This was then centrifuged in analytical ultracentrifuge using swinging bucket rotor, at 40,000 rpm for 36 hrs.

4) The upper band consisting of linear bacterial chromosomal DNA as well as nicked circular plasmid DNA were observed under U.V. light.

5) Plasmid DNA was purified by collecting the lower band of DNA into a sterile glass tube through sterile P-21 hypodermic needle inserted into the side of the tube.

6) Removal of ethidium bromide

a) Equal volume of 1-butanol saturated with water or isoamyl alcohol was added and mixed by pipetting vigorously.

b) This was then centrifuged at 1500g for 3 min.

c) The lower aqueous phase was transferred into a clean glass tube.

d) Extraction was repeated 4-6 times till all the pink colour disappeared from the aqueous phase.

7) The aqueous phase was dialysed against several changes of TE (pH 8.0).

Preparation of chromosomal DNA from Bacillus Sp. I :

(Maniatis et al, 1982)

Cells were harvested by growing in raffinose broth as mentioned in plasmid DNA preparation. Rest of the procedure was as follows :-

1) 1.0g cells were washed with TE buffer and suspended in 10 ml ice-cold spheroplast medium.

2) 1.0 ml lysozyme solution (prepared freshly) was added to give final concentration of 200 µg/ml. 2.5 ml of 0.2 M EDTA pH 8.0 was added immediately, and incubated at 37° for 30 min and suspension was transferred to 250 ml flask.

3) 6.0 ml 10% SDS was added and mixed gently. Lysis of the cells was carried out by heating at 60°C for 10-15 min.

4) Volume of the suspension was measured and treated twice with equal amount of saturated phenol solution.

5) The aqueous layer was treated with equal volume of chloroform + isoamyl alcohol (24:1) twice and the aqueous layer was removed.

6) The volume of the aqueous layer was measured and placed in a sterile beaker, to this two volumes of chilled distilled alcohol was slowly added without mixing the layers. Threads of DNA were separated by swirling around a sterile glass rod.

7) DNA was then separated and kept in a tube. This was then dried in vacuum desicator and dissolved in desired amount of TE buffer. RNase and pronase treatments were given to the preparation before restriction analysis.

Agarose gel electrophoresis :

The method of Sharp et al. (1973) was used for separation, identification and confirmation of purity of DNA preparations using slab gel horizontal agarose electrophoresis.

Horizontal mini slab gels (0.5 x 6 x 9 cm) of 0.7 or 1% (w/v) agarose in TEA (40 mM Tris-acetate;1 mM EDTA, pH 8.0) buffer, were mixed with one third of their volumes of loading buffer (0.25% bromophenol blue in 40% sucrose) and were loaded (5-20 µl) into the wells (slots) of the submerged gel with micropipette. Electrophoresis was carried out for 3-4 h at 50 V.

Staining DNA in agarose gels :

The most convenient method of visualising DNA in agarose gel is by use of the fluorescent dye, ethidium bromide (Sharp et al, 1973). This substance contains a planar group that intercalates between the stacked bases of DNA. The fixed position of this group and its close proximity to the bases, causes dye bound to DNA to display an increased fluorescent yield compared to dye in free

solution. UV irradiation absorbed by plasmid DNA at 260 nm and transmitted to the dye or irradiation absorbed at 300 nm and 360 nm by bound dye itself is emitted at 590 nm in the red-orange region of the visible spectrum. Thus to detect DNA bands, gels were stained with ethidium bromide (0.5 µg/ml) and DNA bands were observed with a short wave-length U.V. transilluminator (Fotodyne) or U.V. lamp.

Curing of plasmids from Bacillus Sp. I using chemical mutagens and temperature treatment :

Curing (removal of resident plasmids from the bacterial strains) was carried out to find out the location of the desired genes in bacteria. Bacillus Sp. I which could degrade raffinose family of oligosaccharides showed the presence of plasmids. To remove the plasmids, bacteria were treated with different mutagens viz. mitomycin-C, acridine orange, novobiocin, ethidium bromide and SDS, as follows :

Curing with mutagens

1. Isolates to be used in curing experiments were streaked on BM + raffinose agar plates. A single colony was then transferred to 20 ml LB medium, and incubated at room temperature for overnight. This culture was used as an inoculum.

