SUMMARY

The major plant foods available to the poor in developing countries are cereals, millets and legumes. Legumes are excellent sources of proteins (20-40%) and carbohydrates (50-60%) and fairly good sources of thiamin, niacin, riboflavin, calcium and iron. They can be considered as a relatively concentrated source of protein, although compared with the FAO (1973) provisional amino acid scoring pattern, they are not ideal, being low in sulphur containing amino acids. However, the high lysine content of most legumes means that they are complementary to cereals which are low in lysine and moderately good sources of the sulphur containing amino acids. A significant problem associated with the use of legumes is poor consumer acceptability due to the presence of antinutritional factors and toxins. Raw pulses contain a wide range of antinutritional (antiphysiological) factors and toxic substances such as hemagglutinins, protease inhibitors, amylase inhibitors, cyanogens, lathyrus factors, saponins, antivitamin factors, metal binding constituents, flatulence factors etc. The presence of these factors is one of the main drawbacks limiting the nutritional and food qualities of the legumes.

Legumes are notorious inducers of flatulence owing to the presence of substantial amounts of flatulence producing oligosaccharides of the raffinose family of sugars. Raffinose family of sugars are galactosides having one or more
α-D-galactopyranosyl groups linked to sucrose moiety in their structure. These sugars (verbaseose, atachyosa and raffinose) are well known to produce flatus in man and animals. Owing to the absence of enzyme α-galactosidase capable of hydrolysing the α-1-6 galactosidic linkage, these oligosaccharides accumulate in the lower intestine and undergo anaerobic fermentation by bacteria with the production of hydrogen, carbon dioxide and methane.

In India, Bengal gram, red gram, green gram and black gram are the chief legumes cultivated and consumed. *Lathyrus sativus* or Kesari-dhal forms a staple item in the diet of some part of poor population in India and other countries. Poor people whose staple is *L. sativus*, which contains neurotoxins like β-N-oxalyl-L-α,β-diaminopropionic acid (ODAP), N-β-D-glucopyranosyl-N-α-L-arabinosyl-α,β-diaminopropionitrile, dioxalyl-diaminopropionic acid and phenolic compounds, are known to suffer from a dreadful paralytic disease called "neurolathy­rism". Studies during the last decade have indicated that ODAP is the chief factor responsible for neurolathy­rism. Recent studies indicate that this toxin (ODAP) may interfere with glutamate uptake in brain.

In spite of its toxicity, it is difficult to completely stop the cultivation and consumption of *L. sativus* because it is a hardy crop which can thrive even under extreme drought conditions. Moreover, the chemical composition of this legume compares well with other legume regarding protein, vitamins, minerals etc. However, along with the neurotoxin,
it also contains phytate, trypsin inhibitor and flatulence factors.

Thus, to utilize the protein rich legumes as a more acceptable source of inexpensive proteins, it is desirable to reduce the antinutritional factors and toxins. Therefore, to improve the nutritive value of legumes, the different methods of food processing viz. cooking/boiling, roasting, parching, germination and fermentation are employed before they are consumed. Among all these processing methods, fermentation is the most advantageous and popular method because it also improves digestibility and nutritive values by increasing vitamins, free sugars, aminonitrogen and by decreasing some antinutritional factors. Microbial communities with their combined physiology and interactions and their enzymatic activities are responsible for the major biochemical and nutritional changes that occur in substrates of most fermented foods (Steinkraus, 1982). Earlier studies carried out in this laboratory on the type of microorganisms involved in Indian fermented foods suggested that the micro-organisms involved during fermentation originate from the grains used for preparing the fermented foods. These studies as well as changes found during fermentation raised the question whether some of the micro-organisms present in fermented foods or stored grains can also breakdown flatulence producing oligosaccharides and neurotoxin (Clostridium perfringens). Hence present investigations were undertaken to find out whether any bacteria involved in or present in stored grains, fermentation can hydrolyse flatulence producing oligosaccharides and neurotoxin (Clostridium perfringens) and if so to study the mode of hydrolysis of these compounds by the bacteria.
Since the major aim of the present investigations was to study the mode of breakdown of neurotoxin and flatulence producing oligosaccharides by bacteria, attempts were made to isolate and characterize bacteria from stored grains and fermented *L. sativus* dhal batter, which can hydrolyse these compounds. A fermented product was prepared using *L. sativus* dhal, for studies on breakdown of neurotoxin and flatulence producing oligosaccharides during fermentation. When this fermented product was analysed for its microbial profile, it was found that only bacteria were present and not yeast or any other fungi during fermentation. The bacteria present during fermentation were characterized as *Leuconostoc mesenteroides*, *Lactobacillus fermenti*, *Lactobacillus sp.* I, *Pediococcus sp.*, *Bacillus sp.* III and *Streptococcus sp.* II. Among these bacteria, *L. mesenteroides* and *L. fermenti* were present as dominant bacteria in a fermented batter resembling other Indian fermented foods. It was possible to isolate bacteria capable of degrading flatulence producing oligosaccharides or neurotoxin from stored grains used for preparation of fermented foods. These bacteria were characterized as *Bacillus sp.* I, *Bacillus sp.* II, *Streptococcus sp.* I, *Klebsiella aerogenes*, *Spirillum sp.*, and *Lactobacillus sp.* II. When all the above stated bacteria from fermented *L. sativus* dhal batter and stored grains were screened for their capacity to hydrolyse the toxins, it was found that *Bacillus sp.* I (isolated from stored soybean grains), and *Streptococcus sp.* I and *Bacillus sp.* II (isolated from stored *L. sativus* grains) were the most efficient organisms for utilization of the oligosaccharides and neurotoxin (GOAP) respectively.
For studies on degradation of flatulence producing oligosaccharides, thin layer chromatographic (TLC) and gas chromatographic (GC) methods were standardized for quantitative and qualitative analyses of the sugars. During GC method, trimethylsilylated derivatives of oligosaccharides and their breakdown products could be separated and estimated rapidly with very high sensitivity. When the effect of different processing methods were studied on oligosaccharides content of *L. sativus* seeds/dhal, it was found that soaking plus boiling or autoclaving, germination and fermentation cause the significant loss of raffinose, stachyose and verbascose. Germination and fermentation can be considered as better methods of processing *L. sativus* because they increase digestibility and nutritive value along with the degradation of antinutritional factors and toxins. When bacteria present during fermentation of *L. sativus* were screened for their capacity to degrade raffinose, it was found that *Pedicoccus sp.* I, *L. mesenteroides, L. fermenti* and *Bacillus sp.* III could hydrolyze raffinose.

