Fructooligosaccharides (FOS) are a class of oligosaccharides which possess 1-3 fructosyl units bound to β-2, 1 position of sucrose (Yun et al, 1997). They are produced by the transfructosylation of sucrose using fructose-transferring enzyme: Fructosyl transferase and β-fructofuranosidase. Fungi are potential sources for the enzyme Fructosyl transferase (FTase). Many fungal strains were screened for the FTase production that can produce more FOS yields. Aspergillus oryzae (MTCC5154), Aureobasidium pullulans (ATCC20524), Aspergillus niger (ATCC2601), Saccharomyces cerevisiae (ATCC36858) and Penicillium citrinum (38065) were sources for FTase activity and this research work deals with the production of FOS by using cell immobilization technique of enzyme (FTase) from the five fungi.

3.1 Microorganisms and culture conditions

The five fungi strains have been collected from culture collection of Food and Drug Administration, Vadodara (Gujarat). All the fungi were maintained in Nutrient Potato dextrose agar slants at 37°C in incubator for their proper growth for 24 hours. After the incubation period all the fungi slants were maintained at 4°C in refrigerator. The various fungal strains used in the present research for production of fructooligosaccharides are listed below along with ATCC/MTCC numbers.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Name of fungal</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Aureobasidium pullulans</em></td>
<td>ATCC : 20524</td>
</tr>
<tr>
<td>2.</td>
<td><em>Aspergillus niger</em></td>
<td>ATCC : 2601</td>
</tr>
<tr>
<td>3.</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td><em>(ATCC :36858)</em></td>
</tr>
<tr>
<td>4.</td>
<td><em>Penicillium citrinum</em></td>
<td><em>(ATCC :38065)</em></td>
</tr>
<tr>
<td>5.</td>
<td><em>Aspergillus oryzae</em></td>
<td>MTCC :5154</td>
</tr>
</tbody>
</table>
Fig: 3.1. showing the Growth of fungal species on potato dextrose agar (PDA), Czapek extract Agar (CEA) and Malt extract agar (MEA) media 
(A) Aspergillus niger (ATCC2601)  
(B) Aspergillus oryzae (ATCC5154)  
(C) Penicillium citrinum (ATCC38065)  
(D) Saccharomyces cerevisiae (ATCC36858)  
(E) Auriobasidium pullulans (ATCC20524)
3.2 Optimization of culture media, Temperature and pH on growth of fungi

In order to find out the effect of different culture media on the growth of five fungi viz., *Aspergillus oryzae* (MTCC5154), *Aureobasidium pullulam* (ATCC20524), *Aspergillus niger* (ATCC2601), *Saccharomyces cerevisiae* (ATCC36858) and *penicillium citrinum* (ATCC38065), they were grown separately on 5 different nutrient media, viz., Czapek's - Dox, Potato Dextrose, Richard's Asthana and Hawker's and Abrus sucrose media (developed by Ishrani et al, 2007), the composition of which has been given in the respective chapter. The dry weights of the mycelial mats were recorded at the end of the incubation period, i.e. after 5 days. Similarly the effect of eight different temperatures ranging between 10° C and 45° C on the growth of these fungi was studied.

In order to study the effect of pH on the growth of these fungi, the pH of the basal medium was fixed at 15 different levels ranging from 2.5 to 9.5. pH adjustment was done by adding suitable quantities of 1 N NaOH and 1N HCl. the growth and sporulation response of fungi 25 ml. of the liquid medium was poured into 150 ml. Erlenmeyer flasks. Inoculated flasks were incubated for 5 days at 25 ± 2° C in BOD incubator. One week old cultures were used for inoculation. Each experiment was run in triplicate. At the end of incubation period mycelial mats were removed by filtration over previously weighed and dried what man’s filter paper no. 42. The pH of the medium was determined by pH meter. The mycelial mats were thoroughly washed with distilled water several times and finally with warm distilled waters. Filter papers containing
mycelial mats were kept in the electric oven at 70°C for 48 hours and then transferred to dessicators and weighed. At the time of weighing fused CaCl₂ tubes were placed inside the balance for removing the ambient atmospheric moisture. The process was repeated till the weight of the mycelial mats were constant. Dry weight results were statistically analysed. The replicates showed only non-significant differences hence only average values have been recorded.

3.3 Statistical Analysis

The dry weights of the different experiments into good, moderate and poor, the general mean of the experiment ± critical difference (CD) at 5% level has been reported as moderate. The dry weights higher than the moderate have been designated as good and lower ones as poor. The results of each experiment have been expressed only by recording the standard error (SE) and critical difference (CD) at 5% level. The standard error in all the cultural experiments dealing with the amount of growth of the fungi was calculated by following formula.

\[
\text{Standard Error} = \sqrt{\frac{\text{mean square of the error}}{\text{Number of replicates}}}
\]

Critical difference was calculated by the formula:

\[
\text{CD} = SE \times P \times 2.
\]

Where P represents probability at 5% level.

