2. REVIEW OF LITERATURE

The term ‘fermentation’ is derived from the latin verb, *fevere*, to boil. Fermentation technology is one of the oldest food technologies that have been used for several thousand years as an effective and low cost means for preserving foods and beverages (Borgstrom 1968). Food fermentation is of prime importance in the developing countries where the limitation of resources encourages the use of locally available fermented food products for additional nutrition. These fermented products are more common among people belonging to rural areas, without much awareness about the microflora involved in their production. In the past few years, great emphasis has been given to identify unknown microflora associated with these products. This microflora involves a combination of bacteria, yeast and fungi which have been reported by several workers from various fermented foods viz. kinema (Kim-Bong-Joon 2000), bushera (Muyanja et al. 2003) and togwa (Mugula et al. 2003).

The most important organism associated with fermentation is yeast. Yeasts as a group of micro-organisms have been commercially exploited as a fermentative species to carry out alcoholic fermentation, especially *Saccharomyces cerevisiae*. The importance of this microorganism has urged many scientists to study the factors governing its growth, survival and biological activities in different ecosystems (Heard and Fleet 1985). *S. cerevisiae* plays a prominent role in controlling the quality and flavor of the final product in wine fermentations (Joshi et al. 2009) and that’s why, it has received considerable attention in fermentation industry. To obtain the best strain, knowledge of *S. cerevisiae* diversity associated with a particular fermented product in a given area, is of prime importance.

A brief review of literature pertaining to the present research problem is presented under the following sub-headings:

2.1 Traditional fermented foods of Himachal Pradesh

2.1.1 Cereal based traditional fermented foods

2.1.2 Traditional alcoholic beverages
2.2 Microflora associated with fermented foods
   2.2.1 Diversity of yeasts in fermented foods

2.3 *Saccharomyces cerevisiae*

2.4 Diversity analysis of *Saccharomyces cerevisiae* strains using molecular approaches
   2.4.1 ITS region sequencing
   2.4.2 PCR-RFLP of ITS region

2.5 Baking traits
   2.5.1 Specific growth rate
   2.5.2 Acid tolerance
   2.5.3 Maltose adaptation
   2.5.4 Invertase activity
   2.5.5 Latent time

2.6 Brewing traits
   2.6.1 Alcohol production and tolerance
   2.6.2 Molasses concentration
   2.6.3 Attenuation and fermentation efficiency
   2.6.4 Flocculation

2.7 Killer activity

2.8 Allele Mining
   2.8.1 *ADH1* gene
   2.8.2 *ATF1* gene

2.9 Organoleptic studies using apple cider

2.10 Bio-emulsifier production

2.11 Genetic diversity among *Saccharomyces cerevisiae* strains
2.1 Traditional fermented foods of Himachal Pradesh

In spite of scientific and technological revolution, the art of fermentation practiced by common man has continued, but largely remained confined to the rural and tribal areas due to (i) high cost or inaccessibility of the industry-made products in remote areas (ii) taste of the people for the traditional fermented products and (iii) their socio-cultural linkages with such products (Thakur et al. 2004). Indigenous fermented foods are an intrinsic part of diet of the ethnic tribes in the Himalayan belt of India, being the oldest and most economic methods for biological enrichment of food products by the manipulation of different microbial population (Nehal 2013). Most of the traditional fermented foods are prepared by processes of solid substrate fermentation in which the substrate is allowed to ferment either naturally or by adding starter cultures. Majority of fermented foods and beverages involves filamentous fungi and are produced in East and South-East Asia (Tamang 1998).

The traditional fermented foods and beverages form an important constituent of staple diet of the people belonging to the tribal belts of Lahaul & Spiti, Kinnaur, Chamba and rural areas of Kullu, Shimla, Mandi and Kangra districts of Himachal Pradesh (India) where wide range of such type of fermented products are prepared and consumed (Kanwar et al. 2007). Traditional fermented foods are generally nutritious and form the basic components of the diet as staple, adjunct, condiment and beverage, providing calories, proteins, vitamins and minerals to the people (Tamang et al. 1996).

Savitri and Bhalla (2007) studied a wide range of traditional foods and beverages unique to tribal and rural belts of Himachal Pradesh (Kinnaur) which constitute a part of staple food consumed during marriages, local festivals and special occasions. Bhatoooru, Siddu, Marchu, Seera, Chilra, Manna, Aenkadu, Sepubari, Patande, Doo, Baari, Dosha, Malpude, Babroo, Bedvin Roti, Madrah, Tchati, Churpa, Sura, Chhang, Kinnauri, Angoori, Chulli, Lugri, Arak/Ara, Rak, Chukh and pickles made from different fruits, vegetables and cereals are some of the popular traditional products in rural areas of Himachal Pradesh.
2.1.1 Cereal based traditional fermented foods

Most of the traditional fermented foods are cereal-based (wheat/barley/buckwheat) and among these, some of the products like Bhaturu, Siddu, Chilra, Marchu, Manna, Dosha, Pinni/Bagpinni, Seera etc. are unique to Himachal Pradesh (Kanwar et al. 2007). In the tribal districts of Himachal Pradesh, large variety of fermented foods are prepared either daily, during special occasions or for the consumption during journey (Sharma et al. 2013). Traditional starter cultures like ‘Malera’ and ‘Treh’ are used as inocula in these fermented foods. However, the natural fermentation method is also used in the production of several products like Seera, Sepubari, Borhe etc. (Thakur et al. 2004). Some of the cereal based traditional fermented products are listed below.

Siddu, also called Khobli in Shimla district, is a traditionally fermented steam cooked, oval or disc shaped dish prepared as a delicacy in rural areas of Kullu and Shimla districts. In Siddu preparation, wheat flour, spices, mixed paste of opium seeds/walnut/black gram are the main ingredients. Inoculum used is ‘Malera’ (previously fermented left over dough), but nowadays, yeast powder is also used for fermentation. Siddu is served hot with desi ghee or chutney and is prepared as a special/occasional dish in the rural areas of Kullu and Manali (Thakur et al. 2004).

Chilra, also known as Lwar in Lahaul, is more or less like ‘Dosa’ but differs from it in terms of ingredients and shape. Major ingredients of Chilra are wheat/barley and buckwheat flour. Inoculum used for its preparation is called ‘Treh’ (previously fermented and left over wheat flour slurry). The traditional bucket shaped wooden vessel used for fermentation is called ‘Lwarenza’ (Kanwar et al. 2007).

Marchu, the major ingredient is wheat flour and is prepared during the local festivals (Phagli, Halda) in Lahaul valley. Inoculum used for fermentation is called ‘Malera’, which is previously fermented left over dough. The Marchu are roties, made on a wooden base with carving to give designed imprints on them. These are prepared during festivals, religious and marriage ceremonies as a snack/breakfast food to be taken with tea (Thakur et al. 2004).
Bhaturu, also known as *Sumkeshi roti* in Lahaul, is an ‘indigenous bread’ or *roties* which constitute a staple diet of the Himachalis living in rural areas of Kullu, Mandi, Kangra, Chamba and Shimla districts. Rural migrants in urban areas also prepare Bhaturu. The starting material is wheat flour or sometimes barley flour. Inoculum used is called ‘*Malera*’. Normal fermentation time is 2-3 hours in summer and 4-5 hours in winter. In order to reduce the fermentation time, people knead flour with more inoculum and warm water. After completion of the fermentation, one can see the rise of dough. Bhaturu serves as a staple diet for rural people who even take it during meals along with vegetables or curries (Sharma et al. 2013).

