Chapter - 2
CHAPTER 2.0

Strain selection and its biochemical characterization for ethanol production under VHG conditions

2.1 Introduction

It was necessary to select an appropriate yeast strain suitable for very high gravity fermentation. *Saccharomyces cerevisiae*, the yeast commonly used for ethanolic fermentation, is not very tolerant to diminished water activity (Edgley and Brown, 1983), yet at 20 °C it can completely ferment wheat mashes containing 38 to 39% (w/v) dissolved solids, yielding over 23% (v/v) ethanol (Thomas *et al.*, 1993). Ethanol tolerance was thought to be independent of the nutritional conditions or the physiological state of the yeast and purely a reflection of its genetic makeup. It is now known that by altering nutritional conditions, it is possible to increase ethanol yield as well as the survival of yeast at high concentrations of ethanol (Casey *et al.*, 1983, 1984; Kolmokoff and Ingledew, 1985). For example, during sake fermentation, concentrations of ethanol up to 20% (v/v) can be achieved. This high yield results from the unique fermentation conditions used and not because of inherent genetic differences among the yeast strains (Kawaharada *et al.*, 1970).

However, nutrients alone cannot contribute to the yeast ethanol tolerance. Ethanol tolerance and the ability to accumulate high ethanol concentrations are also strain-dependent characteristics (Kosaric and Vardar-Sukan, 2001) especially under VHG conditions. In addition, environmental parameters such as temperature, osmotic pressure and carbon dioxide levels may directly affect yeast growth and ethanol productivity (Jones *et al.*, 1981). Successful VHG fermentation is therefore dependent not only on the optimal composition of a fermentation medium, but also on the yeast strain.

2.1.1 Role of reserve carbohydrates

Trehalose and Glycogen are the main reserve carbohydrates in yeast cells and has received extensive work recently (Francois and Parrou, 2001) to prove how important these carbohydrates are for the viability, vitality and physiological activity of yeasts. Sahara *et al.*, (2002) observed that the accumulation of the carbohydrate reserves
trehalose and glycogen is induced during late cold response, and also when the nutrients become limited. Several carbon sources like glucose, galactose or ethanol may serve as precursors for trehalose and glycogen. These two reserve carbohydrates in the yeast *Saccharomyces cerevisiae* can represent up to 25% of the dry cell mass, depending on the environmental conditions (Lillie and Pringle, 1980).

![Trehalose and glycogen metabolism diagram]

**Figure 2.1** Overview of trehalose and glycogen metabolism in *Saccharomyces cerevisiae*.

Many carbon sources can serve as precursors for trehalose and glycogen. As indicated in figure 2.1, trehalose is synthesized by the conversion of glucose-6-phosphate and UDP-glucose into trehalose-6-phosphate, which is catalyzed by trehalose-6-phosphate synthase. Subsequently, trehalose-6-phosphate phosphatase mediates the conversion of trehalose-6-phosphate into trehalose and free phosphate. Trehalose can be degraded by the action of two types of trehalases. The first type, cytoplasmic neutral trehalase, has its maximal activity at pH 6.8 – 7.0. The second type of trehalase is found in vacuoles, optimally active at pH 4.5 – 5.0, therefore designated as acidic trehalase.

Growth rate-regulated accumulation of reserve carbohydrates is observed when yeast cells are grown by batch cultures in shake flasks. During exponential growth on fermentable carbon sources, like glucose, galactose or fructose, cells
exhibit high growth rates and have low trehalose and glycogen levels. Upon depletion of glucose, the growth rate of the cells decreases, resulting in elongation of the G1 phase duration and accumulation of trehalose and glycogen. Several studies indicate that the trehalose accumulation pattern differs from that of glycogen (Francois et al., 1991; Parrou et al., 1999). Conditions like growth at increased temperature, hydrostatic pressure, desiccation, osmotic or oxidative stress, and exposure to toxic chemicals are also associated with decreased growth rates of cells, which may be a general mechanism to regulate reserve carbohydrate accumulation.

