Chapter-II

Review of Literature
REVIEW OF LITERATURE

2.1 History of product

The ancient art of alcohol fermentation has been documented for many years and the well-known fermentation process for converting sugar to alcohol by using yeast and bacteria can be found readily in literature (Section 2.3). The substrate is the main cost component for industrial ethanol production and it is essential that ethanol production should be carried out with cheaper substrates (Lee et al., 1983; Elisson et al., 2001). The ethanol production should be at low cost to be affordable to the poor sector of the community and should be able to use locally available raw material.

Ethanol is now produced by fermentation of grain, especially corn (Sachs, 1980) and because of rapid increase in demand for grains, both as food and feed, there is an urgent need for substitution of these substrates for alcohol as well as for various other industrial fermentations (Azhar and Hamdy 1979). Ethanol production by fermentation faces competition with ethanol production from petroleum-based products as feed stocks. But with the increasing value of these petroleum feed stocks, fermentation of ethanol is bound to receive more attention (Ameh et al., 1988).

In the natural environment, plant and biological materials are a variety sources of alcohols (Obisanyo et al., 1987; MacDonald and Fall, 1993; Kistrine et al., 1998; Lamanna and Goldstein, 1999; Ndip et al., 2001; Ezeronye, 2004). In this regard use of renewable materials would be more economical, since they are cheaper and easily available.

Rapid fermentation and high ethanol levels are desirable to minimize capital costs and distillation energy, while good yields are necessary for process economics. Many tropical fruits such as mango, jackfruit, banana, papaya, guava and cashew
apple have been shown to be suitable for fermentation, mainly because of their appropriate taste, flavor, availability, high sugar content and overall chemical composition. Some research results on the fermentation of fruits, fruit waste and various crops (Table 2.1 and 2.2) have been observed for the production of alcoholic beverages. Fruit juices are consumed for their characteristic flavors and are also considered to be sources of vitamins, minerals and soluble and insoluble fibers (Righetto et al., 1999)\textsuperscript{324}.

2.2 History and distribution of cashew (\textit{Anacardium occidentale})

Anacardiaceae are found throughout the globe and millions of people and animals are aquatinted with them. The English name “cashew” is derived from the Portuguese “caju” which came from the Tupi Indian “acaju” (Rosengarten and Frederic, 1984)\textsuperscript{333}. In one of the Indian languages i.e., in Telugu it is known as “Zeedimamidi”.

Cashew is produced in around 32 countries of the world. The world production figures of cashew crop, published by UN’s Food and Agriculture Organization (FAO), were around 3.1 million tons per annum. The major cashew producing countries with their production figures in 2006 (as per the FAO) are Vietnam (941,600 tons), Nigeria (636,000 tons), India (573,000 tons), Brazil (236,140 tons) and Indonesia (122,000 tons). World’s total area under the cultivation of cashew is around 3.39 million hectares. India ranks first in area utilized for cashew production, though its yields are relatively low. The world’s average yield is 817 pounds per acre (916 kg/hectare) of land.
Table 2.1: Production of alcoholic beverages from fruit and fruit waste by microorganisms

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Producing Microorganism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple pomace</td>
<td>Ethanol</td>
<td>Yeast</td>
<td>Sandhu and Joshi, (1997)³⁴¹</td>
</tr>
<tr>
<td>Banana</td>
<td>Alcohol</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Hammond <em>et al.</em>, (1996)¹³⁶</td>
</tr>
<tr>
<td>Banana</td>
<td>Wine</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Onwuka and Awam, (2001)²⁷⁷</td>
</tr>
<tr>
<td>Banana peels</td>
<td>Ethanol</td>
<td>Yeast</td>
<td>Joshi <em>et al.</em>, (2001)¹⁰⁶</td>
</tr>
<tr>
<td>Black Mulberry</td>
<td>Alcoholic beverages</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Darias-Martin <em>et al.</em>, (2003)⁹¹</td>
</tr>
<tr>
<td>Carica papaya agro waste</td>
<td>Ethanol</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Akin-osanaiye <em>et al.</em>, (2005)¹⁰</td>
</tr>
<tr>
<td>Cashew</td>
<td>Wine</td>
<td>Saccharomyces cerevisiae</td>
<td>Silva et al., (2007)</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------</td>
<td>--------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Cashew juice</td>
<td>Wine</td>
<td>Yeast strain NCYC 125</td>
<td>Osho and Odunfa, (1999)</td>
</tr>
<tr>
<td>Chardonnay grapes</td>
<td>Chardonnay wines</td>
<td>Saccharomyces cerevisiae</td>
<td>Herjavec et al., (2003)</td>
</tr>
<tr>
<td>Decayed fruits and Vegetables</td>
<td>Ethanol</td>
<td>Saccharomyces cerevisiae</td>
<td>Javed Iqbal Qazi, (2005)</td>
</tr>
<tr>
<td>Fruit and Sugar beet extracts</td>
<td>Ethanol</td>
<td>Zymomonas mobilis</td>
<td>Kolios et al., (1989)</td>
</tr>
<tr>
<td>Grape must</td>
<td>Wine</td>
<td>Saccharomyces bayanus</td>
<td>Berovi et al., (2003)</td>
</tr>
<tr>
<td>Fruit</td>
<td>Product</td>
<td>Yeast Species</td>
<td>Authors and Year</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Jerusalem artichoke juice</td>
<td>Ethanol</td>
<td><em>Kluyveromyces marxianus</em></td>
<td>Duvnjak et al., (1981)99</td>
</tr>
<tr>
<td>Mango</td>
<td>Wine</td>
<td><em>Saccharomyces cerevisiae</em> and <em>Schizosaccharomyces spp.</em></td>
<td>Obisanyo et al., (1987)267</td>
</tr>
<tr>
<td>Orange juice</td>
<td>Alcohol</td>
<td>Yeast strains</td>
<td>Okunowo et al., (2005)274</td>
</tr>
<tr>
<td>pineapple cannery waste</td>
<td>Ethanol</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Nigam, (1999)258</td>
</tr>
<tr>
<td>Pineapple Juice</td>
<td>Ethanol</td>
<td><em>Zymomonas mobilis</em></td>
<td>Hilary, (1999)144</td>
</tr>
<tr>
<td>Pineapple waste</td>
<td>Ethanol</td>
<td><em>Zymomonas mobilis</em></td>
<td>Tanaka et al., (1999)372</td>
</tr>
<tr>
<td>Red grapes</td>
<td>Red wine</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Caridi et al., (2004)38</td>
</tr>
</tbody>
</table>
Table 2.2: Ethanol yields from various crops

<table>
<thead>
<tr>
<th>Crop</th>
<th>Crop yield (tonne ha(^{-1}))</th>
<th>Fermentable carbohydrate (% fresh wt.)</th>
<th>Ethanol yield (1 tonne(^{1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banana</td>
<td>12-50</td>
<td>18-20</td>
<td>93-104</td>
</tr>
<tr>
<td>Cane molasses</td>
<td>2.4-4</td>
<td>50</td>
<td>258-291</td>
</tr>
<tr>
<td>Cashew apple</td>
<td>--</td>
<td>11.6</td>
<td>60-68</td>
</tr>
<tr>
<td>Cassava</td>
<td>8.7</td>
<td>30</td>
<td>172-194</td>
</tr>
<tr>
<td>Cocoa pulp</td>
<td>--</td>
<td>13.4</td>
<td>70-78</td>
</tr>
<tr>
<td>Corn (maize)</td>
<td>3.2</td>
<td>60</td>
<td>345-388</td>
</tr>
<tr>
<td>Mango</td>
<td>--</td>
<td>15</td>
<td>58-66</td>
</tr>
<tr>
<td>Millet</td>
<td>6.0</td>
<td>72</td>
<td>414-466</td>
</tr>
<tr>
<td>Pine apple</td>
<td>30</td>
<td>14</td>
<td>43-49</td>
</tr>
<tr>
<td>Potato</td>
<td>1.6</td>
<td>17</td>
<td>98-110</td>
</tr>
<tr>
<td>Rice</td>
<td>2.6</td>
<td>73.4-80.8</td>
<td>422-475</td>
</tr>
<tr>
<td>Sorghum</td>
<td>1.3</td>
<td>68-74</td>
<td>391-440</td>
</tr>
<tr>
<td>Sugar-cane</td>
<td>5.6</td>
<td>13-14</td>
<td>67-76</td>
</tr>
<tr>
<td>Sweet potato</td>
<td>8.3</td>
<td>27</td>
<td>154-173</td>
</tr>
</tbody>
</table>

Source: Adams and Flynn (1982)\(^3\)
The cashew apple contains much tannin and is perishable. For this reason, in many parts of the world, the false fruit is simply discarded after removal of the cashew nut. Cashew apples can be juiced easily since they do not contain seed, however, juicing must be done immediately after harvest.