*Seera* is prepared by soaking wheat grains in water for 2-3 days so as to allow the fermentation to occur by natural microflora. After 2-3 days, the grains are ground, steeping is done to allow the starch grains and some proteins to settle down, and then bran is separated. Starch and proteins are removed, and grounded material is sun-dried. This dried material is known as ’*Seera’*. The dried material is made into slurry by soaking in water which is then poured into hot ghee. Sugar is added, cooked and served as sweet dish/snack. *Seera* is considered to be nutritious, easily digestible and fast snack food. It is prepared occasionally or offered to the guests as a sweet dish in the rural/urban areas of Kullu, Kangra, Mandi and Chamba districts (Kanwar and Sharma 2011).

### 2.1.2 Traditional alcoholic beverages

A number of cereals (rice/barley), fruits (grapes/wild apricot/apple), jaggery and millet-based indigenously made fermented beverages are popular among the people of Himachal Pradesh. These fermented beverages are prepared and consumed regularly and also during special occasions. Some of the very common traditional fermented beverages of Himachal Pradesh are *Sura, Chhang/Lugri, Daru/Chakti, Anoori/Kinnauri, Chulli, Arak/Ara* and *Behmi* (Kanwar et al. 2011). In the preparation of these fermented beverages (except *Sura*), ‘Phab’ is the inoculum used for their fermentation, but, *Sura* is a product made by natural fermentation (Thakur et al. 2004). Some of the traditional alcoholic beverages are discussed here.

*Sura*, a millet based (*Eleucine coracana*) fermented beverage mostly prepared in Lug valley of Kullu district. Millet, locally called *Kodra/Kached*, has long storage life and because of this, it was popular as the ‘famine grain’. No specific inoculum is used for
its preparation. Natural microflora carries out ethanol forming (fermentative) and starch hydrolyzing (saccharolytic) activities. Also, a herbal mix in Sattu base is added during fermentation. Herbal mix or Dhehli preparation is a community effort, in which elderly people go to forests on the 20th day of Bhadrapada month (usually 5 or 6th September) and collect approximately 36 fresh herbs. Next day, the herbs are crushed in ‘Ukhal’ (stone with a large conical cavity) using ‘Mussal’ (a wooden bar) and the extract as well as the plant biomass are added into the Sattu and are roughly kneaded. This is added into a wooden mould to give the shape of a brick, dried and is known as Dhehli. It is divided among the villagers and is used, whenever Sura is to be prepared. The Dhehli provides bioactive compounds as well as stimulatory effect. Sura is consumed during local festivals like Shoerisaja and marriage ceremonies (Thakur et al. 2004).

Chhang/Lugari is an indigenous rice beer made in the tribal belt of Lahaul & Spiti. The preparation of Chhang involves solid-state fermentation as no additional water is added to the ingredients i.e. cooked rice and ‘Phab’ (the traditional inoculum) (Sharma et al. 2013). Chhang is also prepared from barley; however, it takes longer time (one week) to ferment. The traditional vessel, made of metal or stone used to store Chhang, is called ‘Uthi’ in Lahaul. Chhang/Lugari is a very popular fermented beverage which is served during Phagli (traditional new-year of Lahulis) and marriage ceremonies to guests. Chhang is called Jhol, Chakti in Kullu. Distilled form is known as Sra in Lahaul valley. This beverage has a religious significance, as it is sprinkled on guests as shagun (tribal custom). Chhang serves as a tonic in winters as it contains vitamins, amino acids and sugars besides alcohol, and is considered to provide protection against cold (Bassapa 2002).

Daru is a jaggery-based traditionally fermented beverage prepared and consumed in rural areas of Shimla, Kullu and other regions of Himachal Pradesh. Daru is also called Chakti in Kullu valley. This is one of the popular alcoholic beverages prepared in the rural areas especially for entertaining guests during local festivals and marriage ceremonies. Babool wood locally called kikar (Acacia nilotica) is added to give taste and aroma to Chakti (Nehal 2013).
Dried wild apricots, locally called *Chulli*, are used for making the alcoholic beverage. It is a traditional fermented beverage, indigenous to the tribal district of Kinnaur. It forms an integral part of the social life of Kinnauris, as it is served to the guests during local festivals, fairs and marriage ceremonies (Thakur et al. 2004).

In the preparation of *Angoori*, grapes (red and the usual green ones) are used. A number of grape varieties are cultivated in the Kinnaur district and are used in the preparation of this local alcoholic beverage. *Angoori* is also called *Kinnauri* in Kinnaur district. It is one of the very popular traditional fermented beverages consumed during local festivals and marriage ceremonies by the people of that area (Thakur et al. 2004).

### 2.2 Microflora associated with traditional fermented foods

Use of microorganisms in preparing foods from locally available plants is a traditional practice since pre-historic times (Ross et al. 2002). A variety of microorganisms are responsible for carrying out fermentation in the fermented products by playing an essential role in bringing out the biochemical changes during fermentation (Basappa and Venkataramu 1994).

Microorganisms associated with traditional fermented foods are present either in or on the ingredients and utensils, in the environment and are selected through adaptation to the substrate and fermentation conditions (Soni and Sandhu 1990). The indigenous natural fermentation carried out by a mixed colony of microorganisms such as moulds, bacteria and yeasts. Paraggio (2004) studied the biodiversity of natural fermentation of Aglianico del Vulture must and found the predominance of *S. cerevisiae* and *Hansenia sporauvarum*. Farinde et al. (2014) examined the comparative microbial assessment of fermented Lima bean and Locust bean in the production of Daddawa and found the dominance of *Bacillus* species. The quality of fermentation product is based on the type of microorganisms involved in the process. Some of the compounds formed during fermentation include organic acids (e.g., palmitic, pyruvic, lactic, acetic, propionic and butyric acids), alcohols (mainly ethanol) aldehydes and ketones (acetaldehyde, acetoin, 2-methyl butanol) (Herraiz et al. 1990).
In India, mostly due to wide variation in agro-climatic conditions and diverse form of dietary culture of the various ethnical groups, the following three major types of microorganisms are recorded with the traditional fermented foods and beverages (Tamang 1998; Joshi 2000):

- Filamentous fungi: species of *Aspergillus, Amylomyces, Actinomucor, Monascus, Mucor, Neurospora, Penicillium* and *Rhizopus*.
- Yeasts: *Debaromyces, Candida, Geotrichum, Hansenula, Kluyveromyces, Pichia, Saccharomyces, Saccharomycopsis, Torulopsis* and *Zygosaccharomyces*.
- Bacteria: Species of lactic acid bacteria (LAB); *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Tetragenococcus* and *Streptococcus* and species of *Klebsiella, Acetobacter, Citrobacter, Bacillus, Brevibacterium* and *Propionibacterium*.

### Table 2.1: Microbial diversity found in traditional fermented food products of Indian Himalayan belt

<table>
<thead>
<tr>
<th>Fermented food</th>
<th>Microorganisms responsible for fermentation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bhaturu, Marchu and Chilra</strong></td>
<td><em>Lactobacillus plantarum, L. acidophilus, Leuconostoc, Bacillus sp., Lactococcus lactis, S. cerevisiae, Debaromyces hansenii</em></td>
<td>Kanwar et al. 2007</td>
</tr>
<tr>
<td><strong>Soibum</strong></td>
<td><em>Enterococcus durans, Streptococcus lactis, Bacillus subtilis, B. licheniformis, B. coagulans, Candida sp., Saccharomyces sp., Torulopsis sp.</em></td>
<td>Tamang and Tamang 2009</td>
</tr>
<tr>
<td><strong>Daru and Jann</strong></td>
<td><em>S. cerevisiae, Candida famata, C. valida, Kluyveromyces maxianus, Saccharomycopsis fibuligera</em></td>
<td>Thakur et al. 2004; Das and Pandey 2007</td>
</tr>
</tbody>
</table>
2.2.1 Diversity of yeasts in traditional fermented foods

Miguel et al. (2013) characterized different kefir yeast species from Brazil, Canada and the United States of America and showed the highest growth rate of *S. cerevisiae* and *Kluyveromyces marxianus* in kefir. Jimoh et al. (2012) studied the diversity of yeasts in locally fermented beverages sold in Nigeria and found the predominance of *S. cerevisiae, Rhodotorula mucilaginosa, Candida* sp. and *Cryptococcus albidus*. Kuriyama et al. (1997) screened the yeasts isolates obtained from traditional fermented foods of Indonesia for their amylolytic activity and the main yeasts identified were *Endomyces fibuliger* and *Pichia anamola* with highest activity.