3.4 Sporulation Gradation

For determining sporulation gradation, flasks containing the cultures were vigorously shaken. One drop of the culture liquid from each flask was
taken and mixed up, a droplet from which was then spread over a clean slide. It was examined under low power of the microscope and the number of spores per microscopic field was counted. The degree of sporulation was recorded as given below:

<table>
<thead>
<tr>
<th>No. of Spores</th>
<th>Sporulation</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>1 to 10</td>
<td>Poor</td>
<td>+</td>
</tr>
<tr>
<td>11 to 20</td>
<td>Fair</td>
<td>2⁺</td>
</tr>
<tr>
<td>21 to 30</td>
<td>Good</td>
<td>3⁺</td>
</tr>
<tr>
<td>31 and more</td>
<td>Excellent</td>
<td>4⁺</td>
</tr>
</tbody>
</table>

### 3.5 Inoculum development of fungi

The inoculums was prepared by transferring a full loop of spores from 5-day-old culture to 100 ml medium containing 1% sucrose and 0.2% yeast extract (pH 5.5) in 250 ml shake flasks autoclaved at 121°C for 15 min. The flasks were then incubated at 28 ± 2°C for fungi growth on a rotary shaker incubator (Lark Innovative Germany) at 120 rpm for 24 h.

### 3.6 Production of Fructosyltransferase enzyme

A 24 h old inoculums (15% v/v) of each of the five fungal species was transferred to 100 ml of fermentation medium containing 20% sucrose, 0.5% yeast extract, 1% NaNO₃, 0.05% MgSO₄ 7H₂O, 0.25% K₂HPO₄, 0.25%
KH$_2$PO$_4$, 0.5% NH$_4$Cl and 0.25% NaCl with an initial pH 5.5 in 250 ml flasks. These flasks were incubated at 28 ± 1° C on a rotary shaker incubator (Lark Innovative Germany) at 120 rpm for 120 h. at the end of respective fermentation period flasks were withdrawn sample at regular time interval and their contents for FTase enzyme activity (unit/ml) in substrate (60% w/w sucrose solution)

3.7 Enzymes assay

The fructosyltransferase activity of all the five fungi viz., *Aspergillus oryzae* (MTCC5154), *Aureobasidium pullulam* (ATCC20524), *Aspergillus niger* (ATCC2601), *Saccharomyces cerevisiae* (ATCC36858) and *Penicillium citrinum* (ATCC38065) of extra cellular enzyme (culture fluid) was determined by incubating with 250 $\mu$ of the enzyme with 750 $\mu$ of sucrose solution (60 % w/w) in 0.1 M citrate buffer (pH 5.5) and reaction were carried at 55° C for 18 h in a orbital shaker incubator (Haake SWB20, Haake, Germany).

At the end of incubation, the reaction was arrested by keeping the reaction mixture in boiling water bath for 15 minutes. The enzyme activity was determined on the basis of amount of glucose released using glucose-oxidase kit (Span Diagnostic Ltd., Surat, India). Fructosyl transferase activity was estimated as the amount of enzyme required to liberate 1 $\mu$ mol of glucose under the specified conditions. (Hidaka et al, 1987; Yun et al, 1992).

3.8 Cell immobilization (Enzyme Immobilization)

Mycelia of all the five fungal species were immobilized using sodium alginate powder by dissolving 0.3g/l in 100 ml of warm water (50° C) and the fungal mycelia (intracellular Ftase enzyme) after centrifugation were mixed in
sodium alginate solution. The suspension was for 30 minutes without disturbing to eliminate the air bubbles.

100 ml of CaCl₂ solution, prepared by dissolving 2 g of CaCl₂. 2H₂O in 100 ml of distilled water, was poured in above prepared sodium alginate suspension drop wise from a height of about 20 cm with the help of syringe to make beads of 2.0 to 3.0 mm in diameter. The beads were left in CaCl₂ solution for 3 hours in order to complete the exchange reaction of calcium ions for sodium ions to form calcium alginate beads. The gel of calcium-alginate (beads) thus formed entrapped the fungal mycelia in their matrices and the resulting beads were used for reaction with sucrose solution for the production of fructooligosaccharides (FOS).

Fig : 3.2. showing the FTase enzyme of A. pullulans entrapped on alginate beads

3.9 Effect of temperature on FOS production by immobilization technique

In order to find out the effect of four different temperatures, 45° C, 50° C, 55° C and 60° C on the capacity of fructosyl transferase production by present five fungi, the cultures were incubated separately in shaker incubator. For this a 60% sucrose solution (w/v) was transferred to 250 ml conical flasks
and the pH was adjusted to 5.5 utilizing 1N HCl solution. 10 gm of immobilized beads of each fungal species were added in sucrose solution and were incubated separately at four different temperature range, i.e. 45°C, 50°C, 55°C and 60°C in orbital shaker incubator at 120 rpm. The samples were taken out at 24 hrs of the incubation separately and filtered through 0.4 μm millipore membrane.

3.10 Effect of pH on FOS production by immobilization technique

The effect of five different pH values (5.5, 6.0, 6.5 and 7.0 and 7.5) on the capacity of fructosyl transferase production by present five fungi was studied. For this purpose a 60% sucrose solution was prepared and transferred to five different conical flasks of capacity 250 ml, with different pH ranging from 5.5 to 7.5. 10 gm of immobilized bead of each fungal species were added separately in each flasks with different pH values, and the flasks were incubated in orbital shaker incubator at 55°C with rotation of 120 rpm. For estimating FTase activity the samples were taken out at 24 hrs of the incubation separately and filtered through 0.45 μm millipore membrane.

