Fructooligosaccharides (FOS) are oligosaccharides (*Oligo* means few, and *saccharide* means sugar) of fructose containing a single glucose moiety and have a simple molecular structure rather than the complex molecule from its original sucrose. It is also known as neosugar, inulin, oligofructose or oligofructan. Fructooligosaccharides can be produced by the action of fructosyltransferase from many plants and microorganisms (Yun, 1996; Hidaka et al., 1988).

Fructooligosaccharides are composed of 1-kestose (GF2), nystose (GF3), and 1-β- fructofuranosyl nystose (GF4), in which fructosyl units (F) are bound at the β (2 → 1) position of sucrose molecule (GF) (Sangeeta *et al*., 2005; Kaplan et al (2000) and Yun, et al 1996 and Kucbauch, et al., 1972). When the polymeric grade of fructo-oligosaccharides is low, it has a better therapeutic properties compared than the high polymeric grade.

Fructooligosaccharides derived from sucrose using microbial enzymes have attracted special attention due to their sweet taste being very similar to that of sucrose, a traditional sweetener. They are about 0.4 and 0.6 times as sweet as sucrose and have been used in the pharmaceutical industries as a functional sweetener (Biedrzycka *et al*, 2004). Its use emerged in the 1980s in response to consumer demand for healthier and calorie-reduced foods. They are useful for diabetics and are used as prebiotics to stimulate the growth of bifidobacteria in the human colon (Modler et al, 1994).

These oligosaccharides having low caloric values exhibit several beneficial effects such as anti-carcinogenic properties, decrease level of
phospholipids, triglycerides and cholesterol (Biedrzycka and Bielecka, 2004). It was found that FOS supplementation also led to a reduction in the absolute pH of the lower intestine, this increase in acidity made the environment very difficult for “bad” bacteria populations - thus offsetting the likelihood of disease due to unrestricted bacterial multiplication.

1.1 Fructosyltransferase producer microorganisms

Fructooligosaccharides are industrially produced from sucrose by microbial enzymes with transfructosylating activity. Fructosyltransferase is produced intra- and extracellularly by several microorganisms including bacteria and fungi. The most investigated microorganisms in this subject are fungi belonging to genera *Aspergillus* (Van Balken et. al., 1991; L’Hoeine et. al., 2000; Chien et. al., 2001), *Penicillium* (Dhake and Patil 2007), *Aureabasidium* (Sangeetha et. al., 2003) and *Arthrobacter sp.* and *Fusarium* (Sangeetha et al. 2005). This enzyme is naturally found in some foods also such as onion, garlic, Jerusalem artichokes, asparagus, bananas, rye, wheat, tomato and in trace amounts as natural components in fruits, vegetables and honey. Despite the large number of microbial FTase producers, only a few of them have the potential for industrial application and were focused in several studies about FOS production. The survey for FTase producers has been the focus of several studies in this field. Intensive studies have done till date on FTase production by different microorganisms which have been documented in Table 1.1.
Table 1.1: Summary of studies on FTase production by different microorganisms:

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Microorganisms</th>
<th>Carbon Source</th>
<th>Activity (U/mL)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Penicillium purpureogenum</em></td>
<td>Sucrose (10g/L)</td>
<td>600</td>
<td>Dhake and Patil 2007</td>
</tr>
<tr>
<td>2.</td>
<td><em>Aureobasidium pullolans</em></td>
<td>Sucrose (200-500g/L)</td>
<td>120</td>
<td>Vandakova et. al., (2004)</td>
</tr>
<tr>
<td></td>
<td><em>CCY 27-1-94</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td><em>Aspergillus Oryzae CRF</em></td>
<td>Sucrose (10g/L)</td>
<td>NA</td>
<td>Sangeetha et. al., (2005 c)</td>
</tr>
<tr>
<td>4.</td>
<td><em>Aspergillus japonicas</em></td>
<td>Sucrose (200g/L)</td>
<td>NA</td>
<td>Chein et. al., (2001)</td>
</tr>
<tr>
<td></td>
<td><em>TIT-KJ1</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td><em>Bacillus macerans EG-6</em></td>
<td>Sucrose (10g/L)</td>
<td>6</td>
<td>Kim et. al., (2000)</td>
</tr>
<tr>
<td>6.</td>
<td><em>Aureobasidium pullolans</em></td>
<td>Sucrose (100g/L)</td>
<td>121</td>
<td>Shin et. al., (2004)</td>
</tr>
<tr>
<td></td>
<td><em>KCCM12017</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td><em>Aureobasidium pullolans</em></td>
<td>Sucrose (200g/L)</td>
<td>NA</td>
<td>Sangeetha et. al., (2004 a)</td>
</tr>
<tr>
<td></td>
<td><em>CFR 77</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td><em>Aspergillus niger NRRL</em></td>
<td>Sucrose (50g/L)</td>
<td>58.3</td>
<td>Balasubramaniem et. al., (2001)</td>
</tr>
<tr>
<td>9.</td>
<td><em>Aspergillus niger IMI</em></td>
<td>Sucrose (20g/L)</td>
<td>0.022</td>
<td>Nguyen et. al., (2005)</td>
</tr>
<tr>
<td></td>
<td><em>303386</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td><em>Aspergillus japonicas</em></td>
<td>Sucrose (20g/L)</td>
<td>910</td>
<td>Chen 1995</td>
</tr>
<tr>
<td>No.</td>
<td>Organism</td>
<td>Carbon Source</td>
<td>Initial Concentration</td>
<td>Molar Yield</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------------</td>
<td>---------------</td>
<td>-----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>11</td>
<td><em>Aspergillus japonicas</em> JN 19</td>
<td>Sucrose</td>
<td>150-180g/L</td>
<td>55.42</td>
</tr>
<tr>
<td>12</td>
<td><em>Aureobasidium pullolans</em> CFR 77</td>
<td>Sucrose</td>
<td>(200g/L)</td>
<td>616.8</td>
</tr>
<tr>
<td>13</td>
<td><em>Aspergillus japonicas</em> TIT 90076</td>
<td>Sucrose</td>
<td>266g/L</td>
<td>660</td>
</tr>
<tr>
<td>14</td>
<td><em>Aureobasidium pullolans</em> KFCC10542</td>
<td>Sucrose</td>
<td>200-280g/L</td>
<td>101.2</td>
</tr>
<tr>
<td>15</td>
<td><em>Penicillium citricum</em> FERM P-15944</td>
<td>Sucrose</td>
<td>(100g/L)</td>
<td>NA</td>
</tr>
<tr>
<td>16</td>
<td><em>Aspergillus foetidus</em> NRRI 337</td>
<td>Maltose</td>
<td>10g/L</td>
<td>0.52</td>
</tr>
<tr>
<td>17</td>
<td><em>Bacillus macerans</em> EG-6</td>
<td>Sucrose</td>
<td>15g/L</td>
<td>11</td>
</tr>
<tr>
<td>18</td>
<td><em>Penicillium citricum</em> KCT18080P</td>
<td>Sucrose</td>
<td>(200g/L)</td>
<td>0.053</td>
</tr>
<tr>
<td>19</td>
<td><em>Aspergillus niger</em> A50023</td>
<td>Sucrose</td>
<td>175g/L</td>
<td>420</td>
</tr>
</tbody>
</table>
1.2 Mechanism of Action of Fructosyltransferases

The reaction mechanism of the fructosyltransferase depends on the source of the enzyme. In plants and some microorganisms, a series of enzymes act together whereas a single enzyme works in most other microorganisms. In Jerusalem artichoke (*Helianthus tuberosa*) fructosan metabolism is established by two enzymes: sucrose: sucrose 1-fructosyltransferase (SST) and β (2→1) fructans: B(2→1) fructans 1-fructosyltransferase (FFT). In the first instance SST converts sucrose into glucose and an oligofructosides but is unable to promote polymerization above the trisaccharide level; further higher polymers are consecutively synthesized by FFT. The overall reaction mechanism was expressed as follows (Edelman and Jefford, 1968):

\[
\text{GF} + \text{GF} \rightarrow \text{GF} - \text{F} + \text{G} \quad \text{by SST} - 1 \\
\text{GF} - F_n + \text{GF} - F_m \rightarrow \text{GF} - F_{n-1} + \text{GF} - F_{m+1} \quad \text{by FFT} - 2
\]

Where GF is a sucrosyl group and n is the number of extrasucrosyl fructose residues.