Thakur et al. (2004) characterized some traditional fermented foods and beverages of Himachal Pradesh and found some of the predominant yeast species i.e. *S. cerevisiae, Candida* sp., *Zygosaccharomyces bisporus* and *Kluveromyces thermotolerance*. The predominant microflora (yeasts) was characterized on the basis of microscopic, physiological and biochemical characteristics.

Published reports showed that most of the Indian fermented foods are associated with the yeasts belonging to genera *Saccharomyces, Candida, Debaromyces* and *Hansenula* and these organisms are contributed by the raw ingredients, workers and surroundings (Venkatasubbiah et al. 1984; Soni and Sandhu 1990).

In one of the studies, Pathania et al. (2010), while exploring various fermented food products (*babru, bhaturu* and *chilra*), alcoholic beverages (*chhang, lugari, aara, chiang*, apple wine and *chulli*) and traditional inocula (*khameer, phab* and *dhaeli*) of tribal areas of Himachal Pradesh representing regions of Lahaul & Spiti, Sangla, Bharmour, Pangi, Chauntra and Kinnaur showed the predominance of yeast (forty-three) microflora. As these isolates are of different geographical origin and from different sources, they suggested a strong possibility about the existence of strain level differences among them.

On the basis of molecular characterization, out of forty-three yeast isolates, twenty-three isolates from alcoholic beverages, fermented foods and traditional inocula were identified as *S. cerevisiae*, four isolates from *chulli, khameer, bhaturu* and *phab* were identified as *Saccharomyces fermentati*, one isolate from *dhaeli* as *Endomyces fibuliger,*
six isolates from beverages and fermented foods as *Debaromyces hansenii*, two isolates from fermented foods as *Schizosaccharomyces pombe*, five isolates from apple wine as *Issatchenkia orientalis*, and two isolates from fermented foods were identified as *Brettanomyces bruxellensis* and *Candida tropicalis* (Pathania et al. 2010). Earlier workers also encountered similar type of yeast strains in various traditional fermented foods and beverages in other parts of the country (Basappa and Venkataramu 1994; Soni et al. 2001; Shrestha et al. 2002; Muyanja et al. 2003; Mugula et al. 2003).

2.3 *Saccharomyces cerevisiae*

Taxonomists grouped yeasts into 81 genera and 590 species of which only 19 are considered relevant to wine (Ribereau-Gayon et al. 2006). *S. cerevisiae* is the most important yeast species associated with fermentation and during the course of time, number of yeast species were assigned from and to the *S. cerevisiae* group (Barnett 1992; de Barros Lopes et al. 1998; Pretorius et al. 1999). However, it was found that not all yeasts within this group were suitable for wine fermentations (Kurtzman and Fell 1998).

Progressively, a molecular approach divided *Saccharomyces* into genotypically distinct species namely *S. bayanus*, *S. castellii*, *S. cerevisiae*, *S. diasensis*, *S. exiguus*, *S. kluyveri*, *S. paradoxus*, *S. pastorianus*, *S. servazzii* and *S. unisporus* (Quesada and Cenis 1995) and later, into newly defined species such as *S. kunashirensis*, *S. martiniae* (James et al. 1997; Kurtzman and Robnett 2010) and *S. cariocanus*, *S. nikatae* & *S. kudriavzevii* (Naumov et al. 2000). Collectively, *S. cerevisiae* and closely related species, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. mikatae*, *S. kudriavzevii* and the recently described *S. arboriculosis* (Wang and Bai 2008) are known as the *Saccharomyces sensu stricto complex* (Tornai-Lehoczki et al. 1996; Vaughan-Martini and Martini 1998; Ribereau-Gayon et al. 2006; Kurtzman et al. 2011). The yeast species, *S. exiguus*, *S. castellii*, *S. servazzii* and *S. unisporus* are known as the *Saccharomyces sensu largo* group, while *S. kluyveri* forms a group on its own. The *Saccharomyces sensu largo* and *S. kluyverii* are also collectively known as the *Saccharomyces lato* group (Kurtzman and Robnett 2003). Earlier *S. dairenensis* was also included in this group (Petersen et al. 1999).
*S. cerevisiae* is the most useful yeast, having been instrumental in wine making, baking and brewing since ancient times. It is believed that it was originally isolated from the skin of grapes and is most intensively studied eukaryotic model organism in molecular and cell biology. Its cells are round to ovoid, 5–10 µm in diameter. It reproduces by a division process known as budding (Feldmann 2010). All the strains of *S. cerevisiae* can grow aerobically on glucose, maltose and trehalose, but fail to grow on lactose and cellobiose. However, growth on other sugars is variable. Galactose and fructose have been shown to be the best fermenting sugars. The ability of yeasts to use different sugars can differ depending on whether they are grown aerobically or anaerobically. Some strains cannot grow anaerobically on sucrose and trehalose.

*S. cerevisiae* can survive and grow in two forms: haploid and diploid. The haploid cells, asexual form of the yeast, undergo a simple life cycle of mitosis. The diploid cells (preferential 'form' of yeast) similarly undergo a simple life cycle of mitosis and growth, but under stress conditions can undergo sporulation, entering meiosis and producing four haploid spores, which can subsequently mate. This is known as sexual mode of replication. Under adequate supply of nutrients, *S. cerevisiae* cells can double their population every 100 minutes (Herskowitz 1988; Friedman 2011) and mean replicative life span is about 26 cell divisions (Kaeberlein et al. 2005; Kaeberlein 2010).

*S. cerevisiae* was the first eukaryotic genome to be completely sequenced (Goffeau et al. 1996). The genome sequence was released to the public domain on April 24, 1996. The *S. cerevisiae* genome is composed of about 12,156,677 base pairs and 6,275 genes, compactly organized on 16 chromosomes. Only about 5,800 of these genes are believed to be functional. It is estimated that at least 31% of yeast genes have homology to the human genome. The availability of the *S. cerevisiae* genome sequence and a set of deletion mutants covering 90% of the yeast genome have further enhanced the power of *S. cerevisiae* as a model for understanding the regulation of eukaryotic cells (Botstein et al. 1997).