2. Tubes of LB containing various concentrations of drugs were inoculated with the diluted inoculum 10^5 cells/ml. These tubes were incubated on a shaker at 30° for 18 h, and minimum inhibitory concentration (MIC) was determined for each drug.

3. Samples from the tubes showing some growth were then plated on LB + glucose agar plates to get isolated colonies after serial dilution and plates were incubated at 30° for 18 h.

4. Number of colonies obtained after the treatment with mutagenic agents and SDS were then transferred to BM + raffinose agar, BM + melibiose agar, BM + glucose agar and BM + sucrose agar plates with the help of sterile toothpicks to check the degradative capacity of each colony. These plates of selective media were incubated for 24-48 h at 30°. BM + glucose agar plates served as master plate. α-galactosidase activity was also checked from treated colonies.

Curing trials at elevated temperature :

Curing of plasmids was accomplished through growth at an elevated temperature by the method of Steele and Mc Kay (1986).

Bacillus sp. I was grown overnight in raffinose broth. A 1% inoculum was then added to 25 ml LB medium containing different concentration of acridine orange/acriflavine. All the tubes were incubated at 37°, 42° and 45° for 24 h on shaker incubator. The culture were serially diluted with 0.9% saline and plated on nutrient agar plates. Replica plating of the colonies obtained was done as stated earlier.

Detection of α-galactosidase activity in drug treated colonies:

Each colony after treatment with drug was transferred into 1.0 ml Luria broth and allowed to grow in shaking condition for overnight. Cells were then toluenized by adding 0.1 ml of toluene to the culture, and kept at 37° for 10 min. To this, 0.1 ml of 1M sodium phosphate buffer was added. Enzyme reaction was started by addition of 100 ul of 0.01 M α-PNPG solution (1 µmole). Reaction was carried out at 37°C for 10 min and terminated by addition of 1.0 ml. 1M Na_2CO_3 solution. Tubes were then centrifuged at 5000 rev/min for 10 min. and supernatant was read at 420 nm against media blank on spectrophotometer.

Gene Cloning Experiments : (Maniatis et al, 1982)

Shot gun cloning of gene coding for raffinose degradation from Bacillus Sp. I was carried out using a vector plasmid pBR-322 in 5 steps as follows :

1) Generation of DNA fragments (both chromosomal and plasmid DNA) by treating the DNA sample with a site specific restriction endonuclease that generates DNA fragments having termini with an appropriate polynucleotide sequence.

2) Linkage of the fragments to be cloned to a vector plasmid pBR-322 by treatment of the DNA fragment/vector mixture with T_4 DNA ligase, viz. construction of a recombinant plasmid.

3) Introduction of DNA products of ligation into a suitable host cell system viz. E. Coli K12 (C-600) by transformation.

4) Detection of transformed clones containing required hybrid DNA species.

Restriction enzyme analysis of DNA and plasmid DNA of Bacillus Sp. I or pBR-322.

It was necessary to do restriction enzyme analysis of chromosomal and plasmid DNA of Bacillus Sp.I, as well as pBR-322 DNA, so that the fragments generated after digestion by the enzymes can be used for further cloning experiment. Plasmid DNA from Bacillus Sp. I and pBR-322 DNA from E. Coli were purified using caesium chloride density gradient centrifugation as described earlier. The purity of DNAs were checked by agarose gel electrophoresis.

The restriction enzymes used were Pst-I, Eco-RI and Bam-HI (Bethesda Research Laboratories, Inc., Gaithersburg, MD). The protocol followed for digestion with these enzymes was of Maniatis et al. (1982), as follows :

Enzyme reactions contained 0.2-2 µg of DNA in a volume of 20 µl or less.

1 (a) Assay system for cutting pBR-322 DNA consist of 20 µl DNA, 8 µl of 10 x medium buffer, 48 µl of sterile water and 4 µl of restriction enzyme (Bam-HI, Pst-I or Eco-RI).