Cashew is propagated mainly by seeds, resulting in high levels of genotypic and phenotypic variability (Philip and Unni, 1984). Tree yields in farmers' field have been found to be highly varied such that, some trees may yield nothing whilst the best usually produce over 20 kg of nuts (Martin and Kasuga, 1995). It tolerates a wide range of rainfall regimes and is adapted to many soil types including marginal degraded land on which it can be planted for reforestation and protect the adjacent fertile agricultural land from drifting sand (Morton, 1960; Patro and Behera, 1979; Odunsi, 2002). Cashew trees normally flower at the end of rainy season, when new shoots emerge. The flowers develop at the ends of the shoots. Medicinal uses of cashew bark, leaves and apple juice are plentiful and were well known prior to recorded history. The ripening process takes place from September to January, harvesting period is between January and April with peak between February and March, and a 4-year old tree can produce from 100 to 150 kg of cashew apple per year (Tassara and Silva, 2002).

2.2.1 Cashew nut:

The market of cashew nut grows at 10 percent every year. Cashew nut kernel has an indisputably exclusive fine taste and a commercial attractiveness of its own. It is employed as a cocktail delicacy or in the food industry for the manufacture of biscuits, ice creams and chocolates. The cashew nut shell liquid, which is extracted
from the shell surrounding the kernel is an astringent corrosive oil useful in the manufacture of clutch, brake linings, paints and plastics (Odunsi, 2002). Modern uses of shell oil include facial peels and scalp conditioners and shampoos.

2.2.2 Cashew apple:

The edible cashew apple is thick receptacle or "false fruit" to which the cashew nut or true fruit is attached. Although there is growing awareness surrounding the economic importance of cashew production in Asian and African countries, the present practice in most established large-scale plantations is to allow the apples to fall from the trees naturally before harvesting the nuts and then the nuts are detached from the cashew fruits. This contributes a gross wastage of this versatile cashew apples due to lack of good preservative techniques (Aroyeun, 2004; Mohanthy et al., 2006).

Cashew "apple" contains high nutritive values (high content of vitamin C, minerals, i.e., Ca, P, Fe (Ogunmoyela, 1983; Moura, 1998), carotenoids and phenolic compounds (Fenech, 2001; Melo Cavalcante et al., 2005). Cashew apples yield about 60-70% of their weight as juice. It has poor flavors, due to tannins and oils. Juice can be frozen or sterilized at 121°C in an autoclave and stored at room temperature for several weeks.

The present consumption of this apple is about 10% of production. To reduce this waste, it was thought worthwhile to assess the utilization of this material for human consumption in local cottage industries. In Brazil, the apple juice is given some slight processing before being bottled and sold (Lopez et al., 1985). Cashew apples are variously processed for jam, jelly, vinegar, pectin, nonalcoholic and...
alcoholic beverages (Chacrabory et al., 1977; Ogunmoyela, 1983; Maini and Anandh, 1993; Moura, 1998; Oduwole et al., 2001; Muniz et al., 2006).

2.2.2.1 Carbohydrates

The use of the cashew apple as a source of high added-value sugar may present an attractive economic alternative to the Northeast region (Azevedo and Rodrigues, 2000). Cashew apple juice has been reported in the literature (Lopes, 1972; Lima, 1988), to have 80-100g/l of reducing sugars present in the aqueous phase of cashew apple juice. The content of soluble solids (°Brix) ranged from 10.2 to 12.6 for all varieties of cashew apple (Assuncao and Mercadante, 2003).

According to Silva (1998), ripe cashew apple should present a Brix values from 9.8 to 14.0. The reducing sugars of cashew pulp were 5.74% reported by Oliveira et al., (1999). The cashew flour had crude fibre (ca. 20-33%) and carbohydrate (ca. 16-47%) values (Adariye, 1992). Azevedo and Rodrigues, (2000), who worked together proposed a potential economic alternative for the cashew apple juice as a fructose source. Juice has properties allowing itself to be fermented to ethanol easily. Cashew apple has been considered a good substrate for ethanol fermentation since it has high sugars and easily cultivable in the world.

2.2.2.2 Vitamins

The cashew apple has more vitamin C than Guavas, Mangoes and Oranges (Behrens, 1996) and higher amounts of minerals than other fruits. Vitamin C, a necessary constituent of human diets and deficiency causes scurvy and delays the healing of wounds. Its use will increase the nutritional health of the rural
communities. It is important to note that the vitamin C content of cashew juice is higher than that of orange juice. As cashew apple is an excellent source of vitamin C, the ascorbic acid content was determined as a quality parameter to evaluate the final product (Azoubel and Murr, 2003)\textsuperscript{31}. The ascorbic acid content of unsteamed cashew apple juice was \textit{287mg/100ml}. Steaming of the cashew apple prior to juice extraction resulted in a decreased (\textit{230mg/100ml}) content of ascorbic acid (Inyang and Abah, 1997)\textsuperscript{155}.

#### 2.2.2.3 Anti-microbial activities

Studies have shown cashew apple juice to have anti-bacterial, anti-fungal and anti-tumor activities (Kubo \textit{et al.}, 1993\textsuperscript{195}, 1993\textsuperscript{194}; Kozubek \textit{et al.}, 2001\textsuperscript{191}) as well as anti-oxidant effects (Melo Cavalcante \textit{et al.}, 2003)\textsuperscript{236} and antimutagenic activity (Santos \textit{et al.}, 2002)\textsuperscript{342}. Kubo \textit{et al.}, (1999)\textsuperscript{196}, reported that the cashew fruit exhibited antibacterial activity against the Gram-negative bacterium \textit{Helicobacter pylori}, which is now considered to cause acute gastritis and stomach ulcers. The antioxidant capacity of anacardic acid-1 is more related to inhibition of superoxide generation and xanthine oxidase than the scavenging of hydroxyl radicals (Trevisan \textit{et al.}, 2006)\textsuperscript{382}.

#### 2.2.3 Cashew apple products

Cashew wine is made in many countries throughout Asia and Latin America. It is a light yellow alcoholic drink with an alcohol content of 6 to 12 percent. Cashew apple has been reported to be good for wine production and whose commercial production has started in India (Osho and Odunfa, 1999)\textsuperscript{279}. Alcoholic beverages from cashew apple are also prepared in Nigeria in small-scale industries (Ogunmoyela,
An undistilled alcoholic beverage (wine) was prepared by fermenting cashew apple juice with wine yeast, *Saccharomyces cerevisiae* var. *bayanus*. The cashew apple wine as quite acceptable as an alcoholic beverage, has significant differences existing between the cashew wine and the commercial grape wine particularly in taste, aroma, flavor because of probably high tannin content in the cashew wine (Mohanthy *et al.*, 2006).

Abreu, (1997) produced a sparkling wine from cashew apple with an alcoholic content of 7.72%; TSS (°Brix), 11.53. Similarly, Osho and Odunfa (1999) fermented cashew apple juice using commercially available wine yeast (*Saccharomyces cerevisiae*) and three other wine yeasts. Fermentation of cashew juice produced wine of alcoholic contents of 10% with *S. cerevisiae* (Owuama and Saunders, 1990). The concentration of alcohol could reach to 100 g/l at the end of 48 hours, resulting in a desired alcoholic grade of cashew wine (Silva *et al.*, 2007).

The esters methyl 3-methyl butyrate, ethyl 3-methyl butyrate, methyl butyrate, ethyl butyrate, trans-ethyl crotonate and methyl 3-methyl pentanoate were important to the sweet, fruity and cashew like aroma of the beverage (Garruti Deborah *et al.*, 2006). The wine produced using *Saccharomyces cerevisiae* NCYC 125 was found acceptable by the consumer panelists on the basis of color, aroma, bouquet and taste (Mohanthy *et al.*, 2006). The panelists rated aroma and flavor scores between 2.5-3.0 (like moderately-like much) because of probably high tannin content in cashew wine, which imparted a somewhat astringent flavor. Nevertheless, the cashew wine was acceptable to all the panelists (Mohanthy *et al.*, 2006).
Cashew cake had a calorific value of 293.8/100 g and may be useful in feeding diabetic patients who require low carbohydrate foods (Aderiye et al., 1992)\(^5\). It has been reported that cashew apple was used as a nutritional additive in the production of yogurt (Aroyeun, 2004)\(^{24}\) and the juice as a substrate for obtaining single cell protein (Layokun, 1984\(^{205}\); Osho and Odunfa, 1999\(^{275}\)). Cashew apples are used in various food preparations and to make the popular alcoholic beverage known as ‘fenny’ (Rale, 1985)\(^{307}\), a biosurfactant production (Rocha et al., 2006)\(^{327}\).