*S. cerevisiae* is used in baking & brewing and sometimes called as top-fermenting or top-cropping yeast because during fermentation process, its hydrophobic surface causes the flocs to adhere to CO₂ and rise to the top of the fermentation vessel. Top-fermenting yeasts are active at higher temperatures than the bottom-fermenting yeast, *S. pastorianus* and the resulting beers have a different flavor.
2.4 Diversity analysis of *Saccharomyces cerevisiae* strains using molecular approaches

### 2.4.1 ITS region sequencing

Advances in DNA sequencing have provided a platform for understanding primary structures of genes and deduce gene based on sequence information. Various sequencing methodologies have been developed in the past few years and high-throughput sequencing is believed to become a major molecular tool for strain typing in near future. Up to now, this technique has been mainly used for sequencing of specific genome areas after PCR (Fernandez-Espinar et al. 2006). Regions of interest for sequencing include the domains D1 and D2 of the 26S gene (Kurtzman and Robnett 1998), 18S (James et al. 1997) and the 5.8S rRNA gene (Las Heras-Vazques et al. 2003). Various publications reported the usefulness of ITS regions for rapid identification of fungi including non-Saccharomyces (*Candida, Pichia, Hanseniaspora, Torulaspora, Metschnikowia, Saccharomycopsis*) and *S. cerevisiae* yeast (Turenne et al. 1999; Arias et al. 2002; Masoud et al. 2004; Jespersen et al. 2005) from food and beverages. The online (http://www.ebi.ac.uk/Blas2/index.html) availability of these sequenced regions has made it easier to assign unknown yeast strains to specific genus or species (Fernandez-Espinar et al. 2006).

For molecular identification and discrimination of yeasts, ITS region sequencing is preferred due to its simple procedure and it can be fully automated (Ciardo et al. 2006). ITS region is composed of two noncoding regions ITS1 and ITS2, which are separated by the highly conserved 5.8S rRNA gene (White et al. 1990) and is located between 18S and 28S rRNA genes. Since the variability of rRNA genes is limited, it can be difficult to differentiate between species or strains (Fell et al. 2000). The ITS region is more promising for species or strains discrimination because of its higher variability (Iwen et al. 2002). Therefore, ITS region has been adopted as the primary fungal barcode marker (Schoch et al. 2012). Although attempts to identify fungi by focusing on either the ITS1 or the ITS2 region may be successful for some species and genera (Chen et al. 2000; Chen et al. 2001), however, analysis of the complete ITS region offers greater promise for molecular identification (Chen et al. 2001). ITS region sequencing is useful both at inter- and intra-species level (Peay et al. 2008) and has been considered as consistent
classification method for yeast strains (Foschino et al. 2004). Sugita et al. (2000) revealed the intra-species diversity of *Cryptococcus laurentii* by ITS region and 28S rRNA gene sequencing, suggesting the high intra-specific heterogeneity of the ITS region.

### 2.4.2 PCR-RFLP of ITS region

This technique highlights possible differences in largely homologous DNA sequences and can be detected by the presence of fragments of different lengths generated by digesting with restriction endonucleases. Internal transcribed spacer refers to a piece of non-functional RNA situated between structural ribosomal RNAs on a common precursor transcript. PCR-RFLP of this region has been proved useful in assigning genetic diversity among *S. cerevisiae* strains (Couto et al. 1996). The PCR-RFLP region includes 5.8S, 18S and 26S ribosomal genes which are grouped in tandem to form transcription units. These transcription units are repeated between 100-200 times in the genome. Other regions include the internal transcribed spacer (ITS) and external transcribed spacers (ETS), the regions which are transcribed, but not processed. The transcription units are also separated by intergenic spacers (IGS). These ribosomal regions have become tools for identifying phylogenetic relationships among all living organisms (Kurtzman et al. 2011) and in yeasts (Kurtzman and Robnett 1998). According to Li (1997), the transcribed units are more likely to be similar for strains of the same species than for different species. In general, the specific regions on the subunits commonly referred to as domain D1/D2, on the 18S (James et al. 1997) and 26S (Kurtzman and Robnett 1998) have been sequenced.

According to Kurtzman and Robnett (1998), for assigning unknown yeast or yeast strains to a specific species, the nucleotide sequences in these regions can be used to measure homology to known or related yeasts. Furthermore, the amplification and restriction profiling of these regions and the use of fluorescent dyes have yielded notable results in identifying more strains within specific species. Dlauchy et al. (1999) used specific primers NS1 and ITS1 to amplify regions of the 18S gene, which were then digested with four restriction enzymes; *Alul*, *HaeIII*, *MspI* and *RsaI*. Capece et al. (2009) differentiated non-Saccharomyces wine species in a mixed fermentation based on PCR-RFLP analysis of ITS region of different yeast strains. White et al. (1990) used primers ITS1 and ITS4 to amplify regions of the 5.8S gene. These 5.8S regions were also used
extensively for identification and discrimination of yeast strains used in wine or related industries with relative success (Guillamon et al. 1998; Esteve-Zarzoso et al. 1999; Fernandez-Espinar et al. 2000; de Llanos et al. 2004). This technique has also been useful in the studies of reference strains (Ramos et al. 1998; Fernandez-Espinar et al. 2000; Cadez et al. 2002; Esteve-Zarzoso et al. 2003; Naumova et al. 2003; Pham et al. 2011). The non-transcribed regions, 18S gene, ITS region and 26S gene have also been widely used by various authors to identify species and strains in the Saccharomyces sensu stricto group (Couto et al. 1996; Smole-Mozina et al. 1997; Tornai-Lehoczki and Dlauchy, 2000; Caruso et al. 2002; Capece et al. 2003; Vasdinyei and Deak 2003; Fernandez-Espinar et al. 2006). The markers based on restriction analysis of ITS with Dral and HaeIII have also been used to identify and characterize yeast populations with oenological significance as well as species in the larger Saccharomyces sensu stricto group (Esteve-Zarzoso et al. 1999; Granchi et al. 1999; Redzepovic et al. 2002; dos Santos et al. 2007). Perez-Travez et al. (2014) evaluated the complexity of 46 different strains of Saccharomyces by means of PCR-RFLP analysis and by sequencing of 34 gene regions and one mitochondrial gene.

2.5 Baking traits

The potential characteristics of a particular baker’s yeast are determined by its strain. There are six hundred different species of yeast that have been identified in nature but only S. cerevisiae is commonly used for baking. An unlimited number of S. cerevisiae strains are possible and there are several thousand that have already been selected for baking. All S. cerevisiae yeasts have certain similarities, including the substances they use for growth, how they reproduce and their appearance under the microscope. In an individual strain differences like how much sugar it can tolerate, how quickly it can grow and how sensitive it is to calcium propionate are also important. Commercial S. cerevisiae strains are domesticated under artificial selection conditions. These domestication events are dependent on the desired function of the yeast: baking, brewing, wine making, or bioethanol production (Fay and Benavides 2005; Legras et al. 2007).

2.5.1 Specific growth rate

Baker’s yeast strains are domesticated strains of S. cerevisiae that have been selected for broad traits such as robustness, large cell size and high growth rate under
carbon limiting conditions. Sugar signaling is responsible for several adverse effects on the performance of industrial yeasts. One of the most common adverse effects is the reduction in biomass yields due to the tendency of baker’s yeast cells to produce ethanol when sugars are present in excess, even under aerobic conditions. This ethanol formation can be limited by growing yeast cells under conditions of low sugar feeding in aerated reactors (Valentinotti et al. 2003). Nevertheless, in commercial scale bioreactors (100–400 m$^3$), it was difficult to ensure the complete nutrient distribution and aeration at full capacity for ethanol formation. For example, increased sugar concentration at the feeding point simultaneously promotes alcohol formation and alcohol consumption, reducing the theoretical biomass production from 0.50 to 0.39 g per gram of glucose (Devarapalli et al. 2009).

The actual growth characteristics of baker’s yeast from a particular strain are determined by the composition of the environmental conditions. The growth conditions affect how fast the yeast multiplies and how much protein and carbohydrate it accumulates. Rapid growth usually means more protein, more enzymes, and more initial activity. Slow growth usually means more carbohydrate, lower initial activity, and better stability (Lallemand Baking Update 2011).