Since *Bacillus sp.* I isolated from stored soybean grains had maximum capacity to degrade the oligosaccharides, this strain was chosen for further studies on degradation of flatulence producing oligosaccharides. It was found that this strain possesses cytoplasmic \( \alpha \)-galactosidase and invertase for the degradation of raffinose into glucose, galactose and fructose. This strain also could utilize other \( \alpha \)-D-galactosides such as stachyose and melibiose as sole 'C' source. At pH 6.5-7.0 this strain could produce...
maximum amount of raffinose hydrolysing enzymes when grown at 30° for 14-16 h in raffinose broth containing 0.5% raffinose as sole "C" source. Nutritional requirement studies on this bacteria (Bacillus sp. I) showed that common amino acids and vitamins present in legumes does not affect the production of α-galactosidase or invertase in Bacillus sp. I.

Investigations on regulation of production of raffinose hydrolysing enzymes in Bacillus sp. I suggested that in this strain, α-galactosidase is an inducible enzyme, synthesis of which is not regulated by catabolite repression, whereas production of invertase is constitutive. Galactose as well as α-galactosides viz., raffinose, stachyose and melibiose served as inducers for the synthesis of α-galactosidase in Bacillus sp. I. The effect of rifampicin (an inhibitor of RNA synthesis in prokaryotes) on the induction of α-galactosidase was studied with a view to understand whether the induction of this enzyme was regulated at the level of transcription. When rifampicin was added to the culture during the growth in raffinose broth, it was found that at a concentration of 50 μg/ml, it inhibited the induction of α-galactosidase. Thus the repressive effect of transcriptional inhibitor on the inducible appearance of α-galactosidase activity in the cells of Bacillus sp. I is indicative of "de novo" synthesis of the enzyme in this bacteria.

It is reported that in E. coli, plasmid coded α-galactosidase has different properties from that coded by homologous chromosomal DNA (Schmid and Schmitt, 1976). In the present
investigation α-galactosidase and invertase were purified and characterized from *Bacillus sp.* I grown on raffinose broth. Later, the properties of raffinose induced α-galactosidase were compared with that of melibiose induced enzyme from *Bacillus sp.* I. Using different purification techniques, viz., ammonium sulfate fractionation, gel filtration through Sephadex G-200 and ion-exchange chromatography using DEAE cellulose, hydroxylapatite and QAE-Sephadex columns, α-galactosidase and invertase were purified from cell-free extract of *Bacillus sp.* I to 304 and 232 folds respectively, over the crude extract. The recovery of α-galactosidase was 22% and that of invertase was 20% after purification. The purified enzymes were demonstrated to be homogeneous when SDS polyacrylamide disc gel electrophoresis (PAGE) was carried out.