2.3 Microorganisms

Apart from *Saccharomyces cerevisiae* there are many other microorganisms, which have been used for ethanol production. Ethanol production can be carried out using bacteria as the fermentative agent. *Zymomonas mobilis* was used by many workers (Vuuren and Meyer, 1982\(^{393}\); Fein et al., 1983\(^{108}\); Suntinanalert et al., 1986\(^{366}\); Nain and Rana, 1987\(^{253}\); Jones and Doelle, 1991\(^{160}\); Moreau et al., 1997\(^{243}\); Sreekumar et al., 1999\(^{358}\); Ingram et al., 2000\(^{154}\); Ratnam et al., 2006\(^{318}\); Sootsuwan et al., 2007\(^{356}\); Bai et al., 2008\(^{33}\)). Mixed culture of *Zymomonas mobilis* and *Candida tropicalis* were also useful for ethanol production (Patle and Lal, 2007)\(^{284}\). But the drawbacks in using this organism include its inability to utilize the broad spectrum of substrates and it requires a completely sterile environment. Different organisms including bacteria were used on various substrates for the production of alcohol such as *Kluyveromyces* spp. (Duvnjak et al., 1981)\(^{99}\), *C. thermosulfurogenes* (Hyun, 1986)\(^{152}\); *Clostridium thermocellum* (Knutson et al., 1999)\(^{184}\), *Pichia stipitis* (Nigam, 2001\(^{289}\); Talli-Okur and Eken-Saracoglu, 2008\(^{371}\)), *Zymobacter palmae* (Yanase et al., 2005)\(^{912}\), *Lactobacillus buchneri* (Liu et al., 2007)\(^{212}\), *Pachysolen tannophilus* (Cheng et al., 2008)\(^{69}\) and Engineered *E.coli* (Zhou et al., 2008)\(^{421}\).
2.3.1 Yeast biology

Yeasts, which have been known to humans for thousands of years as they have been used in fermentation process like the production of alcoholic beverages, are a form of eukaryotic microorganisms (Ramize et al., 1999\textsuperscript{311}; Wartmann and Kunze, 2000\textsuperscript{400}; Veno et al., 2003\textsuperscript{388}). Previously few investigators have done good piece of work using yeast. They used apple pieces, grape skins and quince and pear pieces as solid supports for wine production in batch and continuous fermentation (Birch and Walker, 2000\textsuperscript{46}; Mallouchos et al., 2002\textsuperscript{223}; Kourkoutas et al., 2003\textsuperscript{190}; Mallios et al., 2004\textsuperscript{222}). Many scientists worked on yeast taxonomy and finally found it close to fungi. Yeasts are a phylogenetically diverse group of unicellular fungi. Hence it is placed under the division Mycota. In monograph, yeasts comprise 83 genera and 590 species (Barnett et al., 1990\textsuperscript{37}).

2.3.2 Degradation of glucose by \textit{Saccharomyces cerevisiae}

Yeasts may oxidize glucose by two major routes, 1) HMP pathway and 2) via the classical Embden-Meyerhof pathway. The oxidation of glucose via the hexose monophosphate (HMP) pathway was very well documented by Horecker and Mehler (1955)\textsuperscript{147}. Reports also exist with evidences of acetate formation during glucose uptake and that HMP pathway is mainly responsible for the oxidation of 0 to 30\% glucose (Blumenthal et al., 1954)\textsuperscript{51}.

However, Eaton and Klein (1954)\textsuperscript{100} have indicated that it is not an important pathway of glucose degradation in young cells. Base studies using [3:4-C\textsuperscript{14}] glucose confirmed that during aerobic degradation of glucose by resting cells of \textit{Saccharomyces cerevisiae}, 80 to 90\% of the carbon at positions 3 and 4 of glucose
were converted into CO$_2$ and the remaining was found as ethanol (Eaton and Klein, 1957)$^{101}$. Studies on the growth of *Saccharomyces cerevisiae* anaerobically on glucose in chemostat culture have shown that in addition to alcohol, substantial quantities of glycerol may also be formed along with some pyruvate (Fiechter *et al.*, 1981)$^{110}$.

### 2.3.3 Biochemistry of fermentation

Gay-Lussac in 1815 established the fundamental changes in fermentation, which he summarized as one molecule of glucose yields two molecules of ethanol and two molecules of carbon dioxide.

$$\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{C}_2\text{H}_5\text{OH} + 2\text{CO}_2$$

Buchner in 1897 showed that the juices from yeast cells could perform the same fermentation process. In 1950, Biochemists Embden, Meyerhof, Robison, Neuberg, Coris and Paranas succeeded in elucidating the mechanism of alcoholic fermentation which has been named as the Embden-Meyerhof-Paranas pathway or the glycolytic pathway (Fig. 2.1) (Nord and Weis, 1958)$^{262}$. The sequence of reactions is shown in figure. As per the Gay-Lussac’s equation, alcoholic fermentation of hexoses likes glucose, fructose, galactose and mannose when fermented: 100 kg of sugar will yield 51.142 kg (=64.442 L) of 100 percent alcohol. The disaccharides like sucrose, maltose, lactose and melibiose which contain two hexoses on fermentation: 100 kg of sugar will yield 53.833 kg (=67.834 L) of 100 percent alcohol. In the case of polysaccharides like the starch (or cellulose): 100 kg of sugar will yield 56.824 kg (=71.603 L) of 100 percent ethanol (Albert Hansen *et al.*, 1948)$^{12}$. 
Fig. 2.1: The Embden-Meyerhof-Parnas pathway of ethanol fermentation.
The metal ions required for the expression of the enzymes activity are $K^+$, $Mg^{2+}$, $Mn^{2+}$ etc. Yeast cells have to be provided with the consistent conditions of pH and temperature necessary for the enzymatic activity. The over all reaction may be summarized as below.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + \text{energy}.$$ 

The reaction is actually very complex. Impure cultures of yeast produce varying amounts of other substances, including glycerin and various organic acids. In the production of beverages, such as whiskey and brandy, impurities supply the flavor.

2.3.4 Explanation of the biochemical reactions

The transport of glucose across the cell membrane is facilitated by a permease, which is a transport protein. Once the glucose enters the cells, it undergoes the following sequence of reactions.

1. Glucose is phosphorylated to Glucose-6-Phosphate by the enzyme hexokinase. ATP donates the phosphate ($P$) ion.

2. Glucose-6-P is converted to its isomeric form fructose-6-P by the enzyme phospho hexose isomerase.

3. Phospho fructo kinase adds one more phosphate from ATP resulting in fructose-1, 6-diP.

4. This 6 carbon molecule is cleaved into two-3-carbon molecules, dihydroxy acetone phosphate and glyceraldehydes-3-phosphate by the enzyme aldolase. The interconversion of the two molecules is catalyzed by
Phospho triose isomerase. As and when glyceraldehydes-3-P gets consumed during oxidation steps, dihydroxy acetone phosphate gets converted to Glyceraldehyde-3-P, which participates in the next steps.

5. Glyceraldehyde-3-P is oxidatively phosphorylated by glyceraldehydes-3-Phosphate dehydrogenase to yield 1,3-diphospho glyceric acid and a molecule of NADH$_2$.

6. Phospho glycerol kinase dephosphorylates the molecule to -3phospho glyceric acid with the release of an ATP.

7. Phospho glycerol mutase transfers the position of phosphate group and results in the formation of 2-phospho glyceric acid.

8. Enolase catalyses the removal of water from 2-phospho glyceric acid to form phosphoenol pyruvic acid.

9. This is dephosphorylated to yield pyruvic acid by the enzyme pyruvate kinase.

10. Pyruvate decarboxylase removes CO$_2$ from pyruvic acid in the presence of thiamine pyrophosphate and results in the formation of acetaldehyde.

11. Alcohol dehydrogenase catalyses the final steps to form alcohol from acetaldehyde. A molecule of NADH$_2$ is consumed in this reaction.
The ATP consumed and released in the reactions are:

<table>
<thead>
<tr>
<th>Step</th>
<th>Energy from release(+)/ consumed(-)</th>
<th>ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATP consumed</td>
<td>-ATP</td>
</tr>
<tr>
<td>3</td>
<td>ATP consumed</td>
<td>-ATP</td>
</tr>
<tr>
<td>5</td>
<td>NADH(_2) released</td>
<td>(2 \times 2) ATP</td>
</tr>
<tr>
<td>9</td>
<td>(2) ATP released</td>
<td>(2) ATP</td>
</tr>
<tr>
<td>11</td>
<td>NADH(_2) consumed</td>
<td>(2 \times 2) ATP</td>
</tr>
</tbody>
</table>

Net yield \(2\) ATP

Thus two ATP are produced per molecule of glucose and the reaction is exothermic as 56,000 calories (240KJ) of energy per mole of glucose are potentially available and \(2\times8000\) calories are retained as ATP. The efficiency of the process is around 29%. This ATP is used both to maintain itself and to produce new cells. If no respiration occurs, the amount of glucose is oxidized (Warren et al., 1990)\(^{399}\). The characteristic rise in temperature during fermentation is due to the energy not retained by the cells and which is lost as heat.