### 2.5.2 Acid tolerance

Baking recipes and ingredients affect yeast performance. Recipes with sours, fruit, calcium propionate, and with natural mold inhibitors require yeasts with good acid tolerance. Sweet, salty, and low absorption doughs also inhibit different yeasts to different degrees because the amount of available water is limited (Kanwar and Keshani 2014). Sourdough fermentation requires a specific knowledge on the effects of process parameters, raw materials and microorganisms in order to attain a specific and reproducible bread quality (Brandt 2007). In manufacturing of bread, pH of the sourdough is usually below 4.7 and furthermore, preservatives are also added in the dough for its long shelf life. The baking yeast which is resistant to acetic acid is also supposed to be resistant to inhibiting doses of propionic or sorbic acids (Clement and Hennette 1982). Hence, strains resistant to the acidic conditions (acetic acid) are generally expected to perform well in the sourdough, as most of the sourdoughs attain a pH of around 3.5 - 4.2 at the end of fermentation (Almeida and Pais 1996).
2.5.3 Maltose adaptation

Yeasts produce carbon dioxide from sugar by a series of enzymatic reactions known as glycolysis (Barnett 2003). In some recipes, sugar comes from high fructose corn syrup or sucrose but in lean doughs, the primary sugar available to yeast is maltose. The way an yeast strain utilizes maltose affects how well it works in lean dough (Jiang et al. 2008). Maltose consists of two glucose molecules linked together and is formed from the damaged starch in flour by the naturally occurring enzymes alpha- and beta-amylases:

\[
\text{Damaged starch} \xrightarrow{\text{alpha-amylase}} \text{Dextrins} \\
\text{Dextrins} \xrightarrow{\text{beta-amylase}} \text{Maltose}
\]

Utilization of maltose by yeast requires two enzymes; maltose permease and maltase. The permease transports maltose into the yeast cell from outside, and maltase cleaves it into two glucose molecules (Higgins et al. 1999). The yeast then uses glucose for glycolysis just as it would sugar from any other source:

\[
\text{Maltose permease} + \text{Maltose} \xrightarrow{\text{Maltase}} \text{2 Glucose}
\]

\[
\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{2 Ethanol + 2 CO}_2
\]

All *S. cerevisiae* yeasts are able to ferment maltose but there are two kinds of strains; adaptive and constitutive. Most of the strains are “adaptive” because their maltose utilization depends on environmental factors i.e. these strains would not make maltose permease and maltase if glucose is present. So they can only use maltose after glucose has been used up. A major limiting factor in dough fermentation is the repression of synthesis of maltose-utilizing enzymes and inactivation of the maltase by glucose (Needleman 1991). Some baker’s yeast strains are known as “constitutive” because their maltose utilization is independent of environmental factors. Constitutive strains tend to have better performance in lean dough (Lallemand Baking Update 2011).
2.5.4 Invertase activity

To a great extent, yeast industry had studied baker’s yeast strains, not only with a view of making them more rapid but also to adapt them as much as possible to different types of baking processes. When sucrose is added into the dough, invertase enzyme catalyzes the hydrolysis of sucrose into glucose and fructose, and thus increases the osmotic pressure to the yeast cells. Therefore, yeast strains with low invertase activity are preferred for making sweet dough applications like pastries, sweet bread, cakes etc. (Romano et al. 2006). When sucrose or glucose are added to the dough, they are directly fermented before maltose indicating the primary utilization of these sugars by the yeast strains (Voica and Codina 2009).

Zhang et al. (2010) studied the relationship between invertase activity and leavening activity of sweet dough. The leavening activity and the invertase activity in sweet dough were determined in several strains of *S. cerevisiae* to investigate their correlations. There was a general tendency that the yeast cells with higher invertase activity showed less leavening ability in sweet dough especially containing sucrose. The results have shown that less invertase level is undoubtedly helpful in the improvement of leavening ability of baker’s yeast. Hernandez-Lopez et al. (2003) also showed that osmotolerance trait in yeast is correlated with a low invertase activity and a slow rate of trehalose mobilization.

2.5.5 Latent time

*S. cerevisiae* latency period also affects baking processes. Straight and no-time doughs work best with fast yeasts that adapt quickly to give good oven spring i.e. the yeast strains with less latent period are best for such kind of preparations (Kanwar and Keshani 2014). Sponge and dough systems work best with slower yeasts to retain sufficient activity for the final proof. Frozen dough systems work best with slow yeasts that retain their activity well i.e. the yeast strains with more latent time are suitable for sponge and frozen doughs (Lallemand Baking Update 2011). It has been reported that shorter the latent time, more likely the *S. cerevisiae* strains to be osmotolerant, i.e. active within sweetened dough (Clement and Hennette 1982).
2.6 Brewing traits

Pure brewer’s yeast cultures are produced at industrial level to meet the demands of brewing industry. Usually two *Saccharomyces* species are used: *Saccharomyces uvarum*, which is used for the production of several types of beer with bottom fermentation (lager yeasts), and *S. cerevisiae* which conducts top fermentation (ale yeasts). Top fermenting yeasts are used for the production of ales like stouts, porters, wheat beers etc. and bottom fermenting yeasts are used for lager beers like Pilsners, Bocks, American malt liquors etc. (Goldammer 2000). Selection of a yeast strain with the required brewing characteristics is vital from both economic and product quality point of view. The criteria for yeast selection will vary according to the requirements of brewing equipment and beer style, but they are likely to include: rapid fermentation, stress tolerance, flocculation, rate of attenuation, beer flavour and stability against mutation and degeneration (Kanwar and Keshani 2014).

2.6.1 Alcohol production and tolerance

A rapid fermentation without excessive yeast growth is important, as the objective is to produce a beer with the maximum attainable ethanol content consistent with the overall flavor balance of the product (Goldammer 2008). Thanonkeo et al. (2011) studied the growth and ethanol production of four *Zymomonas mobilis* strains isolated in Thailand from Jerusalem artichoke (*Helianthus tuberosus* L.) juice and were compared with those of the typed strain *Z. mobilis* ZM4 (NRRL B-14023) at different temperatures. Among the strains tested, TISTR 548 gave the highest ethanol concentration at 30 to 35 °C as compared to the others.

The yeast strain should be tolerant to alcohol, osmotic shock and temperature because several factors influence the overall rate of brewing including osmotic pressure, ethanol concentration of media and ethanol tolerance of yeast strain employed (Stewart et al. 1988). High ethanol tolerance of *S. cerevisiae* in comparison to other yeasts is due to unique lipid composition of its plasma membrane as it synthesizes ergosterol rather than cholesterol and phospholipids containing very high proportion of unsaturated fatty acyl residues (Ingram and Buttke 1984). Gupta et al. (2009) evaluated various strains of *Saccharomyces* sp. for alcohol tolerance limits and reported 3-12% of tolerance in
various indigenous yeast strains. However, for checking alcohol tolerance, most of the studies were conducted on yeast survivability (Mehdikhani et al. 2011) rather than on alcohol production in presence of high ethanol concentration as recommended by Keo (1967).

### 2.6.1 Molasses concentration

In order to fulfill the increasing demand for ethanol, increased production of ethanol is necessary. To achieve this target through fermentation, it is essential to evolve potential *S. cerevisiae* isolates and to study the production capacity of selected strains from cheap and readily available raw materials like molasses (Kamnird et al. 1983; Rotimia et al. 1986). About two-third of the total molasses (about 50 thousand tonnes) obtained as a by-product of sugar mills of Bangladesh alone, and it remains unutilized every year due to lack of modern technologies employed in production of ethanol through fermentation processes (Khan et al. 1989).