(b) Assay system for cutting chromosomal DNA of Bacillus Sp. I consisted of 40 µl of DNA, 16 ul of 10 x medium buffer, 100 µl of water and 4 µl of restriction enzyme (Bam, Pst-I or Eco-RI).

(c) Plasmid DNA of Bacillus Sp. I was cut in an assay system of 10 µl of DNA, 4 µl of 10 × buffer, 24 ul sterile water and 2 µl of enzyme (Bam-HI, Pst-I or Eco-RI).

2. Digestion was carried out for 1 h at 37° and the reaction was stopped by heating at 68° for 5 min.

3. The efficiency of digestion with enzymes was checked by analysing 5 µl of the cut DNA on gel, by agarose gel electrophoresis. Rest of the DNA was precipitated with double the amount of distilled alcohol and preserved at -20°.

Construction of recombinant plasmid and transformation

Recombinant plasmids were produced by joining chromosomal DNA or plasmid DNA with fragments of a vector DNA pBR-322. The principle of insertional inactivation was used in construction of recombinant DNA and selection of the same. The pBR-322 vector specifies resistance to ampicillin (Amp^{+}) and tetracycline (Tet^{r}). The Amp^{+} determinant contains the unique Pst-I site of the plasmid and Tet^{r} determinant contains unique Eco-RI, Bam-HI, Sal-I, Sph I and Xma-III sites. When pBR-322 was used for cloning of pst-I fragments transformants were selected on tetracycline containing plates. Transformant clones carrying hybrid molecules selected were Tet^{r} Amp^{+}.

Ligation of foreign DNA with vector DNA :

The chromosomal or plasmid DNA of Bacillus Sp. I cut with Pst-I and Bam-HI were ligated with pBR-322 DNA cut with Pst-I/Bam-HI as described below, in a ratio of 4:1 µg final concentration :

Assay systems for ligation were as follows :

Plasmid DNA (Bacillus Sp. I)	-	20 µl
pBR-322 DNA	-	20 µl
Ligation salts	-	5 µl
Ligation buffer	-	5 µl
Ligase (T_4-DNA ligase)	-	5 µl

Chromosomal DNA (Bacillus Sp.I) - 60 µl

pBR-322 DNA	-	20 µl
Ligation salt	-	10 µl
Ligation buffer	-	10 µl
Ligase (T_4-DNA ligase)	-	10 µl

Ligation of DNA was carried out at 12° for overnight and ligation efficiency was checked by analysing DNA on gel by agarose gel electrophoresis.

Transformation of recombinant plasmid DNA into E. Coli C-600 using calcium chloride procedure :

Transformation of recombinant plasmid was carried out in E. Coli K12 C-600 by following method of Mandel and Higa (1970) :

1) 2.0 ml of LB medium was inoculated with C-600 strain of E. Coli culture was incubated at 37° for overnight.

2) Next day, 0.5 ml of this inoculum was added to 10 ml LB medium and grown for 90 min at 37° by shaking vigorously.

3) The cells were centrifuged in a sterile centrifuge capped tubes and were suspended in 5 ml of 10 mM $CaCl_2$, incubated at 0° for 3 to 5 min.

4) Cells were centrifuged and suspended in 0.5 ml of 75 mM $CaCl_2$, 50 mM 'Mops' mixture, pH 6.8 at 0° and then 0.2 ml of this were used for transformation and rest of the suspension was used as control.

5) 0.2 ml of suspension of these cells was mixed with 20 µl of each ligated DNA and incubated at 0° for 45 min. LB medium containing proper antibiotic was added to this and incubated at 37° for 2 h.

6) An appropriate quantity of cells were spreaded onto selective media by spread plate method. Plates were incubated at 37° for 24-48 h.

7) Transformants were selected using the principle of insertional inactivation for further experiment. Number of transformants were counted from each plate for checking frequency of transformation.

Controls from Step-4 were also plated on LB agar as well as LB agar containing antibiotics.

Controls as well as experimental suspension were plated on agar plates containing different media as follows, when recombinant plasmid preparation was carried out using DNA fragments act with Pst-I enzyme.

1) LB medium + tetracycline
2) LB medium + amplicillin + tetracycline
3) Minimal medium + raffinose + tetracycline (composition of the minimal medium is given in Table 66A-67)
4) Minimal medium + glucose + tetracycline.