When *Saccharomyces* respire (when air is supplied) complete oxidation of glucose takes place with the liberation of 686,000 calories of energy (2.9 MJ) per molecule of glucose. This happens only when sugar concentration is very low (<1.0\%). There can be a modification of the permease affinity for glucose in cultures when sugar is totally exhausted and the cells start to use ethanol as the substrate (Walsh, 1994)\(^{395}\). In yeast the diauxic shift is found, since glucose is primarily metabolized fermentatively, even in the presence of ethanol and oxygen. Only
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glucose is exhausted while the cells shift to metabolism of ethanol, the quantitative aspect of the signaling is illustrated by the fact that in glucose-limited chemostats, where the steady-state concentration of this compound remains low, both glucose and ethanol are metabolized simultaneously. Rizzi et al (1996)\textsuperscript{326}, studied the effect of glucose-6-phosphate within the cells on glucose transport at different process conditions in continuous cultures.

2.4 Methods for ethanol production

Ethanol can be produced mainly by two methods. One method is by chemical synthesis and the other is via fermentation. Two process definitions that are important to the production of ethanol are as follows:

2.4.1 Chemical synthesis method

Synthesis of ethanol by chemical method for commercial purposes started around the year 1945 and dominated the world market. Michael Faraday prepared ethanol by the acid-catalyzed hydration of ethylene in 1828, a process similar to that used for industrial ethanol synthesis today. Ethylene is a byproduct in recovery of natural gas and also in gasoline production. The increasing crude oil prices of the organization of petroleum exporting countries is now drawing the attention of the importing countries back to the fermentative method of ethanol production.
2.4.2 Fermentative method

The process of conversion of sugars to energy was known to the ancient Egyptians and Mesopotamians, who brewed beer almost 5000 years ago (Scheller and Mohr, 1977). Ethanol for use in alcoholic beverages and the vast majority of ethanol for use as fuel is produced by fermentation. When certain species of yeast and bacteria, most importantly *Saccharomyces cerevisiae*, metabolize sugar in the absence of oxygen, they produce ethanol and carbon dioxide. Ethanol can be made from a wide variety of biological materials. Carbohydrates are the principle raw materials used in fermentation method and these are subjected to conversion using yeast. These raw materials may be broadly classified into three categories based on the type of carbohydrates present.

1. Saccharine materials
2. Starchy materials
3. Cellulosic materials

1. Saccharine materials

Carbohydrate (the actual substance from which alcohol is made) is present in the form of simple, directly fermentable six and twelve carbon sugar molecules such as glucose (Gerald and Charles, 1977), fructose (Suntinanalert *et al.*, 1986) and maltose. Such materials include, cane molasses (McGinnis, 1951), fruit juices (Holzberg *et al.*, 1967), pulverized cherokee rose fruit (He Quanjian *et al.*, 1997), whey permeate (Patrice Vienne and Urs Von Stockar, 1983), banana (Rosario and Pamatong, 1985), apple pomace (Hours *et al.*, 1985), mango fruit juice (Blaggi *et al.*, 1986), pineapple juice (Hilary, 1999), lactose
(Szczodrak, 2004)\textsuperscript{370}, palmyra jaggery (Ratnam \textit{et al.}, 2001\textsuperscript{315}, 2005\textsuperscript{319}, 2007\textsuperscript{317}), sucrose (Carvalho \textit{et al.}, 2008)\textsuperscript{60}, skim milk and sugar beets etc.

2. \textbf{Starchy materials}

It contains more complex carbohydrates such as starch and inulin that can be broken down into the simpler six and twelve carbon sugars by hydrolysis with acid or by the action of enzymes in a process called malting. Such materials include barley (Underkofler \textit{et al.}, 1946)\textsuperscript{384}, millets (Novellie, 1976)\textsuperscript{264}, sweet potato (Chua \textit{et al.}, 1984)\textsuperscript{73}, roots of cassava (Del Rosario Ernesto \textit{et al.}, 1984)\textsuperscript{95}, sugarcane bagasse (Salomon \textit{et al.}, 1988)\textsuperscript{340}, wheat (Raur \textit{et al.}, 1990)\textsuperscript{320}, jerusalem artichoke (Koren and Duvnjak, 1991)\textsuperscript{187}, potato pulp (Yunoki \textit{et al.}, 2004)\textsuperscript{415}, starch (Knox \textit{et al.}, 2004)\textsuperscript{183}, sago starch (Ratnam \textit{et al.}, 2003\textsuperscript{316}, 2006\textsuperscript{318}), corn (Lehman and Rosentrater, 2007\textsuperscript{207}; Nowak \textit{et al.}, 2008\textsuperscript{265}), potato starch (Young \textit{et al.}, 2008)\textsuperscript{414}, sunflower seed (TallI-Okur and Eken-Saracoglu, 2008)\textsuperscript{371} etc.

3. \textbf{Cellulose materials}

Sugars for ethanol fermentation can be obtained from cellulose, which includes complex molecules made up of glucose, derivatives of glucose and pentoses. Few examples are cellulose (Ramachandram and Hassim, 1990)\textsuperscript{308}, hemi cellulose (Perego \textit{et al.}, 1990)\textsuperscript{290}, saw dust (Taragonksi \textit{et al.}, 1985)\textsuperscript{375}, wood (Parekh \textit{et al.}, 1986)\textsuperscript{283}, rice straw (Peris, 1987)\textsuperscript{291}, wastes from paper mill (Xin \textit{et al.}, 1993)\textsuperscript{410}, Moringa oleifera leaves (Makkar and Becker, 1996)\textsuperscript{221}, lignocellulose (Ingram, \textit{et al.}, 2000)\textsuperscript{154}, wheat straw (Nigam, 2001)\textsuperscript{259}, cellulosic
biomass (Ramesh et al., 2005)\textsuperscript{310}, barley straw (Linde et al., 2006)\textsuperscript{210}, cellulosic material (Rabinovich et al., 2006)\textsuperscript{303}, agricultural wastes (Chen et al., 2007\textsuperscript{68}; Patle and Lal, 2007\textsuperscript{284}), garcinia kola pulp (Nzelibe et al., 2007)\textsuperscript{266}, sugar cane bagasse (Cheng et al., 2008)\textsuperscript{69} etc., which contain material that can be hydrolyzed with acid, enzymes or otherwise converted into fermentable sugars called "glucose".

2.4.3 Different fermentation processes and methods

Bioreactor offers possibility to provide an optimally controlled environment for microbial fermentation processes required for optimal yield (Williams, 2002)\textsuperscript{406}. Fermentation is the process by which living cells are able to obtain energy through the breakdown of glucose and other simpler sugar molecules without requiring oxygen. Fermentation is achieved by somewhat different biochemical sequences in different species of organisms. Generally fermentation may be carried out as a batch, continuous and fed-batch processes (Stanbury and Whitaker, 1984)\textsuperscript{360}. Ethanol can be produced by different ways viz., batch, fed-batch, continuous, and semi continuous processes (Caylak, 1998)\textsuperscript{62}, (Zanin et al., 2000)\textsuperscript{417}. However, most of the ethanol is produced by batch fermentation process because it is economical (Caylak and Vardar Sukan, 1998)\textsuperscript{62}. 

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2.4.3.1 Batch fermentation

Batch fermentation can be considered to be a closed system, in which the composition of the culture medium, the biomass concentration, and the metabolite concentration change constantly with time as a result of metabolism of the cells, throughout the fermentation process. In batch fermentation four phases of growth are observed, i.e., lag phase, log phase, stationary phase and death phase. Batch production can reduce initial capital outlay because a single production line can be used to produce several products. Batch fermentation has been widely used for ethanol production from various substrates through shake flasks (Brayan and Silman, 1991)\textsuperscript{55}, batch stirred tank reactor (Chithra and Baradarajan, 1992)\textsuperscript{71}, membrane separation bioreactor (Gryta et al., 2000)\textsuperscript{131}, batch bioreactor (Pramanik, 2003; Laopaiboon et al., 2007\textsuperscript{199}; Riverol and Cooney, 2007\textsuperscript{325}; Xiaojing et al., 2008\textsuperscript{409}).

2.4.3.2 Fed-batch fermentation

It is a system intermediate between batch and continuous process. The term describes batch cultures that are fed continuously, sequentially with fresh media along without removal of culture fluid, volume of the culture increases with time. The controlled addition of the nutrient directly affects the growth rate of the culture and allows to avoid overflow metabolism (ethanol in \textit{Saccharomyces cerevisiae}), oxygen limitation (anaerobiosis). Substrate limitation allows metabolic control, avoid osmotic effects, catabolite repression and overflow metabolism of side products. Fed-batch fermentation is a potential operation mode for effective ethanol production (Curto and Tripodo, 2001\textsuperscript{86}; Shengdong et al., 2006\textsuperscript{346}). Fed-batch fermentation has been used
widely for the production of ethanol through fungal fermentations (Ballesteros et al., 2002)\textsuperscript{36}, other products (Pernas et al., 2000\textsuperscript{292}; Zhong-Ce et al., 2007\textsuperscript{420}).