In order to develop a practical process based on molasses, clarification or pre-treatment of molasses is required to reduce inhibitory components that interfere with the ethanol production (Patil et al. 1986). Sheela et al. (2008) studied ethanol production from molasses by using yeast isolates of the genera *Saccharomyces*, *Zygosaccharomyces* and *Kluyveromyces* and reported maximum ethanol production at 15% sugar concentration. It has also been reported in the literature that high initial sugar concentrations can affect yeast growth by increasing the lag phase, decreasing the growth rate and reducing the ethanol tolerance in later stages of fermentation (Nishino et al. 1985). Usually, yeast strains need 11-15 % sugar in the molasses medium. Too much of sugar reacts adversely thus, creating undesirable osmotic stress on the yeast cells which results in lowering of ethanol production (Beuchat 1983).

### 2.6.2 Attenuation and fermentation efficiency

Attenuation refers to the percentage of sugars converted to alcohol and carbon dioxide, as measured by specific gravity. Most yeasts ferment glucose, sucrose, maltose, and fructose. To achieve efficient conversion of sugars to ethanol (good attenuation), the yeast strain capable of completely utilizing these sugars is required. Brewing yeasts vary significantly in the rate and extent to which they use these sugars (Goldammer 2008).
High attenuation yeasts generally give dry, clean, fully fermented finish, but they may take a long time to completely clear the product unless fining agents are used. Low attenuation yeasts result in a fuller bodied, more complex beer because they may not fully ferment complex sugars, but comparatively they settle down more quickly (Smith 2010). Hence, selection of the suitable yeast strain with appropriate attenuation to match the beer style is important.

Fermentation efficiency is an expression of how much alcohol is actually produced in relation to the theoretical amount which could be produced. Fermentation efficiency is affected by several factors like sugar concentration, temperature, pH, nitrogen supplement etc. Arrizon and Gschaedler (2002) observed an increase in fermentation efficiency at high sugar concentrations by supplementing an additional source of nitrogen during exponential phase. Although it has been shown that yeasts with low nitrogen requirements are more effective for fermentation (Manginot et al. 1998), the nitrogen concentration used in the above study was lower than as reported by others (Albers et al. 1996; Manginot et al. 1998).

### 2.6.3 Flocculation

In selecting a yeast strain for beer production, a brewer considers a number of factors. Yeast's flocculation ability is one of the major concerns. The number of cells suspended in wort during primary and secondary fermentation is a key factor influencing fermentation speed, beer flavor, maturation and filtration. Although centrifugation can be applied to separate suspended cells, flocculation is still an important and necessary process for the removal of yeast (Jina and Speersb 1998). The term "flocculation" refers to the tendency to form clumps of yeast called flocs. The flocs (yeast cells) descend to the bottom in the case of bottom-fermenting yeasts or rise with carbon dioxide bubbles to the surface in the case of top-fermenting yeasts. The flocculation characteristics need to be matched to the type of fermentation vessel used i.e. a strongly cropping strain is ideal for skimming from an open fermenter, but unsuitable for a cylindroconical fermenter (Goldammer 2008). Flocculation and attenuation are inversely correlated with each other i.e. a low flocculator yeast strain is usually a good attenuator (Smith 2010).
2.7 Killer activity

Killer activity is an easy and effective non-molecular method for yeast biotyping by determining the killer sensitivity patterns (KSPs) of a yeast strain towards a panel of selected killer toxins (Buzzini et al. 2007). Killer toxins (mycocins) were first discovered by Makover and Bevan (1963) in strains of *S. cerevisiae*. Toxin-producing strains are conventionally labeled as ‘killer’ when they produce a mycocin which is able to kill other strains (labeled as ‘sensitive’) belonging to the same or to other species. Conversely, strains which do not secrete or are not sensitive to any toxin are labeled as ‘neutral’. Killer activity has been in more than 90 yeast species belonging to both ascomycetous and basidiomycetous genera reported by various workers (Young 1987; Golubev 2006; Rodriguez-Cousino 2011).

Apart from use for biotyping of yeasts isolates, several applications have been proposed for yeast killer toxin systems. For example, different toxins have been used as models for studying polypeptide secretion mechanisms in eukaryotic cells, while those encoded by plasmids are useful for studying the biology of eukaryotic ‘viruses’ (Magliani et al. 1997; Schmitt and Breinig 2002). Another field of application is the fermentation industry. In many studies, isolation of toxin-producing, killer-sensitive and killer-neutral strains of *S. cerevisiae* from fermenting grape must (Musmanno et al. 1999; Gurierrez et al. 2001) have been reported. Although many variables affect the expression of killer and killer-sensitive phenotypes during winemaking, there are clear evidences that killer interactions may affect strain activity during fermentation (Fleet and Heard 1993). Moreover, killer spoilage yeasts spontaneously occurring in fermentation environment can potentially compete with commercial starters, particularly, if these are sensitive to killer toxins. As a result, particular attention has been devoted to employ killer strains of *S. cerevisiae* with desirable enological properties as starters in order to prevent the growth of spoilage yeasts during the early stages of wine fermentation (Petering et al. 1991; Marquina et al. 2002; Schmitt and Breinig 2002; Golubev 2006).

2.8 Allele Mining

2.8.1 *ADH1* gene

Two major pathways are involved in the energy metabolism of *S. cerevisiae*, namely glycolysis and aerobic respiration. Ethanol is a key metabolite in energy metabolism, being an end product of glycolysis and ethanolic fermentation while also
serving as a carbon substrate during aerobic respiration, with alcohol dehydrogenases (ADHs) catalysing the inter-conversion of acetaldehyde and ethanol (Smidt et al. 2008). The genes encoding classical ADHs include \textit{ADH1}, \textit{ADH2}, \textit{ADH3}, \textit{ADH4} and \textit{ADH5} (Lutstorf and Megnet 1968; Ciriacy 1975; Walton et al. 1986; Feldmann et al. 1994). Ida et al. (2012) analyzed the effects of the deletions of genes encoding alcohol dehydrogenase (ADH) isozymes of \textit{S. cerevisiae}. They observed that the decrease in ethanol production by \textit{ADH1} deletion alone could be partially compensated by the upregulation of other isozyme genes, while the deletion of all known ADH isozyme genes stably disrupted ethanol production. Yeast ADH was the first pyridine nucleotide-dependent dehydrogenase to be crystallized. Determination of the primary structure of Adh1p in \textit{S. cerevisiae}, marked the first case of gene cloning by functional complementation. Physico-chemical methods revealed that the protein had a molecular weight of 150 kDa with the active enzyme containing four identical reactive sites and four similar, if not identical, polypeptide chains (Harris 1964).

The first biochemical data on Adh1p and Adh2p showed that the kinetic properties of both enzymes favored alcohol production. Under the conditions of high ethanol concentration and the efficient removal of acetaldehyde, both enzymes could function in the oxidation of ethanol (Heick et al. 1969). Adh1p is normally constitutive under laboratory conditions and has a high Km value for ethanol (17000–20000 mmol L$^{-1}$) (Thomson et al. 2005), and therefore, it seems to be chiefly responsible for the production of ethanol during anaerobic growth. \textit{ADH1} (YOL086C) gene is situated on chromosome XV of \textit{S. cerevisiae} at coordinates 160594 to 159548 (crick strand). If levels of intracellular ethanol are low, Adh2p would produce acetaldehyde and NADH at a faster rate than Adh1p (Wills et al. 1982). Kinetic investigation of commercially available ADH showed it to be capable of oxidizing all primary alcohols with chain lengths between 2 and 10 carbon atoms (Schopp and Aurich 1976), and the activity of Adh1p decreased with increasing chain length of the primary alcohols (Ganzhorn et al. 1987). The substrate specificity of Adh1p is restricted to primary unbranched aliphatic alcohols and any branching decreases the activity and efficiency of the enzyme (Leskovac et al. 2002). Cyclic alcohols (benzyl alcohol, cyclohexanol) are not oxidized in detectable amounts (Drewke and Ciriacy 1988) and thiol compounds exert no effect on this isozyme
(Cheng and Lek 1992). It is also reported that overexpressed Adh1p reduces formaldehyde (FA) to methanol in vivo (Grey et al. 1996) and is able to provide a considerable degree of protection against cadmium (Yu et al. 1991). Hasunuma et al. (2014) reported that co-expression of TAL1 and ADH1 in recombinant xylose-fermenting S. cerevisiae improves ethanol production from lignocellulosic hydrolysates in the presence of furfural.