2.1.1.1 Glycogen: Glycogen metabolism is controlled by the activity of glycogen synthase and glycogen phosphorylase. Both enzymes are regulated by phosphorylation and dephosphorylation reactions. While phosphorylation activates phosphorylase and inactivates synthase, dephosphorylation provokes the opposite effects. Several reports indicate that cAMP-dependent protein kinases are involved in the control of these enzymatic activities in yeast (Mishra, 1983; Wingender-Drissen and Becker, 1983).

2.1.1.2 Trehalose: Trehalose is a non-reducing disaccharide of glucose and it was considered for a long time solely as a storage carbohydrate in yeast (Lillie and Pringle, 1980). In Saccharomyces cerevisiae, intracellular trehalose is accumulated when cells are submitted to a shift in temperature above 28 °C or exposed to either ethanol or hydrogen peroxide, suggesting that accumulation might correspond to a general response to physiological stress (Thevelein, 1984). In many organisms capable of surviving several environmental conditions such as freezing, heat shock, and dehydration, trehalose was found, seems to be cell protector (Thevelein, 1984) under such conditions.

Exogenous trehalose restored the viability of yeast cells during freezing by possible protection of the cellular membrane (Diniz-Mendes et al., 1999). At high temperature, trehalose can protect cells by acting as a “chemical chaperone”, which reduces heat-induced denaturation and aggregation of proteins in yeast cells (Welch and Brown, 1996). Trehalose is also capable of protecting DNA and lipids (Benaroudj, 2001). Trehalose synthesis is stimulated by heat shock and osmotic stress (Ribeiro et al., 1994; De Virgilio et al., 1994) and its accumulation correlate with
thermotolerance of yeast cells (Piper, 1998; Singer and Lindquist, 1998a; Singer and Lindquist, 1998b)

Under high gravity conditions yeast cells are exposed to several environmental stresses due to high osmotic pressure and high ethanol concentrations. Moreover, exposure of yeast cells to high osmolarity leads to dehydration, collapse of ion gradients across the plasma membrane and decreased cell viability (Mager and Varela, 1993). These result in changes in biochemical composition of yeast – increased intracellular trehalose and decreased proteins and glycogen (Majara et al., 1996). Trehalose protects the cells by preserving the integrity of biological membranes and stabilizing proteins in their native state (Lucero et al., 2000).

2.1.2 Invertase activity

Sucrose and raffinose are hydrolyzed by the invertase enzyme of yeast *S. cerevisiae*. There are two different forms of invertase. Extracellular invertase is highly glycosylated while intracellular invertase is not glycosylated (Carlson, 1998). The physiological significance of intracellular invertase is not known. Both intracellular and extracellular invertases are expressed from the SUC2 gene (Perlman et al., 1984).

There is evidence that the ability of *S. cerevisiae* to ferment high sucrose concentrations is related inversely to activity of invertase (Evans, 1990). One hypothesis is that high sugar levels lead to depletion of intracellular phosphate, which in turn slows glycolysis (Thevelein and Hohmann, 1995). The hydrolysis of sucrose to free glucose and fructose increases osmotic pressure, and when high invertase activity is present, this occurs rapidly to inhibit yeast.

The aim of this study is to screen-out a microorganism, which can grow and ferment well under high osmolarity conditions; the effect of nutrients supplementation on yeast growth and ethanol yield. And also, yeast cellular biochemical changes during the course of fermentation under VHG conditions.