2.4.3.3 Continuous fermentation

In continuous operation fresh nutrients are added continuously into the reactor and equivalent amount of converted nutrient solution along with microorganisms is simultaneously taken out of system. The loss of biomass at any time is exactly balanced by the cell growth inside the bioreactor, under same conditions and the system is said to be under steady state. Prince and Barford (1982)\textsuperscript{399} have described ethanol fermentation in tower fermentor using natural flocculating yeast. Production of ethanol by means of continuous fermentation with continuous product removal, is performed in vacuferm (Cysewski and Wilke, 1977\textsuperscript{87}; Ramalingam and Finn, 1977\textsuperscript{309}), BIOSTIL (Cook, 1980)\textsuperscript{79}, Hollow fiber fermentor (Mehaia and Cheryan et al., 1984)\textsuperscript{231}, Stirred tank reactor (Hwai-Shen and Hsien-Wen, 1990)\textsuperscript{151}, biofluidized-bed (Melin et al., 1992)\textsuperscript{332}, membrane bioreactor (Kargupta et al., 1998)\textsuperscript{173}, tower reactor (Viegas et al., 2002)\textsuperscript{391}, combined bioreactor system (Bai et al., 2004)\textsuperscript{34}, series fermentation system (Xu et al., 2005)\textsuperscript{411}, Tower type reactor (Tang et al., 2006)\textsuperscript{374}, serial bioreactors (Purwadi et al., 2007)\textsuperscript{301}.

2.4.3.4 Submerged fermentation (SmF) method

Submerged fermentation is the most popularly used technique for the production of a large number of products using wide range of microorganisms. The medium used for submerged fermentation contains relatively high processed ingredients. Sterilization and process control are easier. In submerged fermentation
the substrate is dissolved or suspended as small particles in a liquid, usually water, along with the active microorganisms (Tanaka et al., 1986)\textsuperscript{373}. And these processes were developed utilizing the cheap carbohydrates, both saccharine and starchy materials. The saccharine materials include fruit juices (Quereshi and Tamahane, 1984)\textsuperscript{302}, apple pomace (Hours et al., 1985)\textsuperscript{149} and sweet sorghum (Garcia and Primo, 1986)\textsuperscript{115}. The SmF processes have also been used for the fermentation of other products (Peinado et al., 2004\textsuperscript{289}; Joshi et al., 2006\textsuperscript{168}; Kim et al., 2007\textsuperscript{177}; Rossi et al., 2007\textsuperscript{334}). Most enzymes are manufactured using the conventional technique of SmF, where microbial cells are suspended in a large volume of water that is stirred and aerated using mechanical devices (Vinieggra-Gonzalez and Favela-Torres, 2006)\textsuperscript{392}. The water activity of the medium is high, making it prone to contamination if asepsis is not maintained.

2.4.3.4.1 Bioreactors used in submerged fermentation

Process control through bioreactor in submerged cultures is based on the measurement of physical, chemical and biochemical properties of the broth, such as pH, dissolved oxygen, temperature, agitation rate and others, using dedicated probes followed by the manipulation of the physico-chemical properties of the culture with suitable actuators (Lim and Lee, 1991)\textsuperscript{208}. Some of the key process parameters play an important role in fermentation (Ghosh and Swaminathan, 2003)\textsuperscript{121}. Fermentation is a novel technique which can overcome some of the limitations of conventional fermentation and yield high productivities. Traditionally, industry still uses the surface technique, however, at present a much greater emphasis has been placed on the use of submerged culture, batch techniques in stirred tank reactors (Lockwood, 1975)\textsuperscript{213}.
2.4.3.5 Liquid surface fermentation (LSF) method

It is the oldest fermentation method for ethanol production. The fermentation medium is inoculated with cells of *S. cerevisiae* by blowing air through the sterile solution in the pans with temperature maintained at 28-30°C. Fermentation time is relatively high from 8-10 days and the cells grow in the form of a folded mat on the surface of the liquid medium. LSF is not commercially exploited.

2.4.3.6 Solid state fermentation (SSF) method

Solid state fermentation (SSF) is generally defined as the growth of microorganisms on moist solid material in absence or near absence of free water and it is mainly used in antibiotic production. SSF has been used widely for the production of ethanol (Sandhu and Joshi, 1997) and variety of products through fungal fermentations (Shotwell *et al.*, 1966; Aiba *et al.*, 1976; Joshi and Sandhu, 1996; Kashyap *et al.*, 2002; Babitha *et al.*, 2006; Bhatti *et al.*, 2007; Balkan and Ertan, 2007) and fruity aroma production (Christen, 1997; Adriana Bramorski, 1998). Kirby and Marden (1980) have used the solid state fermentation technique for chopped sugar beet. Over the last 15 years mathematical models have been developed with the intention of using them as tools to identify SSF bioreactor design and operating strategies that can overcome these difficulties (Mitchell *et al.*, 2003). Recent models have not only included water balances, but have also described how the growth rate of microorganism is affected by the amount of water in the solids (Mitchell *et al.*, 2004). The fermenting solids must be taken into account in mathematical models of solid-state fermentation bioreactors (Marques *et al.*, 2006).
2.5 Factors influencing fermentation

Optimization of the physical and chemical conditions for any fermentation is carried out by batch fermentations using free cells (Chen, 1981)\textsuperscript{67}. The physical parameters as well as the chemical parameters effecting the ethanol production by yeast are as follows.

2.5.1 Physical parameters

The physical conditions necessary for efficient conversion of glucose to ethanol by yeast were maintained, such as substrate concentration, pH, temperature, oxygen, fermentation time, seed culture volume and seed culture age.

2.5.1.1 Substrate concentration

It is a known fact that a molecule of glucose yields two molecules of ethanol and two molecules of carbon dioxide. At very low substrate concentration yeast is starved and the productivity decreases. At a reducing sugar concentration of 30\% (w/v), the substrate concentration showed an inhibitory effect on the yeast growth as well as on the rate of ethanol production (Ghose and Tyagi, 1979)\textsuperscript{119}. Many scientists have reported the same inhibitory effect and it is attributed to the high osmotic pressure caused due to high sugar concentrations (Pironti, 1971)\textsuperscript{295}.
2.5.1.2 pH

The optimum pH of the medium is one of the factors effecting growth, product formation of microorganisms and the characters of their metabolism. The \( \text{H}^+ \) or \( \text{OH}^- \) ion concentrations may have a direct effect on the cell or it may act indirectly by varying the dissociation degree of substances available in the medium.

pH is an important factor in alcoholic fermentation, since it is related with infections of the culture, affects the number of fermentation batches with the same yeast culture and therefore affects the ethanol yield (Akrida-Demertzi, et al., 1988)\(^{11}\). Yeast has been reported to excrete nucleotides, which are acidic, during fermentation (Higuchi and Uemura, 1959)\(^{143}\). Organic acids are excreted throughout fermentation (Coote and Kirsop, 1974)\(^{83}\). Most strains of \textit{S. cerevisiae} have exhibited a broad range of pH between 2.4 and 8.6. Optimal growth is usually reported at pH 4.5 (Jones et al., 1981)\(^{164}\). The ethanol production rate was optimum in the pH range of 5.5–6.0, and an ethanol yield of 0.41–0.46 g/g was obtained in the fermentation of wood (Sreenath and Jeffries, 2000)\(^{359}\).

2.5.1.3 Temperature

The optimum temperature differs from different groups of microorganisms. Deviation of temperature from the optimum range will retard the growth of microorganism and decrease or sometimes stop the yield of the ethanol. This may be due to the substantial effect of temperature on the activity of the enzyme, the activity of the transport system, and other important physical and biochemical functions of the microbial cell.
Temperature is an essential parameter that affects the yeast growth and governs its metabolism (Noor et al., 2005)\textsuperscript{261}. The inhibitory effect of ethanol concentration on the yeast growth rate depends strongly on the fermentation temperature (Boulton, et al., 1996)\textsuperscript{52}. Surprisingly few comprehensive studies have been published, although it is clear that both optimum and maximum tolerable temperatures for growth and fermentation are strongly strain dependent (Walsh and Martin, 1977)\textsuperscript{396}. Growth near the maximum temperature has been reported causing destabilization of the plasma membrane (Essar, 1979)\textsuperscript{105} and a rapid decrease in cell viability (Krouwel and Braber, 1971)\textsuperscript{192}. However, both optimum and temperature tolerance for growth and fermentation are strongly strain dependent (Rousseau et al., 1992)\textsuperscript{335}.

The effects of temperature and yeast extract concentration on fermentation were described by Chen (1980)\textsuperscript{65}. Pramanik (2003)\textsuperscript{298}, reported that the optimum ethanol concentration was achieved at 30°C. Yeasts can be stored inactively at low temperature (>0°C) and are readily revived (Stark, 1954)\textsuperscript{361}.