Arnold (1965) suggested that Agave enzyme catalyzes a stepwise transfructosylation reaction to give rise to higher FOS formation where synthesis of FOSs from sucrose takes place as follows:

\[
\text{GF} + \text{Fructosyltransferase} \rightarrow \text{F-Fructosyltransferase} + \text{G} - 3 \\
\text{F-Fructosyltransferase} + \text{GF} \rightarrow \text{GF} + \text{Fructosyltransferase} - 4
\]

In this transfructosylation reaction glucose, not fructose, acts as the acceptor of the fructose molecule from sucrose. GF2, GF3, and GF4 cannot act as donors of the fructosyl moiety for the synthesis of higher oligosaccharides.
but act as acceptors of fructose from sucrose only for the synthesis of higher oligosaccharides. This mechanism is identical with that of chicory enzyme reported by Singh and Bhatia (1971). Dickerson (1972) proposed the reaction mechanism of *Claviceps purpurea* enzyme which produces mainly neokestose–based oligosaccharides. The suggested mechanism is as follows:

\[
\begin{align*}
F2 \rightarrow & 1G + F2 \rightarrow 1G \rightarrow F2 \rightarrow 6G1 \leftarrow 2F + G \quad \text{--- 5} \\
F2 \rightarrow & 1G + F2 \rightarrow 6G1 \leftarrow 2F \rightarrow F2 \rightarrow 1F2 \rightarrow 6G1 \leftarrow 2F + G \quad \text{--- 6}
\end{align*}
\]

Where numbers indicate the position of carbonyl carbon atoms and arrows represent the direction of glycosidic linkage (e.g., F2→1G refers to sucrose).

The hydrolyzing reactions also occur in addition to the above two synthetic reaction. A hydrolysate like F2→6G acts again as fructosyl donor and acceptor for the synthesis of neokestose and its tetraoligomer. Gupta and Bhatia (1980) proposed a model for the fructosyltransferase in *Fusarium oxysporum*. They suggested that fructose is transferred from the donor site to the fructosylated nucleotide bridge and this, in turn, transfers the fructose moiety to the sucrose at the acceptor site to form GF2. GF4 was the highest glucofructosan, suggesting that the acceptor site is perhaps just big enough to accommodate up to GF4. This seems a similar result with the cases of fructosyltransferase from *A. niger* (Hirayama et al, 1989; Hidaka et al, 1988) and *A pullulans* (Yun et al, 1992, 1990 and Hayashi et al, 1991) in that GF4 is the biggest molecule of FOS in both cases. Jung et al (1989) proposed a mathematical model for the mode of action of fructosyltransferase derived from *A. pullulans*. The enzyme reaction mechanism is as follows:
Fig: showing network of the reaction mechanism for the production of fructooligosaccharides from sucrose catalyzed by fructosyltransferase derived from A. pullulans: G, GF, GF2, GF3, and GF4 means glucose, sucrose, 1-kestose, nystose, and 1-fructofuranosyl nystose, respectively.

The reaction mechanism expressed as equation:

\[ GFn + GFn \rightarrow GFn-1 + GFn+1 \]

Where \( n = 1-3 \)

According to this mechanism the enzyme acts on sucrose in a disproportionation type reaction where one molecule of sucrose serves as a donor and another acts as an acceptor. The reaction mechanism of *A. pullulans* is very similar with that of agave fructosyltransferase (Satyanarayana, 1976) except that the first reaction step is irreversible. Duan et al, (1994) proposed a modified reaction mechanism of the fructosyltransferase derived from *Aspergillus japonicas* where glucose inhibition did not occur for 1-kestose and nystose and substrate inhibition for sucrose and the hydrolyzing reaction for nystose were found. In particular, the enzymes from *Auriobasidium* sp. and *A.*
niger have a high regiospecificity which selectively transfer the fructosyl moiety of sucrose to the 1-OH furanoside of the sucrose molecules (self transfer) resulting in the formation of only 1-kestose based FOS.

Most of the microbial Fructosyltransferases thus, may catalyze the reactions of a readily reversible primary step and a subsequent irreversible step:

\[
\text{Fru} + \text{Enz} \xleftrightarrow{} \text{Fru} - \text{Enz} + \text{R} \quad \text{---8}
\]

\[
\text{Fru} - \text{Enz} + \text{Acceptor} \leftrightarrow{} \text{Fru} - \text{Acceptor} - \text{Enz} \quad \text{---9}
\]

Where Fru is fructose, Enz is fructosyltransferase, and R represents a carbonyl of an aldose. The aldoside part of the substrate molecule is possibly replaced by an enzyme–linked group, and partial decomposition of this FOS precursor to aldose and ketose may furnish the energy necessary for FOS synthesis.