2.2 Materials and Methods

2.2.1 Strain selection

Four yeast strains were procured from different sources. The strain *Saccharomyces cerevisiae* 101 was obtained from the Central Food Technological Research Institute
(CFTRI), Mysore, India. A flocculating, non-amylolytic yeast *Saccharomyces bayanus* was kindly provided by Dr. Roberto Ambrosoli, university of Turin, Italy. Fission yeast *Schizosaccharomyces pombe* *NCIM* 3457 was obtained from National Collection of Industrial Microorganisms, National Chemical Laboratory (NCL), Pune. They were shipped in glass slants containing MPYD (Wickerham) agar. Baker’s yeast *Saccharomyces cerevisiae* was procured from a local bakery store.

![Schizosaccharomyces pombe NCIM 3457](image1) ![Saccharomyces bayanus](image2)

*Figure 2.2* Four yeast strains used in VHG fermentations.

### 2.2.2 Maintenance of yeast cultures

The procured yeast strains were maintained (preserved) on conventional slants containing wickerham (MPYD) agar (Wickerham, 1951) by periodic transfer method. The slants were stored at refrigerated temperature (4-5°C) in order to reduce the metabolic activity of cultures; thereby increasing the duration of periodic sub-culture to 3-6 months.
Wickerham agar medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Malt extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Agar agar</td>
<td>22.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Suspended the ingredients in water and heated to boiling to dissolve completely. pH was adjusted to 5.0 and required volume dispensed to test tubes. The tubes were plugged with cotton and sterilized by autoclaving at 121 °C for 20 min; suitable slants were prepared by allowing the tubes to cool on a slant board.

2.2.3 Monitoring of cell growth

The culture growth was monitored by measuring the optical density (OD) of the cell suspension at 540 nm using a spectrophotometer (ELICO India Ltd.). For flocculating yeast, a 30 mM/l EDTA was used to ensure floc disruption.

2.2.4 Cell dry weight estimation

A 10 ml volume of the culture broth was centrifuged at 5000 rpm in centrifuge for 15 min to separate the yeast cells, washed repeatedly for three times (centrifugation after each washing) using de-ionized water and the centrifuged cells were dried at 70-90 °C for 12 h, till constant weight attained. This method does not distinguish between dead and live cells.

2.2.5 Total count using haemocytometer

The cell concentration was determined by a direct microscopic cell count using a haemocytometer (Neubauer, Germany). The culture broth was diluted suitable and was mounted on to haemocytometer with the aid of a coverslip and individual cells were counted in four corner squares plus Central Square (each square area - 1/25 mm$^2$ x 0.1 mm depth) and average count per square was taken. The total number of cells per ml was calculated using the formula: Average number of cells per square x 25 x $10^4$. 

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2.2.6 Yeast viability

The yeast cells viability was determined by the methylene blue staining technique (Postgate, 1967). A 200 μl sterile solution of methylene blue (3.3 mM in 68 mM Sodium citrate) was mixed with 200 μl of yeast suspension and diluted to reach an OD of 0.4-0.7 at 620 nm. This mixture was shaken and after 5 min incubation, placed in a Neubauer counting chamber. The number of stained (inactive) and unstained (active) cells were counted in five different fields with a total of 200-300 cells and the percentage of viable (active) cells was noted.

2.2.7 Measurement of ethanol tolerance

The ethanol tolerance of the yeast strains was examined through ethanol shock experiments as described by Fengwu Bai, 2007. The 250 ml flasks containing 100 ml medium composed of 30 g l\(^{-1}\) glucose, 5 g l\(^{-1}\) yeast extract, and 3 g l\(^{-1}\) peptone were prepared and sterilized at 121 °C for 15 min. After cooling, 10 ml fresh culture of each strain was inoculated and incubated overnight at 30 °C and 150 rpm. Then, ethanol shock was exerted onto the cultures by adding 20 ml ethanol into each of these cultures, making the ethanol concentration about 15% (v/v). The cultures were incubated at 30 °C and 150 rpm for two hours, and the percentage of viable cells was counted by the regular methylene blue stain and chamber counting techniques. If the ethanol tolerance of the strains could not be distinguished, the concentration of ethanol shock was increased to 18% (v/v).