The molecular weight of α-galactosidase was estimated to be 1,90,000 by gel filtration on Sephadex G-200 and Sepharose-4B and about 48,000 by SDS PAGE, which proved that intact enzyme is having a tetrameric structure in *Bacillus sp.* I. The molecular weight of invertase, estimated by both gel filtration and SDS PAGE was found to be approximately 80,000. Further studies on kinetic properties of purified α-galactosidase and invertase were also carried out. Alpha galactosidase exhibited the optimum pH 7.0 at 37°C with α-PNPG as a substrate. The Michaelis constants of α-galactosidase during present investigations were 0.5, 8.0, 36.0, 66.0 and 20 mM and Vmax values were 83, 250, 285, 300 and 76 μmoles of product liberated per minute per mg protein for α-PNPG.
melibiose, raffinose, stachyose and p-nitrophenyl-α-D-
fucoside respectively. Substrate specificity studies on
the α-galactosidase showed that it is a highly specific
enzyme for α-D-galactosides and does not act on β-galacto-
sides. The enzyme was found to be inhibited strongly by
melibiose, galactose, raffinose, arabinose and inositol when
α-FNFG was used as a substrate. Galactose and melibiose
behaved as competitive inhibitors of α-galactosidase whose
KI values were 0.5 mM and 10 mM respectively as calculated
from Dixon plots. Metal ions like Ag⁺, Hg²⁺, Cu²⁺ and Pb²⁺
caused 100% inhibition of α-galactosidase in the present
studies. Other inhibitors like PCMB and NEM also caused
complete loss of α-galactosidase activity suggesting that
the enzyme requires 'thiol' groups for its activity.

During present investigation, invertase from Bacillus
sp. I exhibited the optimum pH at 7.0 and optimum temperature
at 37° when sucrose was used as a substrate. The Michaelis
constants for this enzyme were 5.0, 22 and 20 mM for sucrose,
raffinose and maltose respectively. Trehalose and melilotriose
were not attacked by the purified enzyme preparation which
suggest that it is a highly specific enzyme. The activity of
invertase was inhibited by Ag⁺, Hg²⁺, Zn²⁺, Cu²⁺, Pb²⁺ and
sulphydryl binding reagents (PCMB and NEM). Both α-galacto-
sidase and invertase isolated in the present studies were
inhibited by Tris-HCl buffer (pH 7.0) at 10 mM resembling
other glycosidases.
Preliminary investigations on fermentation of *L. sativus* dhal showed that there is some decrease in the Odp content during natural fermentation. Hence in order to study the mode of breakdown of ODP by bacteria, different bacteria present in fermented *L. sativus* dhal and stored grains were screened for their capacity to degrade neurotoxin. *Bacillus* sp. II and *Streptococcus* sp. I isolated from *L. sativus* grains were more efficiently able to utilize ODP as sole 'C' and 'N' source for their growth and the latter culture was used for further studies.

In order to study whether *Streptococcus* sp. I can grow in *L. sativus* dhal batter during fermentation and bring about degradation of ODP, experimental fermentation of *L. sativus* flour was carried out. It was found that when *L. sativus* batter was fermented for 16 h with a mixture of dominant bacteria present during natural fermentation (viz. *L. mesenteroides* and *L. fermenti*) along with *Streptococcus* sp. I, neurotoxin content decreased significantly compared to natural fermentation along with the other desirable changes. This experiment suggested that *Streptococcus* sp. I can grow during fermentation in presence of other bacteria and degrade neurotoxin.

When studies were carried out on enzyme system involved in degradation of ODP, it was found that *Streptococcus* sp. I had intracellular enzymes for degradation of neurotoxin (ODP). The pathway for breakdown of ODP in bacteria was elucidated for the first time using radiolabelled neurotoxin, which is as follows:
Thus it is evident from the above stated pathway that neurotoxin (ODAP) degraded into the final products ammonia and pyruvate by the intracellular enzymes viz. ODAP hydrolase and DAPA-ammonia lyase. Kinetic properties of ODAP hydrolysing enzymes were studied in crude cell-free extract of *Streptococcus sp.* I. It was found that ODAP hydrolase and DAPA-ammonia lyase both exhibited optimum pH 8.0 and temperature 37°C for their activity using ODAP and DAPA as substrates respectively. It was found that DAPA-ammonia lyase was more active than ODAP hydrolase during the conversion of ODAP (neurotoxin) into its final products pyruvate and ammonia, in *Streptococcus sp.* I. The inhibitor studies on these enzymes showed that both were inhibited by sulphydryl and carbonyl group binding agents. It was found that the enzymes require pyridoxal phosphate as a cofactor. Among the inorganic salts studied, Ag⁺, Hg²⁺ and Li²⁺ inhibited both the enzymes whereas NH₄⁺ acted as an activator. The structural analogues of ODAP viz. glutamate and aspartate inhibited the activity of ODAP hydrolase significantly.
When studies on optimization of cultural conditions for production of ODAP hydrolysing enzymes were carried out, it was found that *Streptococcus sp.* I grow well and produce ODAP hydrolysing enzymes in a basal salt medium (pH 7.0) containing 0.4% ODAP as sole 'C' and 'N' source and incubated at 30°C for 24-36 h, with complete breakdown of ODAP.