2.5.1.4 Oxygen

The growth rate of Saccharomyces cerevisiae is also increased with increase in dissolved oxygen concentration. Dissolved oxygen concentration in the range of 5-10 parts per billion was shown to increase the specific ethanol production rate over the rates under anaerobic condition (Grosz Rong, 1981)\textsuperscript{130}. Addition of small amount of oxygen is shown to increase the percentage of unsaturated fatty acids in the plasma membrane as well as the total number of fatty acids (Rogers and Stewart, 1973)\textsuperscript{328}, which increased ethanol tolerance of the cells (Thomas et al., 1978)\textsuperscript{380}. Agitation
creates shear forces, which affect microorganisms in several ways like causing morphological changes, variation in their growth and product formation and also damaging the cell structure (Mittal, 1992). In the case of shake flask cultures the oxygen supply is indirectly a factor of agitation (Ghosh and Swaminathan, 2003). The agitation speed provides the maximum and uniform supply of nutritional materials provided in the medium and also the supply of oxygen which is an essential step to achieve the proper metabolic activity by the activity of mitochondria (Birch and Walkar, 2000). Agitation also favors oxygen supply to the cells that is important for high biomass concentration (Kongkiattikajorn et al., 2007). Excess oxygen in the fermentation medium, on the other hand, will promote respiration and cell growth at the expense of ethanol productivity (Kosaric et al., 1983).

2.5.1.5 Fermentation time

The optimum fermentation period for achieving higher yields of ethanol depends on the organism as well as the fermentation conditions. Fermentation is normally completed depending on substrate concentration. At low sugar concentrations fermentation reaction is fast and needs few hours whereas at high sugar concentration fermentation is slow and requires number of days.

Kargi et al., (1985) conducted solid state fermentation for ethanol production and that reported 72-75 h are required for complete fermentation.
2.5.1.6 Seed culture volume

The fermentation processes is greatly influenced by the yeast inoculums level (Birol et al., 1995). The inoculums level also affects the fermentation rate. A cell number of approximately $10^7$ cells/ml of compressed yeast has to be used to get good results (Rose and Harrison, 1970). At higher yeast concentrations the alcohol content increases but it is also reported to affect the fusel alcohol content (Watson and Hough, 1966). El-Diwany et al., (1992) reported that the increase in cell concentration (inoculums size) of the yeast above $3.6 	imes 10^5$ cells/100 ml decreased the ethanol yield. Whereas the percentage of ethanol yield was increased when the concentration of the yeast was increased from $1.09 \times 10^6$ yeast cell/10ml to $3.56 \times 10^6$ yeast cell/10ml (Akin-osanalaye, 2005).

2.5.1.7 Seed culture age

Tolerance to sugar and alcohol is variable for different strains in yeast (Gray, 1941, 1945). Furthermore, the degree of tolerance to each of these materials in a single strain can change depending on the age of the cultures and the previous conditions of growth (Gray, 1946; Nosiro and Ouchi, 1962). Seed culture age greatly influences fermentation processes (Birol et al., 1995). Sussman and Halvarson (1966), studied the variation of viability with age and they found that the viability of cells vary with age, consequently changes the generation properties. Pramanik, (2003) reported that the fermentation time was reduced from 96 to 63 hours by increasing the inoculums time from 12 to 48 hours.
2.5.2 Chemical and nutritional parameters

Ethanol tolerance of yeasts is strongly dependent on the composition of the medium in which it is evaluated (Casey and Ingledew, 1986\textsuperscript{61}; Kalmokoff and Ingledew, 1985\textsuperscript{170}; Nabais \textit{et al.}, 1987\textsuperscript{249}; Rosa \textit{et al.}, 1988\textsuperscript{329}). It is now admitted that the maximal concentration of ethanol that is characteristic of different alcoholic fermentations (for the production of beer, wine, or sake or for large-scale production of ethanol) is more dependent on medium composition than on the intrinsic ethanol tolerance of the industrial strains (Kalmokoff and Ingledew, 1985\textsuperscript{170}).

For optimal growth and fermentation, yeasts require micro and millimolar concentrations of different inorganic cations (Jones and Greenfield, 1984\textsuperscript{159}). Microelements play an important role in the cellular metabolism, primarily due to their requirements as cofactors for a large number of enzymes (Gadd, 1992\textsuperscript{114}). The optimal concentrations of various cations, which have been determined for several species of the genus \textit{Saccharomyces}, have been given in a review by Jones and Greenfield (1984\textsuperscript{159}). These ionic species could either act on the enzymatic activity by participating on the catalytic center or as activators or stabilizers, or they could play a structural role by shielding the negatively charged membrane phospholipids and cell wall phosphomannans (Jones and Greenfield, 1984\textsuperscript{159}).

2.5.2.1 Carbon source

Carbon source has dual role in biosynthesis as well as in the generation of energy. Apparently some of the biomass was formed from carbon sources other than glucose, namely carbon compounds in the yeast extract. For instance, Belaich and Senez (1965\textsuperscript{40}) reported that when \textit{Zymomonas mobilis} was grown on a complex
medium, 52% of the cellular carbon came from yeast extract or peptone. Most of the studies on the production of ethanol are from different substrates such as Saccharine materials (Section: 2.4.2: 1), Starches (Section: 2.4.2: 2) and Cellulosic materials (Section: 2.4.2: 3).

2.5.2.2 Nitrogen source

Many industrially important compounds, such as ammonia, nitric acid and organic nitrates, contain nitrogen. Nitrogen occurs in all living organisms- it is a constituent element of amino acids and thus of proteins, and also of nucleic acids (DNA and RNA). Most of the industrial microorganisms can utilize both organic and inorganic nitrogen sources. Organic nitrogen sources such as urea provide a good nitrogen source although addition of biotin suppression of the urea transport system is decreased when a better nitrogen source is available (Cooper, 1982)\textsuperscript{80}. Yeast extract is used as nitrogen for the production of ethanol fermentation medium (Cysewski and Wilke, 1976)\textsuperscript{88}. Nutrients such as tryptone, yeast extract and a mixture of purine & pyrididine bases increased the sugar uptake and ethanol production (Thomas \textit{et al.}, 1994)\textsuperscript{381}.

Many nitrogen sources support growth of \textit{S. cerevisiae} strains (Large, 1986)\textsuperscript{202}. Inorganic nitrogen sources, which are necessary may constitute up to 10% of the total assayable nitrogen, reduce catabolic enzyme levels and transport activity for nonpreferred nitrogen sources through a phenomenon known as nitrogen catabolite repression (Cooper and Sumrada, 1983)\textsuperscript{82}. Ammonia salts are used as nitrogen sources for ethanol production (Cysewski and Wilke, 1976\textsuperscript{88}; Kargi \textit{et al.}, 1985\textsuperscript{172}). Addition of ammonium sulfate (0.2 g L\textsuperscript{-1}) increases the production of alcohol to 6.19%
(Nahvi et al., 2002)\textsuperscript{252}. Shang (2006)\textsuperscript{345} demonstrated that growth on good nitrogen sources such as ammonia can harvest more yeast cells.

During fermentation, metabolism of nitrogen compounds by \textit{S. cerevisae} may govern the efficiency of alcoholic fermentation and affect the final product quality (Salmon and Barre, 1998)\textsuperscript{339}. Biosynthetic pools of amino acids are filled and the remaining nitrogenous compounds are utilized as nitrogenous sources (Cooper, 1982)\textsuperscript{81}. Once pools are filled growth begins, where nitrogenous compounds are taken up and degraded in a specific order depending on environmental, physiological and strain-specific factors (Jones, and Pierce, 1964)\textsuperscript{161}.

2.5.2.3 Phosphate source

Increasing the phosphate concentration appears to have stimulated growth of yeast. Phosphorus is assimilated in the form of dihydrogen phosphate ion, H\textsubscript{2}PO\textsubscript{4}\. The inorganic phosphate has been observed to play a key role in the growth of yeast, utilization of carbon sources and production of ethanol. The phosphate addition was accompanied by a morphological change with the increase in cell diameter (Mairella, \textit{et al.}, 1984)\textsuperscript{220}. Major role of phosphorus as a component of sugar phosphates, nucleic acid and nucleoside di or tri phosphates. It is also found condensed in organic phosphates and energy (Kulaev and Vagabov, 1983)\textsuperscript{197}. Cells also use phosphate to transport cellular energy via adenosine triphosphate (ATP). Nearly every cellular process that uses energy obtains it in the form of ATP. ATP is also important for phosphorylation, a key regulatory event in cells. Phospholipids are the main structural components of all cellular membranes. The intracellular concentrations of PO\textsubscript{4}^{2-}. 
regulate the synthesis of lipids and carbohydrates. Corn steep liquor supplemented with K$_2$HPO$_4$ enhanced the production of ethanol (Tang et al., 2006)\textsuperscript{374}.

2.5.2.4 Trace elements

Yeast has become a model microorganism for studying metal transporters and their accumulation in the cells (Nelson, 1999\textsuperscript{256}; Cohen et al., 2000\textsuperscript{76}). Jones and Gadd (1990)\textsuperscript{162} have suggested concentrations of many microelements which can be optimal for the yeast growth and the ethanol or higher concentrations for some biologically important microelements which may be applicable in nutritive medium. All microorganisms require certain trace elements. The trace elements fall into three broad categories (Rose, 1976)\textsuperscript{332}.

a) Macro elements (K, Mg, Ca, Zn, Fe, Mn, Cl) are required in the concentration of 0.1 to 1 mM and are taken up by facilitated diffusion

b) Micro elements (Co, B, Cd, Cr, Cu, I, Mo, Ni, Va) are required in the concentrations of 0.1 to 100 $\mu$M to affect growth and

c) Inhibitors (Ag, As, Hg, Li, Ni, Os, Pd, Se, Te) with concentrations greater than 10 to 100 $\mu$M affect growth and fermentation adversely.

