GENERAL SUMMARY
A great variety of microorganisms are known to occur in or on grains but only a certain group of them is involved in the deterioration of grains in storage and are known as storage fungi. Most of these fungi when invade grain cause unrecoverable loss in the quality and the quantity of most cereal grains. While some fungi elaborate toxic metabolites and consequently poison the cereal grain.

In the present investigation an effort has been made to determine the contaminating fungi in the stored wheat grains collected from different localities of Sagar district. Fifty grain samples were collected from small storage units and were examined by direct plating on three different media i.e., malt salt (containing 2% salt), malt salt (containing 7.5% salt) and malt extract agar. In all 22 fungal species belonging to 12 different genera were collected from the test grain samples by using above three plating media malt extract medium was found most suitable for the recovery of maximum number of fungal contaminants. A total of 19 fungal species belonging to 11 genera were collected using malt extract agar medium where as on malt salt (2% salt) and malt salt (7.5% salt) only 12 and 10 fungal species were recorded, respectively, from the test grain samples.
Aspergillus flavus II was found to be most frequent in its distribution, indicating its occurrence in 82% grain samples. Aspergillus niger and Rhizopus oryzae were recorded in 54% test grain samples; while A. flavus thomii, A. tamarii, A. variecolor and A. nidulans were recorded in 34, 30, 36 and 22% grain samples respectively.

In order to study the seed health, grain samples were also examined by adopting blotter method. A total of 18 fungal species belonging to 11 genera were recorded in the test grain samples when sown on blotters. During germination, only 63.10 ± 3.75 per cent grains showed normal germination on blotters. Percentage of non germinating grains was 18.00 ± 2.521 while 18.90 ± 2.688 per cent seeds indicated abnormal germination.

Colonizing fungi elaborate certain enzymes on the surface of substrate which play a decisive role in determining the extent of damage to the substrate caused by them. To find out the enzyme producing potential of grain contaminating fungi 9 Aspergilli, i.e., Aspergillus candidus, A. flavus thomii, A. flavus II, A. flavus III, A. flavus IV, A. fumigatus, A. niger, A. tamarii, A. variecolor were grown on Czapek's dox liquid medium and were tested for the production of 19 hydrolytic enzymes using API Zym testing system A. flavus
thomii, *A. flavus* II and *A. niger* were found to produce 13, 12 and 10 hydrolytic enzymes respectively. In cultures of *A. tamarii* and *A. variecolor*, activity of only 6 and 4 hydrolytic enzymes was recorded. Remaining fungi were found to produce 4-10 hydrolytic enzymes in their cultures. Activity of acid phosphatase, Naphthol AS-BI-phosphohydrolase and B-galactosidase was found greater in *A. flavus thomii*, *A. flavus* II, *A. flavus* IV, and *A. tamarii*. In addition to these activity of a-glucosidase and B-glucosidase was also found higher (i.e., equivalent to 10 nanomoles of substrate hydrolysis) in cultures of *A. tamarii*.

These fungi have also been tested for the production of six lipases responsible for the hydrolysis of ester linkages in even numbered carbon containing fatty acid esters (carbon - 8 to carbon - 18 chain length) using chromogenic lipid substrates of p-nitrophenyl group i.e., p-nitrophenyl caprylate, p-nitrophenyl caparate, p-nitrophenyl laurate, p-nitrophenyl myristate, p-nitrophenyl palmitate and p-nitrophenyl stearate.

All the fungi indicated the activity of lipase C 8, lipase C 10 and lipase C 12. Four test strains of *Aspergillus flavus* (*A. flavus thomii*, *A. flavus* II, *A. flavus* III and *A. flavus* IV) were also found to produce lipases responsible for the hydrolysis of carbon 14, carbon 16 and carbon 18 chain length fatty acid esters.
A. *fumigatus* indicated poor activity of lipase 14 and lipase 16. It is interesting to note that A. *variecolor* showed positive activity of lipase C 18, however it could not produce lipase C 14 and lipase C 16.

Four Aspergilli i.e. A. *flavus thomii*, A. *flavus* II, A. *tamarii* and A. *variecolor* were grown in five different culture media to test the production of acid phosphatase (pH 4.8), alkaline phosphatase (pH 9.2), acid sulphatase (4.8), alkaline sulphatase (pH 9.2), a-D-glucosidase, B-D-glucosidase, a-D-galactosidase, B-D-galactosidase and N-acetyl B-D-glucosaminidase, by using chromogenic substrate of p-nitrophenyl group.

It was also observed that *Aspergillus flavus thomii* synthesized almost all the test enzymes in all culture conditions with maximum production of acid phosphatase, alkaline phosphatase, a-D-glucosidase and N-acetyl-B-D-glucosaminidase in Czapek's dox medium. Whereas the activity of a-D-galactosidase and B-D-galactosidase was found higher in malt salt (containing 2.0% salt) medium.

Activity of alkaline phosphatase, alkaline sulphatase, a-D-glucosidase and B-D-glucosidase was found higher in culture of A. *flavus II* when grown on Czapek's dox medium. In malt extract medium the activity of N-acetyl-B-D-glucosaminidase was found higher. Whereas
a-D-galactosidase and acid phosphatase activities were found higher in the cultures of this fungus grown in malt salt (2.0% salt) and MYS_{10} culture media, respectively.