### 1.3 Characteristics of Fructosyltransferases

Several fructosyltransferase have been extensively purified and characterized (Hirayama et al, 1989; Shiomi, 1981; Edelman et al, 1963; Nandra and Bhatia, 1980). In general, the enzymes derived from microorganisms are bigger in size and more stable temperature – wise than those from plants. The optimum pH and temperature for fructosyltransferase activity lies between 5-6.5 and 50 – 60 degree Celsius respectively (Jong Won Yun, 1996). This enzyme is convenient for commercial use since the reactions are routinely carried out at fairly high concentration of sucrose solution (700 - 850 g/l); therefore, operation can be conducted without considering a significant contamination problems. The fructosyltransferase from Agave
Americana is activated by Ca++, Mg++, Co and Li+ and inhibited by many minerals such as Ag+, Pb, Hg, Al+++ and Sn (Bhatia and Nandra, 1979) whereas the enzyme of the *Auriobasidium* sp. is inhibited by Hg, Cu, and Pb (Hayashi et al, 1991) but its activators are not established. Although most of the Fructosyltransferases catalyze transfructosylation at rather high concentrations of sucrose, many workers have determined the enzyme activity of transfructosylation at low sucrose concentrations. Fructosyltransferase units have been expressed as the amount of enzyme responsible for transferring one µ mole of fructose per minute or as the amount of enzyme capable of producing one µ mole glucose per min. The specificity of microbial fructosyltransferase depends chiefly on the β-D-fructoside residue of sucrose. Some substrates with terminal fructose (e.g., raffinose and inulobiose) are also suitable for oligofructosides synthesis (Hirayama et al, 1989). Furthermore, 1-kestose, nystose, and 1-fructofuranosyl nystose also act as donor and acceptor of a fructosyl unit as well. Many FOS- producing microorganisms also simultaneously produce a hydrolytic enzyme that degrades FOS (Hayashi et al, 1990; Hirayama, 1989; Patel et al, 1994). This hydrolytic activity may be responsible for the appearance of fructose in the final reaction products. On suppressing the hydrolytic nature of enzyme FOS production can successfully be enhanced.

1.4 Chemical Structure of Fructooligosaccharides

Two different classes of fructooligosaccharides (FOS) mixtures are produced commercially, based on inulin degradation and transfructosylation processes.
FOS can be produced by degradation of inulin, or polyfructose, a polymer of D-fructose residues linked by β (2→1) bonds with a terminal α(1→2) linked D-glucose. The degree of polymerization of inulin ranges from 10 to 60. Inulin can be degraded enzymatically or chemically to a mixture of oligosaccharides with the general structure Glu –(Fru)n (GFn) and Frum (Fm), with n and m ranging from 1 to 7. The main components of commercial products are kestose (GF2), nystose (GF3), fructosynystose (GF4), bifurcose (GF3), inulobiose (F2), inulotriose (F3), and inulotetrose (F4).

The second class of FOS is prepared by the transfructosylation action of a β-fructosidase of *Aspergillus niger* on sucrose. The resulting mixture has the general formula of GFn, with n ranging from 1 to 5.

The structures of FOSs synthesized in cell-free enzyme systems are essentially identical to those produced by whole cell systems. A research group of Meiji Seika co. (1984), the first commercial producer of FOS, introduced the chemical structure of FOS produced from *A. niger* fructosyltransferase. The chemical structure of FOS produced by *Aureobasidium* fructosyltransferase was also identified by methylation, GLC, GC-MS, and NMR analysis (Hayashi et al, 1989). These two representative FOS are now widely known to be oligosaccharides containing 1-kestose, nystose, and 1F-fructofuranosyl nystose. *Aspergillus sydowi* produced six different FOS showing a high degree of polymerization (DP3-13); their chemical structures were well illustrated (Muramatsu et al, 1988). Nagamatsu et al, (1990) identified 1-kestose and neokestose – based oligofructans in *Lycoris radiata* herb tissue. Since the
degree of polymerization and linkages of FOSs vary with the enzyme sources, the structural analysis is important in the study of fructooligosaccharides.