The affect of different cations their stimulatory effect on growth and fermentation by yeast is extensively listed by Jones and Greenfield (1984)\textsuperscript{159}. The role of few inorganic nutrients and their approximate concentrations required for the growth of 25 g of yeast cells per liter listed in Table 2.3.
Table 2.3: Major inorganic nutrients and their role in yeast growth.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Role</th>
<th>Concentration μM/lt</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>Enhances tolerance to toxic ions involved in control of intracellular pH. K⁺ excretion is used to counter balance uptake of essential ions e.g., Zn⁺, Co²⁺, K⁺ stabilizes the optimum pH for fermentation.</td>
<td>20 x 10³</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Levels of Mg²⁺ are regulated by divalent cation transport system. Mg²⁺ seems to buffer the cell against adverse environmental effects and is involved in activating sugar uptake</td>
<td>5 x 10³</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Ca²⁺ is actively taken up by cells during growth and is incorporated into cell wall proteins. Ca²⁺ buffers the cell against an adverse environment. Ca²⁺ counteracts Mg²⁺ inhibition and stimulates the effect of sub-optimal concentration of Mg²⁺</td>
<td>1.5 x 10³</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>Zn²⁺ is essential for glycolysis and for the synthesis of some vitamins, uptake is reduced below pH 5, and two K⁺ ions are excreted for each Zn⁺ taken up.</td>
<td>50</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>Mn²⁺ is implicated in regulating the effects of Zn²⁺. Mn²⁺ stimulates the synthesis of proteins.</td>
<td>15</td>
</tr>
<tr>
<td>Fe²⁺,³⁺</td>
<td>Is the active site of many yeast proteins.</td>
<td>10</td>
</tr>
<tr>
<td>Na⁺</td>
<td>Passively diffuses into cells. Stimulates the uptake of some sugars</td>
<td>0.25</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>Act as a counter ion to the movement of some positive ions.</td>
<td>0.1</td>
</tr>
<tr>
<td>Mo²⁺</td>
<td>Stimulates growth at low concentrations.</td>
<td>0.5</td>
</tr>
<tr>
<td>CO²⁺</td>
<td>Stimulates growth at low concentrations.</td>
<td></td>
</tr>
<tr>
<td>B²⁺</td>
<td>Stimulates growth at low concentrations.</td>
<td></td>
</tr>
</tbody>
</table>

Source: Jones et al., (1981)¹⁶⁴
Magnesium

Magnesium is an important divalent cation in metabolic process and physiological functions, including cell growth, cell division and enzyme activity in yeast (Walker, 1994). Magnesium ions are essential to the basic nucleic acid chemistry of life, and thus are essential to all cells of all known living organisms. Many enzymes require the presence of magnesium ions for their catalytic action, especially enzymes utilizing ATP, or those which use other nucleotides to synthesize DNA and RNA.

Magnesium is an essential cofactor for many of the glycolytic enzymes and has also been identified as a limiting nutrient in fermentation broth containing peptone and yeast extract (Dombek and Ingram, 1986). The supplementation of yeast fermentations with 0.5 mM Mg²⁺ proved to prolong exponential growth and to reduce the decline in fermentative activity (Dombek and Ingram, 1986). Several reports have also demonstrated that magnesium ions are implicated in the amelioration of the detrimental effects of stress (Blackwell et al., 1997; Birch and Walker, 2000). However, in respect to ethanologenic S. cerevisiae, studies on such a protective effect of magnesium ions have hardly been reported.
Chapter-II

Review of literature

Calcium

The calcium forms a wide variety of complexes in biological systems (Glick, 1972)\textsuperscript{124} and competes directly with Mg\textsuperscript{2+} for enzymes binding on pyrophosphatase, pyruvate kinase, enolase, membrane ATPase and many others (Subik and Kolarov, 1970\textsuperscript{364}, Moe and Butler, 1972\textsuperscript{243}). The Mg\textsuperscript{2+} activation is essential to these enzymes. The Ca\textsuperscript{2+} coordinates stronger than Mg\textsuperscript{2+}, giving a more stable complex which is enzymatically inactive (Williams, 1959)\textsuperscript{408}.

Ethanol at high temperature interferes with membrane organization, increasing its fluidity and permeability to ions and small metabolites (Salgueiro, 1988)\textsuperscript{405} and inhibiting the transport of nutrients (Van Uden, 1985)\textsuperscript{385}. Therefore, it is possible that the increase in thermo stability by the presence of optimal concentrations of Ca\textsuperscript{2+} could be extensible to an increase in ethanol tolerance in fermenting yeasts.

Iron

Iron is an essential trace element for most living organisms. However, its availability is limited by the low solubility of Fe(III) and the ability of intracellular free iron to cause the production of toxic radicals (Shin \textit{et al.}, 2001)\textsuperscript{347}. The effect of Fe radicals on cell damage is widely accepted (Raymond and Bryan, 1995)\textsuperscript{321}. Ferritin is an efficient way of storing iron as well as to increase its bioavailability (Goto, 1999)\textsuperscript{126}. There are two forms of iron storage in \textit{Saccharomyces cerevisiae}, the first is a cytosolic ferritin-like molecule and the second implies localization of iron to vacuoles where iron is bound to polyphosphate instead of ferritin (Raguzzi \textit{et al.}, 1988)\textsuperscript{305}. 
Manganese

Manganese enhances the yeast growth, especially in aerobic conditions (Berg et al., 2002). In the metabolism of S. cerevisiae, manganese has also an important role because it is incorporated in some enzymes, such as pyruvate carboxylase, glutamine synthetase and arginase (Welder, 1994) and it is essential for the bud growth. Mn$^{2+}$ is also present in the Golgi, where it activates glycosyl-transfereases, which are involved in the processing of secreted proteins. Transport of Mn$^{2+}$ ions into yeast cells can be passive and driven by the concentration gradient (Kihn et al., 1988) which is energy-dependent, stimulated by glucose (Okorokov et al., 1985; Blackwell et al., 1998). The addition of Mn, Zn & Cu ions enhanced the biomass yield up to 10% and alcohol yield to 20% which is in accordance with some previous results (Jones and Gadd, 1990; Stehlik-Tomas et al., 1997). The addition of Mn ions (0.1 g/l) enhanced the biomass yield and alcohol yield which was in accordance with some previous results (Stehlik-Tomas et al., 1997, 2004). Manganese compounds are less toxic than those of other widespread metals such as nickel and copper.

Zinc

Zinc, in a biologically relevant form of Zn$^{2+}$ ions is essential as catalytic cofactor of many enzymes, including alcohol dehydrogenase, alkaline phosphatase, carbonic anhydrase and several carboxypeptidases. Zinc also plays a critical structural role in enzymes and many noncatalytic proteins (Berg et al., 2002). Deficiency of zinc ions retards cell growth and fermentation activity. On the other hand, high concentration of zinc ions in a nutrient substrate may be toxic, as zinc affects the
permeability of membranes to potassium causing a decrease in both yeast growth as well as fermentation activity (Liu et al., 1997). Zn, Co and Fe promotes the catalytic activity of enzymes of yeast (Veliky and Stefanec, 1964; Faille, 1976). Avci and Donmez, (2006) reported that the addition of 0.17 g L⁻¹ of ZnSO₄·7H₂O to the fermentation medium of T. ethanolicus increased the ethanol concentration by 39%. Addition of 10 mg/l zinc to acid hydrolyzate of wood did not affect peak ethanol production, but it did increase rates of sugar utilization and ethanol production (Sreenath and Jeffries, 2000).

Cobalt

Cobalt in small amounts is essential to living microorganisms and it is a central component of the vitamin cobalamin or vitamin B₁₂. The cobalt content determined is too low to promote the catalytic activity of enzymes (Veliky and Stefanec, 1964; Fuhrman and Rothstein, 1968). In fact, the optimum concentration of cobalt for increasing the catalytic activity of enzymes is 15 ppm (Veliky and Stefanec, 1964). Cobalt is considered to be cofactor for glycolysis (Crane, 1975). These are better described as coenzymes, which are organic substances that directly participate as substrates in an enzyme reaction. Although cobalt is an essential element for life in minute amounts, at higher levels of exposure it shows toxic effects. The catalysts that performed well in the steam-reforming of ethanol under reaction conditions, produced metallic (ferromagnetic) cobalt particles and oxidised cobalt species (Llorca et al., 2003).
Copper

Copper is found in a variety of enzymes. It is an essential element for yeast cell development and also a vital divalent cation in yeast cells, acting as a cofactor of some enzymes such as cytochrome c oxydase, lactase and Cu, Zn-superoxide dismutase (Welder, 1994402; Kihn et al., 1988176). In addition to its enzymatic roles, copper is used for biological electron transport. The blue copper proteins also participate in electron transport. Copper is also reported to influence yeast biomass (Vesna Stehlik et al., 2004)389. In toxicity, copper can inhibit the enzymes. In the media Cu$^{2+}$ concentrations of up to 0.094 mM had no effect on alcoholic fermentation, whereas higher Cu$^{2+}$ concentrations markedly decreased yeast cell growth rate and ethanol production (Mrvcic et al., 2007)246.