Production of acid phosphatase, alkaline phosphatase and a-D-glucosidase was found higher in cultures grown on MYS_{10} medium. While a-D-galactosidase activity was found higher in malt salt (2% salt) medium. This indicates that different culture conditions affect the production of different enzymes by a particular fungus.

The production of a-amylase, carboxymethyl cellulase, protease and esterase by test fungi i.e., *A. flavus thomii*, *A. flavus* II, *A. tamarii* and *A. variecolor* was also noted in almost all the test culture media.

Enzymes in moulding grain includes enzymes present in grain it self and those produced by colonising fungi on grain. Some of the enzymes from grain and from fungi may show similar catalytic property. But some enzymes may be specific to grain and others to fungi colonizing them. Various methods have been devised to detect fungal contaminants in grains. However in the case of certain enzymes which are found to have a higher activity in contaminating grains, or those which are specific to fungal activity on grain but are absent in grains, can be of much use in the detection of deterior-
genic moulds in grains. Studies adopting this approach have been carried out in two phases i.e. to identify those enzymes which are present in clean grains as well as those present in moulding grains. Grains of wheat variety C-306 and four fungal strains i.e., *A. flavus thomii*, *A. flavus II*, *A. tamarii* and *A. variecolor* have been used in present investigation. Enzyme samples from grains (i.e., from grains shakings, grain extracts and from germ extracts) were prepared using clean dry grains (10.15 per cent water content) in 10 mM potassium phosphate EDTA extraction buffer (pH 7.2) and were tested for the activity of 6 lipases using chromogenic lipid substrates of p-nitrophenyl group. Grain extracts and germ extracts showed positive activity of lipase C 8, lipase C 10, lipase C 12 and lipase C 14. While grain shakings indicated a positive activity of only three lipase i.e., lipase C 8, lipase C 10 and lipase C 12.

It is noteworthy that lipase C 18 activity was found to be entirely absent in both grain and germ extracts and grain shakings of clean wheat grains having 10.15 per cent water content. Activity of lipase C 16 has also not been confirmed in grain extracts and grain shaking. While studying extracellular production of lipase, some of the test storage fungi such as *A. flavus thomii*, *A. flavus II*, *A. flavus III*, *A. flavus IV*, and *A. tamarii* were found positive for the activity of lipase C 16 and lipase C 18.
Activity of 19 hydrolytic enzymes has also been tested in grain shaking, grain extract and germ extracts by using API Zym testing system. Out of 19 test enzymes, only 12 and 13 enzymes have been detected in grain and germ extracts, respectively. While the grain shakings showed only 7 out of 19 enzymes. Activity of B-glucosidase was not detected in any of the grain extracts and grain shakings. In a separate set of experiment using API Zym test, activity of B-D-glucosidase was recorded in germinating spores of A. flavus thomii, A. flavus II, A. tamarii and A. variecolor. This indicates that lipase C 18 and B-D-glucosidase are specific to some of the test fungi only and are not a characteristic of clean wheat grains. Hence, the activity of these enzymes can be used as a marker of mould contamination in or on grains particularly of those which showed positive activity of above mentioned enzymes.

It was interesting to note that the extraction of enzymes from wheat grains could be minimized by shaking the grains in extraction buffer (Potassium phosphate EDTA pH 7.2) in comparison to the method of grinding the grain in buffer.

In order to identify certain enzymes specific for mould development in grains, another set of experiment was conducted. For this water content of the unsterilized
clean wheat grains (10.15% WC) was raised to 15, 20 and 25 per cent and were then inoculated with the spore suspensions of each test fungi (i.e. *A. flavus thomii*, *A. flavus II*, *A. tamarii* and *A. variecolor*) and incubated at 28°C temperature in incubator. A set of controls of uninoculated wetted grains having different water content (15, 20 and 25% WC) and dry wheat grain having 10.15% water content were also run alongside with the inoculated grains. Enzyme activity in these grains was assayed after 15, 30 and 60 days storage. For this enzyme samples from above grains were obtained by grinding and shaking of the grains in extraction buffer (pH 7.2) and activity of acid phosphatase (pH 4.8), alkaline phosphatase (pH 9.2), Acid sulphatase (pH 4.8 and pH 6.2), alkaline sulphatase (pH 9.2), α-D-glucosidase, β-D-glucosidase, α-D-galactosidase, β-D-galactosidase and N-acetyl-B-D-glucosaminidase has been tested using chromogenic substrates of p-nitrophenyl group.