**Fig 1:** Chemical structure of fructooligosaccharides. (GF$_2$: $n = 2$, kestose; GF$_3$: $n = 3$, nystose; GF$_4$: $n = 4$, 1F-fructofuranosyl nystose)

**1.5 Physico-chemical properties of Fructooligosaccharides**

The extensive data on the physico-chemical properties of fructooligosaccharides are scarcely available. Gross (1962) reported chemical properties of some kestosides such as 1-kestose, 6-kestose, and neokestose. The specific rotation ([α]$_D$) and melting temperature of 1-kestose are +28.5° and 199-200°C, respectively. It forms fine white crystals fairly rapidly. The relative sweetness of 1–kestose, nystose, and 1-fructofuranosyl nystose to 10% sucrose solution are 31, 22, and 16% respectively (Neosugar Research group, 1984).
FOS are highly hygroscopic; it is difficult to keep the lyophilized products stable under atmospheric conditions for prolonged periods. The viscosity of an FOS solution is relatively higher than that of sucrose when at the same concentration, and the thermal stability is also higher than that of sucrose (Neo-Sugar User’s guide, Meiji Seika Co. Kawasaki, Shi, Japan, 1982). FOS are highly stable in the normal pH range for food (4.0-7.0) and at refrigerated temperatures over one year. FOS resemble sucrose in many properties such as solubility, freezing and boiling points, crystal data etc.

1.6 Measurement of FOS

High performance liquid chromatography (HPLC) technique is applied for measuring FOS. FOS and free sugars were easily separated on an ion – exchange column (e.g., HPX-87C, Biorad, Richmond, VA, USA) which was connected to a refractive index detector. The column temperature was kept constant at 85°C. Water was used as the mobile phase at a flow rate of 0.6ml/min. (Hidaka et al., 1988; Yun et al., 1993, 1994, 1995.). The TLC system for determination of FOS was well described by Collins and Chandorkar, 1971.

1.7 Uses of Fructooligosaccharides:

Fructooligosaccharides have a number of interesting properties (Hidaka et al., 1987; Oku et al., 1984; Mc Kellar and Modler, 1989; Tokunaga et al., 1989).

1. FOS have a low sweetness intensity since they are only about one-third as sweet as sucrose. This property is quite useful in the various kinds of foods where the use of sucrose is restricted by its high sweetness.
2. FOS are calorie free; i.e., they are scarcely hydrolyzed by the digestive enzymes and not utilized as an energy source in the body, thus they are safe for diabetics.

3. They are noncarcinogenic, i.e., they are not used by Streptococcus mutants to form the acids and insoluble β-glucan that are the main culprits in dental caries.

4. FOS encourage the growth of the bifidobacteria and discourage the growth of potentially putrefactive microorganisms that have a tendency to cause diarrhea.

5. FOS decrease the levels of serum cholesterol, phospholipid, and triglyceride (Neosugar research group, 1984).

Takahisa Tokunaga et al., (1993) have studied the effects of fructooligosaccharides intake, in the dose level of 1g, 3g and 5g/day for two weeks on the intestinal microflora and defecation in 27 healthy volunteers (male 21, female 6) and reported that in all the dose levels the number of Bifidobacteria was significantly increased during FOS intake period. They also noticed the significant increase of stool frequency and softening effect on stool in all subjects.

FOS has been a popular dietary supplement in Japan for many years, when the Japanese government installed a ‘Functionalized Food Study Committee “to start to regulate” special nutrition foods or functional foods” that contain the categories of fortified foods i.e., vitamin-fortified wheat flour (O’Donnell & Claudia, 1994), and is now becoming increasingly popular in western cultures for its prebiotic effects.
1.8 Market Trend

FOS were first introduced into market as foodstuffs by Meiji Seika Co. in Japan during 1984. Japan has the largest commercial market; its market volume amounted to over 4,000 metric tons in 1990 (Food chemicals, Japan, 1989). Although FOS have not been marketed in the U.S. and Europe, several companies have been trying to get the Generally Recognized As Safe (GRAS) status for using FOS with plain, unsweetened dairy products. It is evident that consumer interest in low calorie food products is increasing (Stamp, 1990). Thus, consumption of FOS in the sugar market is expected to continue to rise.

1.9 Aim of Present Research

The aim of present research work is to optimize the production parameters like temperature, pH, agitation speed, concentration of carbon source, incubation periods, addition of amino acids and vitamins on FOS production by immobilization technique of *Aspergillus oryzae*, *A. niger*, *P. citrinum*, *S. cerevisae* and *Aureobasidium pullulans*.

1.10 Scope of study

It might be possible to detect particular range of a specific physical or chemical parameter which may have a crucial role to promote enzymatic activity for the enhanced production of FOS. Our work may be helpful to optimize the production of fructooligosaccharides by the microorganism so as to get maximum production.