3.11 Effect of different concentration of carbon source (sucrose) on FOS production by immobilization technique

The effect of four different concentration of sucrose viz., 40%w/w, 50%w/w, 60% w/w and 70% w/w sucrose solution on the capacity of FTase production by present five fungi was studied. For this purpose four different concentrations of sucrose viz., 40% w/w, 50%w/w sucrose, 60% sucrose and 70% sucrose solutions were prepared and transferred separately in 250 ml conical flasks, pH was adjusted to 5.5.
10 gm immobilized bead at each fungal species was added separately in solutions of different sucrose concentrations and were incubated at 55° C in orbital shaker incubator with speed of 120 rpm. The samples were taken out at 24 hrs of the incubation separately and filtered through 0.45 μm Millipore membrane.

### 3.12 Effect of Agitation speed (rpm) of shaker incubator on FOS production by immobilization technique

The effect of four different speeds of shaker incubator viz., 100 rpm, 120 rpm, 150 rpm, and 180 rpm on the capacity of FTase production by present five fungi was studied. For this purpose 10 gm immobilized bead to each of the fungal species selected for present investigation was added to 60% w/w solution of sucrose and the flasks were incubated at four different (rpm) speeds separately in orbital shaker incubator. The samples from each flask were taken out at 24 hrs of the incubation separately and filtered through 0.45 μm milipore membrane.

### 3.13 Effect of different incubation period on FOS production by immobilization technique

In order to find out the effect of five different incubation times viz., 8 hrs, 16 hrs, 24 hrs 32hrs, 40hrs and 48hrs on the capacity of FTase production, 10 gms of immobilized beads of each of the five fungal species was added separately in 60% Sucrose solution and pH was adjusted to 5.5. Samples from each flask were withdrawn at 4 hrs, 8 hrs, 12 hrs, 16 hrs, 20 hrs and 24 hrs intervals, and filtered 0.45 μm milipore membrane.
3.14 Effect of addition of vitamin and amino acid FOS production by immobilization technique

The effect of vitamin (ascorbic acid) and amino acid (leucine) on the capacity of FTase, production by present five fungi was also studied. For this purpose 0.5 gm ascorbic acid and 0.5 gm. leucine were added separately in 60% solution of sucrose. 10 gm of immobilized bead of each microbial species was then added separately to sucrose solutions. The results were recorded after 24 hrs of reaction time. The samples were similarly filtered through 0.45 μm milipore membrane.

3.15 Analysis of Fructo oligosaccharides

The reaction mixture obtained by filtering through 0.45 μm millipore membrane from each set of experiment was subjected to HPLC (High Performance liquid chromatography, also sometimes called High pressure liquid chromatography) analysis for quantification of FOS.

HPLC (Agilent 1200 series, Japan), equipment with a refractive index detector RID6A (Shimadzu, Japan) was used for FOS analysis. by an Aminopropyl column 250×4.6 mm SS Excil amino 5 μm (SGE, Australia). 10 μl of the reaction mixture from each sample was injected using HPLC injector syringe (Hemilton, Nevada, USA) and the analysis was carried out at room temperature (25 ± 2°C) using acetonitrile/water (75:25) with a flow rate of 1 ml/min. Data acquisition was done by AIMIL chromatography Data Station (AIMIL, New Delhi), and processed on computer using WINCADS software (AIMIL, New Delhi).
Fig. 3.3. HPLC instrument from (Agilent 1200 series, Japan)

Calculation of FOS percentage

\[
\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Standard weight}}{\text{Sample weight}} \times \frac{50}{10} \times \frac{\text{Potency}}{10} \times 100
\]

Table peak area of FOS standard and their RT (retention time)

<table>
<thead>
<tr>
<th>FOS components</th>
<th>Peak area</th>
<th>RT (Retention time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose (F)</td>
<td>35138</td>
<td>5.75</td>
</tr>
<tr>
<td>Glucose (G)</td>
<td>290197</td>
<td>6.93</td>
</tr>
<tr>
<td>Succrose (S)</td>
<td>213450</td>
<td>8.24</td>
</tr>
<tr>
<td>Kestose (K)</td>
<td>422677</td>
<td>11.89</td>
</tr>
<tr>
<td>Nystose (N)</td>
<td>246373</td>
<td>16.32</td>
</tr>
<tr>
<td>Fructofuranosynystose (GF4)</td>
<td>32664</td>
<td>22.70</td>
</tr>
</tbody>
</table>
Fig 3.4. HPLC chromatogram of FOS standards

3.16 Chemicals

FOS standards 1-kestose (GF2), 1 Nestose (GF3), 1-fructofuranosylnystose (GF4) and Succrose, Glucose, fructose were obtained from Wako Pure Chemicals Japan and Sigma Aldrich USA. Other chemicals were purchased from Otto, Himedia and Merck (India).