Activity of the test enzymes was found higher in inoculated and uninoculated (naturally moulding) grains having 15, 20 and 25 per cent water content, than that of uninoculated clean wheat grain having 10.15% water content. Amongst test enzymes, activity of N-acetyl-B-D-glucosaminidase was found higher in inoculated grain than that of uninoculated grain having similar water content i.e., 15, 20, 25 per cent. Grain
shakings from inoculated wheat grain having 25 per cent water content indicated 16.5-65.5 times greater activity of N-acetyl-B-D-glucosaminidase in comparison to uninoculated dry grains (10.15% WC) during a period of 15-60 days of storage at 28°C. Enzyme samples obtained by grinding the inoculated grain having 25 per cent water content have also indicated 12.9 to 36.3 times increased activity of this enzyme than uninoculated dry grains (10.15% WC). Activity of a-D-galactosidase was found to be higher in inoculated grain at all water contents, and was maximum (88.0 times) in grains having (25% WC) when inoculated with Aspergillus flavus thomii in comparison to uninoculated dry grains. Shakings from inoculated grains have indicated 4-7 times greater activity of B-D-glucosidase in grains having 15.0% water content, 8-11 times greater in grain having 20.0% water content and 13-14 times greater in grain having 25.0% water content in comparison to grains having 10.15% water content. Grain extract from inoculated grains have also indicated increased activity of this enzyme. But this never exceeded more than 2.6 times in comparison to dry grains (10.15% WC).

Many folds increased activity of above mentioned three enzymes in inoculated and uninoculated grains, in comparison to uninoculated clean dry grains suggests that activity of these enzymes is an indication of mould development in grains in general. However, after the
quantification of mould biomass in relation to the activity of these enzymes in moulding grains. These can be used to determine fungal biomass in grains even when the contaminants are present in incipient level.

A parallel experiment was also run to determine the activity of a-amylase, carboxymethyl cellulase, protease and esterase enzymes in moulding grains. Activity of these enzymes was determined after 7, 15 and 30 days of incubation at 28°C. Both, uninoculated (naturally moulding) and inoculated wheat grains having 15, 20 and 25 per cent water content, have indicated increased amylase activity in comparison to dry grains (10.15% WC). Carboxy methyl cellulase (CMCase) and protease activity was also found higher in inoculated and uninoculated wheat grains having 15, 20 and 25 per cent water content than uninoculated dry grains (10.15%). Inoculated and uninoculated (naturally moulding) grains having similar water content were found to have many folds increased activity of esterase than uninoculated clean grains having 10.15% water content; when examined after 7, 15, 30 days of incubation.

A marked decrease in the germinability of wheat grains was also noted in inoculated grains containing 25% water content. Grains inoculated with *Aspergillus flavus thomii* and *A. flavus* II indicated a loss of germination in 60% seeds after 15 days of incuba-
tion. Percentage of non-germinating seeds increased to 100% in all the inoculated grains containing 25% water content after 30 days of incubation. In grains having 20% water content a loss of germination in 66, 84, 88 and 100 per cent seeds was recorded when inoculated with A. *flavus* II, A. *tamarii*, A. *variecolor*, and A. *flavus thomii* respectively, after 60 days storage. In the case of inoculated seeds containing 15 per cent water content, seed germination was not found to be much more affected as compared to seed having 20 and 25 per cent water content. But percentage of seeds showing normal germination was reduced to some extent.

A number of factors are known to affect the survival and further growth of a particular fungus in/on stored grains. Besides temperature and relative humidity, antagonistic activity amongst microorganism is the major factor that can determine and play a decisive role in the development of particular mould on grain. Antagonistic activity of 4 Aspergilli i.e., A. *flavus thomii*, A. *flavus* II, A. *tamarii* and A. *variecolor* has been tested against 10 other grain contaminating fungi i.e., Acremonium *curtipes*, Alternaria *alternata*, A. *tenuissima*, Bipolaris *australiensis*, Corynascus *sepedonium*, Curvularia *pallescens*, Aspergillus *nidulans*, Fusarium *culmorum*, F. *equiseti* and F. *oxysporum*. It was noted that Aspergillus *variecolor* was found highly active against the growth of most of the test fungal species. Further,
it was also observed that *A. tamarii* was antagonistic to almost all test fungal species but in no case it was found to cause more than 27.7% inhibition in the growth of the test species except *Fusarium culmorum* where the inhibition was 38.8%. However, *A. flavus thomii* and *A. flavus* II were found antagonistic to some of the test fungi; while other test fungi caused inhibition in the growth of these antagonists. Effect of culture filtrates of 6 Aspergilli i.e., *A. flavus thomii*, *A. flavus* II, *A. flavus* III, *A. flavus* IV, *A. tamarii* and *A. variecolor* have also been tested on the germination of wheat grains. It was observed that when wheat grains were given a pre-soaking treatment of culture filtrates, some of the seeds were found to develop abnormal seedlings while some lost their germination potential. All the test fungi were found to synthesize aflatoxins when grown on wheat grains having 18.5% water content. Maximum production of aflatoxin B₁, B₂, G₁ and G₂ i.e., 1.2818, 1.309, 1.973 and 1.217 μ gm per 100 gm grain respectively was found in grains inoculated with *A. flavus* III respectively. *A. flavus thomii* produced aflatoxin B₁, B₂ and G₂ only. It was found to produce more than 2 μ gm of B₂ and G₂ per 100 gm of experimental wheat grains. Other test strains have also produced varied amount of aflatoxins.