When the recombinant plasmid preparation was carried out using DNA cut with Bam-HI, after the transformation, samples were plated on LB agar plates as well as plates containing following different media :

1) LB medium + ampicillin
2) LB medium + ampicillin + tetracyclliline
3) Minimal medium + raffinose + ampicillin
4) Minimal medium + glucose + ampicillin

Selection of 'Raf$^+$' transformants

Raf$^+$ transformants of *E. coli* cells were selected for their ability to grow on ampicillin or tetracycline containing raffinose agar plates.

Production of antisera against ∝-galactosidase of Bacillus Sp. I :

Three rabbits weighing 1.3 to 1.5 kg were used for raising antibodies against purified 'mel' induced ∝-galactosidase and 'raf' induced ∝-galactosidase. Out of three one served as a control and two were injected with two different enzymes subcutaneously. Rabbits were injected according to the protocol given in Table-68. Rabbits were bled using a needle to bleed the middle vein of the ear and 5-10 ml of blood was collected at each bleeding. The protocol followed for bleeding is given in Table-69.

Immunodiffusion test : (Ouchterlony, 1967)

1% Agarose gel prepared in 50 mM sodium phosphate buffer pH 7.0 with a pinch of azide was used to prepare gel cast on slides, and the slides were dried. A coating of 1.0% agarose solution was spread on the slide to form a thin film and allowed to set. Wells were then punched into the gel and the antigens (∝-galactosidase) (10 µl each having protein concentration of 1 mg/ml) and immunized sera (10 µl) were added into different wells and allowed to diffuse for 24 h at room temperature. The slide was kept under moist atmosphere to prevent gels from drying out.

Staining the gels

The gels were washed with saline (containing a pinch of azide) to remove excess unreacted proteins from the gel 10 x 10 cm Whatman paper No.3 was placed over the gel. A pad of absorbent paper and finally a 1.0 kg weight were placed. After 10 min, the weights were removed, gel was dried with a dryer, then the slide was stained with coomassie blue for 2-3 min, stain was poured off. The slide was rinsed in distilled water and then destained with destaining reagent till the clear band was obtained.

Immunotitration of α-galactosidase

Immunoprecipitation was carried out in plastic micro centrifuge tubes by adding variable amounts of antiserum (diluted antisera in total 50 µl of sodium phosphate buffer, pH 7.0) to constant amount of antigen (α-galactosidase having five enzyme units). After mixing the anti sera and enzyme, the tubes were incubated at 4° for 3 hr and centrifuged at 18,000 rpm for 10 minutes in a microcentrifuge and supernatant assayed for α-galactosidase activity as described earlier.

TABLE-68 : Injection protocoal for immunization of animals, against ∝-galactosidase from Bacillus sp. I.

Injection days	Rabbit-I	Rabbit-II	Rabbit-III
0	100 µg protein in 0.5 ml 0.05 M phosphate buffer, pH 7.0 + 0.5 ml complete Freund's adjuvant.	100 µg protein in 0.5 ml 0.05 M phosphate buffer, pH 7.0 + 0.5 ml complete Freund's adjuvant.	--
7	50 µg of protein in 0.5 ml 0.05M phosphate buffer, pH 7.0 + 0.5 ml incomplete adjuvant.	50 µg of protein in 0.5 ml 0.05M phosphate buffer, pH 7.0 + 0.5 ml incomplete adjuvant.	--
14	-do-	-do-	--
28	-do-	-do-	--

Rabbit-I : Injected with 'Mel' induced ∝-galactosidase.
Rabbit-II : Injected with 'Raf' induced ∝-galactosidase.
Rabbit-III : Control;

TABLE-69 : Bleeding Schedule for immunization of animals against α-galactosidase from Bacillus Sp. I.

Days	Bleeding schedule		
	Rabit-I	Rabit-II	Control (Rabbit-III)
0	+	+	+
7	-	-	-
14	-	-	-
28	+	+	+
35	+	+	+
42	+	+	+

(+) indicates animals bled.

(-) indicates animals not bled.