SUMMARY

CHAPTER - IV

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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, flatus 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 flatus 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 (verbascose, stachyose and raffinose) are well known to produce flatus in man and animals. Owing to the absence of enzyme α-galactosidase capable of hydrolysing the $\alpha 1 \rightarrow 6$ 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-$[-\alpha,\beta$-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 flatus
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 micro-
organisms 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 oligo-
saccharides and neurotoxin (ODAP). Hence present investigations
were undertaken to find out whether any bacteria involved in
fermentation can hydrolyse flatulence producing oligosaccharides
                  ^ or bacteria present in stored grains,
and neurotoxin (ODAP) 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 mesent-
eroides, 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 neuro-
toxin 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 (ODAP) 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, trimethyl silylated 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 Pediococcus 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 $\propto$ -galactosidase and invertase for the degradation of raffinose into glucose, galactose and fructose. This strain also could utilize other $\propto$ -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-galacto-
sidase 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-galacto-
sidase 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-galacto-
sidase 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 polyacry-
lamide 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° with
α-PNPG as a substrate. The Michelis constants of α-galacto-
sidase 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
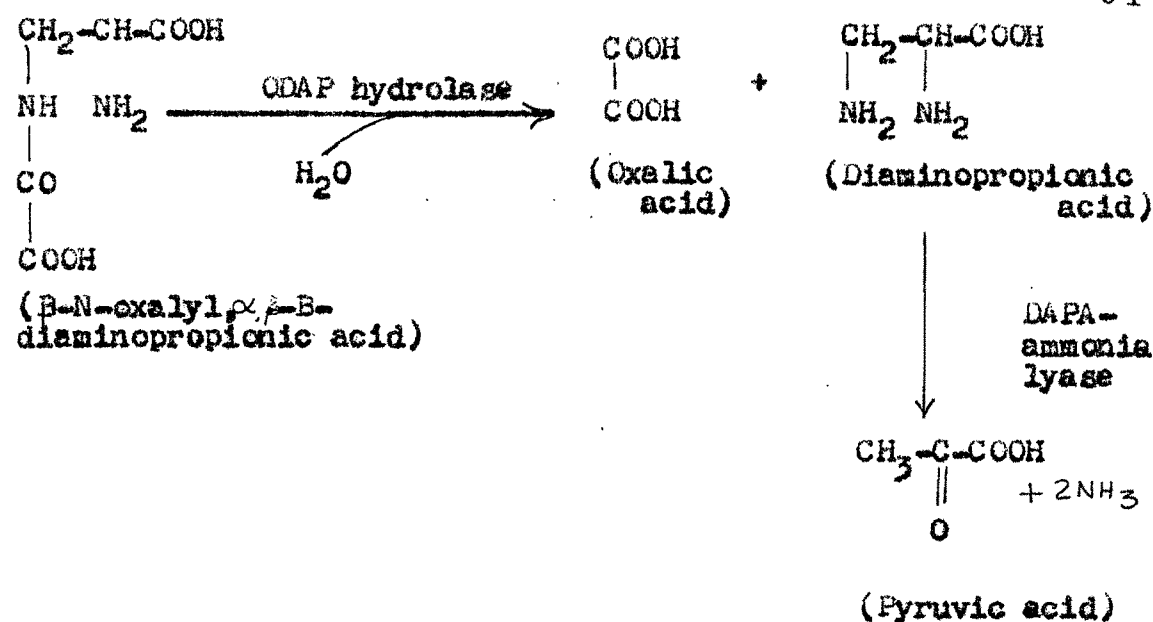of product liberated per minute per mg protein for α-PNPG,

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-galactosides. The enzyme was found to be inhibited strongly by melibiose, galactose, raffinose, arabinose and inositol when α-PNPG 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^+, Hg^{2+}, Cu^{2+} and Pb^{2+} 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 Michelis 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^+, Hg^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and sulphydryl binding reagents (PCMB and NEM). Both α-galactosidase 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 ODAP content during natural fermentation. Hence in order to study the mode of breakdown of ODAP 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 able to utilize ODAP as sole 'C' and 'N' source/for their more efficienctly 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 ODAP, 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 ODAP, it was found that Streptococcus sp. I had intracellular enzymes for degradation of neurotoxin (ODAP). The pathway for breakdown of ODAP in bacteria was elucidated for the first time using radiolabelled neurotoxin, which is as follows :

$$CH_2-CH-COOH$$

$$NH \quad NH_2 \xrightarrow[\quad H_2O \quad]{\text{ODAP hydrolase}} \quad \begin{matrix} COOH \\ COOH \end{matrix} \quad + \quad CH_2-CH-COOH$$

$$CO$$

$$COOH$$

(β-N-oxalyl α,β-B-diaminopropionic acid)

(Oxalic acid)   (Diaminopropionic acid)

DAPA-ammonia lyase

$$CH_3-C-COOH \ + 2NH_3$$
$$\overset{\parallel}{O}$$

(Pyruvic acid)

Thus it is evident from the above stated pathway that neurotoxin (ODAP) gets 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° 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^+, Hg^{2+} and Li^{2+} inhibited both the enzymes whereas NH_4^+ 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° 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 dhal.

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 investi-
gation, 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 mitonycin-C, SDS,
acridine dyes etc., to locate the genes coding for the degra-
dative 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 shot gun 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. α-Galactosidase positive clones were detected
by their ability to grow on minimal medium containing raffinose
as sole 'C' source. The recombinant plasmid possessed in addi-
tion to the vector DNA, a 7.0 Kb Pst-I insertion fragment coding
for α-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 ODAP utilization on bacterial isolates might be useful for further genetic manipulations. Curing studies also provided the plasmid linked raffinose and melibiose utilization in Bacillus 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 Bacillus 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 Bacillus 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 (ODAP), thereby play an important role in degradation of these compounds during fermentation. Moreover, these properties of bacteria, viz. utilization of flatuence producing oligosaccharides and neurotoxin are due to the enzymes coded by degradative plasmids.