2.8.2 ATF1 gene

It is well known that ester formation is highly dependent on the yeast strain used (Peddie 1990), and on certain fermentation parameters such as temperature (Sablayrolles and Ball 1995), pitching rate (D’Amore et al. 1991), and top pressure (Landaud et al. 2001). In addition, the concentrations of assimilable nitrogen compounds (Sablayrolles and Ball 1995), carbon sources (Younis and Stewart 2000), dissolved oxygen (Sablayrolles and Ball 1995), and fatty acids (Thurston et al. 1982) have a profound impact on ester production rates. However, these factors allow only minor adjustments to the final ester concentrations of the produced beverages, so that the overall ester balance after fermentation is often suboptimal, resulting in an inferior end product. In order to obtain better control over ester synthesis, much research has been focused on the elucidation of the biochemical mechanisms of ester synthesis as well as on the factors influencing ester synthesis rates.

Esters are formed intracellularly in an enzyme-catalyzed condensation reaction between two cosubstrates, a higher alcohol and an activated acyl-coenzyme A (acyl-CoA) molecule (Nordstrom 1963). The best-known enzymes involved in ester synthesis are called alcohol acetyltransferases (AATases; EC 2.3.1.84). These enzymes catalyze the formation of acetate esters from two substrates i.e. an alcohol and acetyl-CoA. It has been shown that during fermentation, acetate ester production rates follow a pattern corresponding to the AATase activity (Malcorps et al. 1991). Purification of the acetate ester-synthesizing enzymes has led to the identification of three distinct AATases: AATase I, its closely related homologue Lg-AATase I, and AATase II. These AATases are encoded by ATF1, the ATF1 homologue Lg-ATF1, and ATF2 genes, respectively (Fujii et al. 1994; Yoshimoto et al. 1999). While ATF1 and ATF2 are present in both S. cerevisiae (ale) and S. bayanus (lager) strains, Lg-ATF1 is found only in S. bayanus
strains (Yoshimoto et al. 1998; Dufour et al. 2002). Homology-based searches of the *S. cerevisiae* genome have not revealed other genes with homology to *ATF1* and/or *ATF2*. However, Malcorps and Dufour (1992) speculated that apart from Atf1p, Lg-Atf1p, and Atf2p, one other, yet unidentified enzyme with AATase activity, is present in the yeast proteome.

The only ester-synthesizing enzymes that have already been studied in any detail are Atf1p and its closely related homologue Lg-Atf1p. According to Fuji et al. (1996), in haploid *S. cerevisiae atf1_* strains, isoamylacetate transferase and ethanol acetate transferase activities were 80 and 20 % lower, respectively, than in the wild-type strain. This resulted in an 80 % decrease in isoamyl acetate production and a 30 % decrease in ethyl acetate production as compared to rates observed in wild-type cells. In another study, overexpression of *ATF1* derived from an industrial lager brewer’s yeast strain resulted in a 27-fold increase in isoamyl acetate production and a 9-fold increase in ethyl acetate production as compared to rates in empty-vector transformants (Fujii et al. 1994). Similarly, overexpression of *Lg-ATF1* showed a seven-fold increase in isoamyl acetate and a two-fold increase in ethyl acetate concentrations (Fujii et al. 1994). Lilly et al. (2000) showed that overexpression of *ATF1* in the commercial wine yeasts VIN7 and VIN13 leads to a 3- to 10-fold increase in ethyl acetate concentrations and a 4- to 12-fold increase in isoamyl acetate concentrations in the produced wines. Similarly, Verstrepen et al. (2003) have demonstrated that overexpression of *ATF1* in a commercial brewer’s strain leads to significantly increased concentrations of isoamyl acetate and ethyl acetate in the beers produced. These results indicate that the expression level of *ATF1* is an important limiting factor for ester synthesis under industrial conditions, confirming the hypothesis of Malcorps et al. (1991). *ATF1* gene also reported to have role in yeast survival under oxidative stress conditions (Sanso et al. 2008). Fernandez-Vazquez et al. (2013) showed that the fission yeast Sin3/Elp3 elongator complex is important for oxidative stress survival. They reported that the stress transcriptional program is governed by the Sty1-Atf1-Pcr1 pathway and is found to slightly affect the mutant cells.

2.9 Organoleptic studies using apple cider

The ability to produce palatable effervescent beverage and wine by alcoholic fermentation of natural fruit juices is a demonstration of inherent ingenuity of man. Apple
cider is a fermented beverage made from apples. Apples and several other fruits have the balanced quantities of acid, tannin, nutritive salts for yeast feeding, and water to naturally produce a stable and drinkable beverage. Therefore, alcoholic beverages in most countries are adjusted in one way or the other for fermentation of local fruits to produce their wines (Okunowo et al. 2005).

There is abundance of tropical fruits in India which includes apple, guava, pineapple, plum, orange etc. These fruits are highly perishable, and susceptible to bacterial and fungal contamination as a result they fail to reach the market due to over ripeness, spoilage and mechanical damage (Ihekoroye and Ngoddy 1985). Besides, these fruits are difficult to keep for considerable length of time; hence the ripe fruits are utilized either as fresh or processed into juice and specialty products (Oyeleke and Olaniyan 2007). High rate wastage of these fruits especially during peak season necessitates the need for alternative preservation and post-harvest technologies towards an enhanced utilization of these fruits. The production of alcoholic beverages from common fruits could help reduce the level of post-harvest losses (Alobo and Offonry 2009).

Apple (Malus domestica; Family Rose) is one of the most important fruits in India. It is one of the exotic fruits prized for its very pleasant, sub acid and aromatic nature. Apple fruit is a good source of vitamin C, carbohydrates, proteins, minerals, pectin, calcium and phosphorus. Jalali et al. (2013) reported the total soluble solids, titratable acidity, pH, Juice content, sugar-acid ratio and amount of acid on an average for apple fruit as 10.36 %, 4.26 %, 4.22 %, 0.56 %, 36.35 and 0.29 %, respectively.

The process of fermentation in alcoholic beverage has the catalyst function that turns fruit juice into an alcoholic beverage. During fermentation, yeast interacts with sugars in the juice to produce ethanol and carbon dioxide. During fermentation, there are several factors that winemakers take into consideration. In alcoholic beverage making, the temperature and speed of fermentation are important considerations as well as the levels of oxygen present in the Must at the start of the fermentation. There are number of factors which affect yeast performance in fermentation such as yeast strain employed, fermentation temperature, media composition, mode of substrate feeding, osmotic pressure, ethanol concentration, membrane composition etc. (D’Amore 1992).
Temperature is one of the most important parameters for the performance of alcoholic fermentation because it may affect the kinetics of the process as well as the final quality of the product. Fermentation temperature greatly affects yeast growth, fermentation rates and production of volatile compounds. In general, fermentation rates increase with increasing temperature (>29°C), after which premature cessation is probably due to elevated ethanol toxicity (D’Amato et al. 2006). Beltran et al. (2007) demonstrated that low temperature fermentations altered nitrogen transport and metabolism, and suggested that coordination between carbon and nitrogen metabolisms may be hampered.