2.5.2.5 Metal chelating agents

Though metal ions are known to enhance the ethanol yield (Cysewski and Wilke, 1976)88, metals like Cu, Zn and Mn are very toxic if they are present in higher concentrations (Gadd and Griffiths, 1978)113. In case of crude substrates containing these metal ions, they may be removed by passing the crude substrate through resins (Johnson, 1976)158. Another method, which can be used, is addition of metal-chelating agents, which form a complex with the metal and this metal complex acts as a 'metal buffer' which reversibly dissociates to release ions as they are utilized by the growing organism or combines with the excess metal ions added to the medium. The different metal chelating agents used are potassium ferrocyanide, EDTA, DPTA, sodium potassium tartarate and Zeolite X (Clark et al., 196574; Oderinde et al., 1990269; Kalpana Pandey and Agarwal, 1993171; Mubeccel Ergun et al., 1997247).
2.5.2.6 Vitamins

Vitamins have diverse biochemical functions. The largest number of vitamins functions as precursors for enzyme cofactor bio-molecules (coenzymes), which act as catalysts and substrates in metabolism. Some microorganisms can’t synthesize a full complement of cell components and therefore require preformed compounds called growth factors such as vitamins. Thiamine and pyridoxal increase the specific ethanol productivity, while thiamine may also contribute to increased ethanol tolerance (Rahn, 1952)\(^{306}\). When acting as part of a catalyst, vitamins are bound to enzymes and are called prosthetic groups. For example, biotin is a part of enzymes involved in making fatty acids. Vitamins also act as coenzymes to carry chemical groups between enzymes. Biotin and Calcium pantothenate are widely used in ethanol fermentation (Staphanie Bringer \textit{et al.}, 1984\(^{363}\); Lawford and Sternborg, 1986\(^{204}\); Kademi and Baratti, 1996\(^{169}\)). The role of vitamin B on the rate of alcohol fermentation was extensively studied by Saburo Fukui \textit{et al.}, (1956)\(^{336}\). The vitamin requirements are common to most strains of \textit{Saccharomyces cerevisiae} (Oura, 1974)\(^{280}\). The requirement for various vitamins is also temperature dependent (Amana and Takeuchi, 1961)\(^{14}\).

2.6 Response surface methodology (RSM)

Response surface methodology (RSM) is the most widely used statistical technique, which is a suitable method for identifying the effect of individual variables and for seeking the optimum conditions for a multivariable system efficiently. This method has been successfully applied to optimize ethanol fermentation (Ratnam \textit{et al.}, 2001\(^{315}\), 2003\(^{316}\), 2006\(^{318}\), 2007\(^{317}\), Linde \textit{et al}, 2006\(^{210}\)) and other biotechnological
processes (Maddox and Reichert, 1977\textsuperscript{218}; Bowman and Geiger, 1984\textsuperscript{53}; Sreekumar et al., 1999\textsuperscript{358}; Garcia et al., 2000\textsuperscript{116}; Ambati and Ayyanna, 2001\textsuperscript{15}; Francis et al., 2003\textsuperscript{111}; Hai et al., 2006\textsuperscript{133}). A detailed account of this technique has been outlined (Cochran and Cox, 1968)\textsuperscript{75}.

Statistically designed experiments using response surface methodology was used to get more information about interactions between the parameters (Ghosh and Swaminathan, 2003)\textsuperscript{121}, which it well suited to the study of the main factors and their interaction effects on the bioconversion yields. This method has been applied to optimize vegetable oil bioconversion (Cheynier et al., 1983)\textsuperscript{70}, and biomass production which was effectively promoted by optimizing the medium composition using RSM (Hamedi et al., 2007)\textsuperscript{135}. The central composite design (CCD) was used in order to determine the co-optimum level of the factors, as well as to provide an insight into the interactions amongst these factors during fermentation of ethanol.

2.7 Evaluation of growth kinetics

Monod (1942)\textsuperscript{242} first showed the relation of bacterial growth rate to substrate concentration. In literature, several rate equations for ethanol production by yeast cells have been proposed (Converli et al., 1986\textsuperscript{78}; Alba et al., 1968\textsuperscript{6}; Gose and Tyagi, 1979\textsuperscript{120}).
2.8 Down stream processing

There are several down stream processing procedures developed for the recovery of ethanol. The separation of industrial alcohol from beer was further complicated as the reaction mixture contains many by-products (Hatch, 1962)\textsuperscript{139}. Alcohol is pulled off as a side-stream split to the rectifying column. In this final column, the azeotropic alcohol-water mixture of 95% ethanol is taken off as a top side-stream, condensed and run to storage where it is split into three parts (a) direct sale as potable, government controlled alcohol (b) denatured by small additions of mildly toxic ingredients and solid for industrial uses (c) made anhydrous by ternary azeotropic distillation using benzene or extractive distillation using ethylene glycol. Alcohol purification by extracting the fermented beer with super critical carbon dioxide at 80 atmospheres has been reported (Hartline, 1979)\textsuperscript{138}. Some publications have suggested that ethanol can be recovered by extracting the beer with a mixture of solvents under slight pressure, so that when the pressure is subsequently reduced the solvent flashes off and is recovered for re-use (Paturau, 1982)\textsuperscript{287}. Productivity of the ethanol fermentation can be improved by integrating ethanol recovery with ethanol fermentation. A number of ethanol recovery processes have been studied such as vacuum distillation (Cysewski and Wilke, 1977)\textsuperscript{89}, solvent extraction (Chang et al., 1992)\textsuperscript{64}, gas stripping by CO\textsubscript{2} (Taylor et al., 1995)\textsuperscript{377}, membrane distillation (Gryta et al., 2000)\textsuperscript{131}, double-distillation (Gomez et al., 2003)\textsuperscript{125}, pervaporation (Kaseno et al., 1998)\textsuperscript{174}, Vane, 2005\textsuperscript{386}).
2.9 Qualitative tests for ethanol

The qualitative tests for the estimation of alcohol in dilute solutions are usually simple, rapid and can be used, provided only one is present at a time. There are two important methods worth mentioning, 1) Colorimetric determination using ceric ammonium nitrate (Reid and Truelove, 1952)\textsuperscript{323}, and 2) Permanganate test by spectrophotometer (Rask and Hildebrandt, 1947)\textsuperscript{313}.

2.10 Quantitative determination of ethanol

Many quantitative methods for the determination of ethanol have been developed during these years to give the accurate percentage concentrations. The estimation methods include specific gravity method (William Horwitz, 1980)\textsuperscript{404}, refractometric (William Horwitz, 1980)\textsuperscript{405}, titrimetric (Neish, 1952\textsuperscript{255}; Kubiek, 1963\textsuperscript{193}; Arthur Caputi and Dawson Wright, 1969\textsuperscript{25}) and chromatographic methods. The recent advancement in the development is an automatic multicommutated flow system for ethanol determination which is based on dichromate reduction reaction (Comitre Ana Lucia and Reis, Boe-Ventura, 2000)\textsuperscript{77}.
2.11 Applications of ethanol

Ethanol in the form of alcoholic beverages has been consumed by humans since pre-historic times. Ethanol is an important industrial solvent and it is also an important chemical feed stock for the synthesis of pharmaceuticals, detergents, adhesives, plastics and plasticizers and a host of other chemicals (Fig. 2.2). Fermented beverages can be broadly classified by the foodstuff from which they are fermented. Beers are made from cereal grains or other starchy materials, wines and ciders from fruit juices, and meads from honey. Alcoholic beverages are sometimes used in cooking, not only for their inherent flavors, but also because alcohol dissolves hydrophobic flavored compounds which water cannot. As a beverage it was reported to reduce the risk of atherosclerosis and cataract in people consuming one alcoholic drink per day by 50% (Trevithick et al., 1999)\textsuperscript{383}. Fuel alcohol production is the most interesting area of research in the recent scenario (Patzek, 2004)\textsuperscript{288}. Ethanol can be used as an antiseptic to disinfect the skin before injections are administrated, often along with iodine. It also finds its application as a fuel and in therapeutics (Adeniyi et al., 2007)\textsuperscript{4}. Ethanol was commonly used as fuel in early bipropellant rocket vehicles, in conjunction with an oxidizer such as liquid oxygen. In future, bioethanol will become an attractive commodity from an economic and environmental point of view in large markets world wide and, in all energy markets. Medicinally, ethanol is a soporific, i.e., sleep-producing; although it is less toxic than the other alcohols, death usually occurs if the concentration of ethanol in the bloodstream exceeds about 5\%.
Fig. 2.2: Chemical products derived from ethanol