ODAP hydrolysing enzymes were inducible and not constitutive in this bacteria. The vitamins, amino acids (except glutamic acid and aspartic acid) and sugars which are commonly present in *L. sativus* seeds did not affect the rate of ODAP hydrolysis during growth of bacteria. Since these compounds are generally present in *L. sativus,* it might be justified that this culture can degrade neurotoxin during fermentation of *L. sativus* chal.

Thus these studies indicated that different bacterial strains possess the enzymes involved in the degradative pathways of oligosaccharides and ODAP. Recombinant DNA technology can be a useful tool if one wants to prepare a novel strain having capacity to degrade the toxins and antinutritional factors present in commonly consumed cereal-legume based foods.

A major step in the construction of bacterial strains capable of degrading novel compounds is the recognition that a complete set of genes allowing the degradation of a compound is borne on plasmids or main chromosome. Hence during the present investigation, bacteria capable of degrading the oligosaccharides and neurotoxin, were screened for the presence of plasmids.

*Bacillus sp.* I which can degrade raffinose, *Bacillus sp.* II and *Streptococcus sp.* I which can degrade neurotoxin as well as *L. mesenteroides* which grows as a dominant bacteria during
fermentation of *L. sativus* dhal, showed the presence of plasmids. Therefore, attempts were also made to cure these bacterial strains of plasmids using curing agents like mitomycin-C, SDS, acridine dyes etc., to locate the genes coding for the degradative enzymes. Curing experiments showed that bacteria lost their degradative capacity when they were devoid of plasmids in them. These data suggested that raffinose utilization in *Bacillus sp.* I and ODAP utilization in *Streptococcus sp.* I and *Bacillus sp.* II are plasmid coded functions. Thus these properties can be due to the degradative plasmids, since there are reports on degradative plasmids coding for sugar utilization, pesticide degradation, several xenobiotics-degradation etc.

Further detailed studies were carried out on the genetics of raffinose utilization in *Bacillus sp.* I. Genes coding for raffinose utilization in *Bacillus sp.* I were shotgun cloned in *E. coli* C-600 using a vector pBR-322 plasmid. For this, plasmid DNA from *Bacillus sp.* I was cleaved with the restriction enzyme *pst-I* and ligated to pBR-322 for transformation into *E. coli* C-600. $\alpha$-Galactosidase positive clones were detected by their ability to grow on minimal medium containing raffinose as sole 'C' source. The recombinant plasmid possessed in addition to the vector DNA, a 7.0 Kb *Pst-I* insertion fragment coding for $\alpha$-galactosidase activity. Thus these experiments gave a physical evidence linking 'Raf' phenotype to a plasmid in *Bacillus sp.* I. In other bacteria viz. *Pediococcus sp.* and *E. coli* K_{12} also plasmid coded raffinose utilization is reported by other workers (Gonzalez and Kunka, 1986; Schmid and Schmitt, 1976). It is reported that in *E. coli* K_{12} the 'Raf' character
resides in at least three plasmid-borne functions, namely a specific permease, an α-galactosidase and an invertase, which are commonly controlled by a regulatory gene (Schmitt et al., 1977). Thus this information on plasmid coded raffinose or GDP utilization on bacterial isolates might be useful for further genetic manipulations. Curing studies also provided the plasmid linked raffinose and melibiose utilization in *Pacillus sp.* I. Hence further studies were also carried out to detect whether raffinose induced and melibiose induced α-galactosidase are immunologically related or not in *Pacillus sp.* I. For this investigation, antisera were raised against purified α-galactosidase in rabbits. The results of immunodiffusion and immunotitration provided an evidence that both 'raf'-induced and 'mel'-induced α-galactosidases in *Pacillus sp.* I have common antigenic determinants or they are immunologically identical. Since the molecular, kinetic and immunological properties of both the α-galactosidases were almost similar, it can be concluded that 'raf' coded and 'mel' coded enzymes might be the same proteins or they must be having homology in their structure and functional properties or they both must be the components of the same operon unlike that of *E. coli* system reported by Schmid and Schmitt (1976).

In conclusion, there exists bacteria on the surface of the grains (which are used for the fermentation of legume based foods), which can degrade flatulence producing oligosaccharides and neurotoxin (OGAP), thereby play an important role in degradation of these compounds during fermentation. Moreover, these properties of bacteria, viz. utilization of flatulence producing oligosaccharides and neurotoxin are due to the enzymes coded by degradative plasmids.