The pH of a growth medium is another important parameter for the successful progress of fermentation because it influences yeast growth as well as ethanol formation, besides sensory quality of the alcoholic product. pH has been found to affect malic acid, an important volatile compound that affects titrable acidity. While studying the interaction of pH, alcohol concentration and wine matrix on malolactic fermentation (MLF), wine matrix showed greatest impact on the rate of MLF, followed by pH and alcohol (Paul and Hoger 2003).

Initial sugar level of juice also affects the rate of fermentation. Kaur and Kocher (2002) found 20% sugar concentration to be optimum for ethanol production from sugarcane juice. An increase in the initial sugar concentration decreases the fermentation rate. It has been reported that the fermentation efficiency of the yeast decreased with increase in initial sugar concentration (Attri 2009). Singh and Kaur (2009) reported maximum ethanol production up to the level of 24 °Brix, and there after no change was recorded at higher Brix levels. Singh et al. (1998) also reported similar results in case of kinnow wine with 24 °Brix after five days of fermentation. Moreover, the supplementation of nitrogen and phosphorus sources in the fermenting musts, has been found to increase yeast growth and sugar catabolic rate (Patil and Patil 2006; Ghosh et al. 2010).

2.10 Bio-emulsifier production

Bio-emulsifiers are surface active biomolecules produced by microorganisms. These molecules are capable of reducing surface and interfacial tensions in both aqueous
solutions and hydrocarbon mixtures (Ferraz et al. 2012). High molecular weight bio-\emulsifiers produce stable emulsions without lowering surface or interfacial tension (Bognolo 1999). Bio-emulsifiers have higher biodegradability over chemical surfactants, high selectivity, higher foaming, lower toxicity and stability at extreme temperatures, pH and salinity. Industrial applications of bio-emulsifiers are in the paint, cosmetics, textile, detergent, agrochemical, food and pharmaceutical industries (Banat et al. 2000).

Despite the numerous interesting properties of bio-emulsifiers, high cost of production and low yields compared to commercially available surfactants, are major obstacles for their large scale application. Efforts are being directed towards reducing their production cost and increasing the yields by strain improvement, nutritional and environmental optimization or fermenter design as well as using cheap and renewable substrates (Mulligan 2005). The carbon substrate is an important limiting factor in bio-emulsifier production (Sen 1997). The type of carbon substrate used for production has influenced both the quality and quantity of bio-emulsifier (Panilaitis et al. 2007; Abouseoud et al. 2008; Das et al. 2009).

Mannoprotein extracted from \textit{S. cerevisiae} is an effective bio-emulsifier (Cameron et al. 1988; Torabizadez et al. 1996). The emulsion produced is thick and viscous. Since \textit{S. cerevisiae} is edible (it is used in food and beverage products), the emulsifier is expected to be nontoxic and could have applications in the food and cosmetics industries (Dikit et al. 2010). This product also satisfies the current consumer demand for natural and environmentally safe products. Mannoproteins are freely soluble in water and can be extracted from the cell wall of \textit{S. cerevisiae} in high yields (Ballou 1976; Cabib and Roberts 1982). Thus, strains of \textit{S. cerevisiae} have become important from which bio-emulsifiers could be extracted by using water soluble substrates (Barriga et al. 1999; Torabizadez et al. 1996). These sources offer the advantages of low cost and a high volume of yeast biomass which translates into high bio-emulsifier yields than from synthetic sources.

2.11 Genetic diversity among \textit{Saccharomyces cerevisiae} strains

Vezinhet et al. (1999) carried out ecological studies on \textit{S. cerevisiae} strains isolated from the enological fermentative micro flora from two vineyards (Champagne and Loire Valley) for six consecutive years. The strain identification was performed by PFE
chromosomal patterns or mitochondrial DNA restriction profiles. In both situations, a large diversity in molecular patterns was seen. Some of the strains, which were more frequently encountered over the six-year experiment, seemed to be widely distributed.

Schuller et al. (2005) developed strategies for the preservation of biodiversity and genetic resources as a basis for further strain development. A total of 1620 yeast isolates were identified using mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) and a pattern profile was verified for each isolate, resulting in a total of 297 different profiles, belonging to the species S. cerevisiae. The strains corresponding to seventeen different patterns showed a wider temporal and geographical distribution, being characterized by a generalized pattern of sporadic presence, absence and reappearance.

Blanco et al. (2006) studied genetic diversity of wine S. cerevisiae strains involved in spontaneous fermentations by analysis of mitochondrial DNA restriction patterns. Yeasts were isolated at different stages of fermentations from three different white grapevine varieties. Nineteen different patterns, out of a total of 446 strains analyzed were identified, but only a few of them appeared at high frequencies which were found to be associated with the fermentation process. Some strains were common to all fermentations; however, most of them were in minority being only found at low frequency for one or two specific grape varieties. The dominant strain was different for each variety except in one case, suggesting that some strains are better adapted to certain must conditions.

Landry et al. (2006) predicted that S. cerevisiae, the budding yeast, is the most thoroughly studied eukaryote at the cellular, molecular, and genetic levels, yet there is a little knowledge about its ecology or population and evolutionary genetics. S. cerevisiae occupies numerous habitats and its population harbour important genetic variation. Therefore, there is an increasing interest in understanding the evolutionary forces acting on the yeast genome.

Valero et al. (2007) used commercial wine yeast strains as starters which are grown extensively over the past two decades. In this study, seventy-two spontaneous fermentations were completed from a total of 106 grape samples, and 2160 colonies were isolated. Among these, 608 Saccharomyces strains were identified and 104 different chromosomal patterns were found. The large majority of these strains (91) were found to
have unique patterns, indicating great biodiversity. Difference in biodiversity according to the vineyard and year was also observed showing that the biodiversity of *Saccharomyces* strains is influenced by climatic conditions and specific factors associated with the vineyards, such as age and size.

Legras et al. (2007) analyzed the genetic diversity among 651 strains from 56 different geographical origins, worldwide. Their genotyping based on 12 microsatellite loci revealed 575 distinct genotypes organized in subgroups of yeast types, i.e. bread, beer, wine and sake. Some of these groups presented unexpected relatedness. Bread strains displayed a combination of alleles intermediate between beer and wine strains, and strains used for rice wine and sake were most closely related to beer and bread strains. However, up to 28 per cent of genetic diversity between these groups was associated with geographical differences which suggest local domestications.

Capece et al. (2012) studied the diversity of *S. cerevisiae* strains associated to spontaneously fermenting grapes from an Italian “heroic vine-growing area”. 39 strains were selected on the basis various technological traits among 132 isolates. By using three molecular typing techniques (evaluation of cell wall gene polymorphisms, mtDNA restriction analysis, inter-delta amplification analysis), considerable amount of genetic variability was found. The analysis of principal aromatic compounds produced during inoculated fermentation of two grape musts demonstrated the strain impact on wine flavor and a significant influence of grape must on strain metabolic behavior.

Liu and Zhang (2014) studied the genetic variation among 41 *S. cerevisiae* strains using MF (ALPHA) 1 gene, a pheromone precursor gene. Sequence analysis demonstrated a distinct population structure in *S. cerevisiae* and a distinguishing *S. cerevisiae* strain was obtained from Kunming grape juice which was placed in a different group from rest of the strains. They also observed that the strains were clustered together according to ecological rather than geographical factors.