2.2.8 Growth studies of ethanol tolerant strains in a modified Wickerham’s high gravity medium

In order to identify the osmotolerant yeast among the selected strains, ethanol tolerant yeast strains, viz. *Saccharomyces bayanus*, *Saccharomyces cerevisiae* CFTR1 101 and *Schizosaccharomyces pombe* NCIM 3457 were evaluated for their growth under high gravity conditions. High gravity media were prepared by increasing the concentration of carbon source, glucose, of the Wickerham medium without altering other nutrients concentration, which mimics a simple high gravity medium.
Composition of modified Wickerham's medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>200-300 g/l</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g/l</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0 g/l</td>
</tr>
<tr>
<td>Malt extract</td>
<td>3.0 g/l</td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The media were sterilized by autoclaving at 121°C for 15 min, cooled and inoculated with 0.1 ml of 24 h grown cell suspension of each strain. The turbidity was measured by spectrophotometer at 540 nm after inoculation and subsequently at intervals of 6 h for 42 h. pH of the media were maintained at 5.0 and the growth temperature at 30 °C on a rotary shaker (130 rpm).

2.2.9 Effect of yeast extract supplementation on growth

Yeast extract 12 g/l was supplemented to the modified Wickerham’s (high gravity) medium, as described in the previous section, without altering the other nutrients. The yeast extract concentrations were raised to 1.5% in supplemented media having 200 g/l and also 300 g/l glucose concentrations. The media were inoculated with *Saccharomyces bayanus* yeast strain at an initial cell number of $2.5 \times 10^7$ cells/ml of medium and allowed to grow at 30 °C for 72 h duration on rotary shaker. Yeast cell counts were noted at 12 h time intervals.

2.2.10 Effect of temperature on yeast growth

To assess the effect of temperature on growth of yeast, *Saccharomyces bayanus*, high gravity modified Wickerham’s medium was prepared with glucose concentrations 200, 250 and 300g/l, the growth of yeast was monitored at different temperatures between 15 °C to 35 °C.
2.2.11 Fermentative ethanol production of yeast strains under high gravity conditions

All the three yeast strains were tested for their efficiency in sugars to ethanol conversion. Defined very high gravity sugar medium containing 300 g/l glucose as sole carbon source was used to determine the fermenting ability of the yeast strains.

**Defined very high gravity (VHG) medium**

<table>
<thead>
<tr>
<th>Component</th>
<th>Control</th>
<th>Complete medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>Ammonium sulphate ((\text{NH}_4)(_2)\text{SO}_4)</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.0</td>
<td>3.0 + 9.0 (12 g/l)</td>
</tr>
<tr>
<td>Potassium dihydrogen orthophosphate ((\text{KH}_2\text{PO}_4))</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnesium sulphate ((\text{MgSO}_4.7\text{H}_2\text{O}))</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calcium chloride ((\text{CaCl}_2.2\text{H}_2\text{O}))</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Temperature was maintained at 30 °C and the pH at 4.6-4.8. Fermentations were run for 72 h period. Additional 9.0 g/l yeast extract was supplemented to the control medium in order to provide sufficient nitrogen and other nutrients for yeasts, that form complete defined very high gravity medium.

2.2.12 Glucose estimation

Residual glucose concentration in the fermenting medium was measured using the DNS method (Miller, 1959).

2.2.13 Ethanol estimation

*Gas chromatographic method:*
Ethanol in the fermented wash and fermentation by-products such as aldehydes, methanol, total esters, total fusel oils and acetone contents were estimated by using gas chromatography (GC) as per standard methods (Anthony, 1984).
Agilent systems model 6890 was used and the conditions were as follows: Graphitized packed column 5% carbowax 20M phase, matrix 80/120 carbopack-B, Length 6 ft (1.83m) × 2mm I.D. × 1/4" O.D. Nitrogen was used as carrier gas with flow rate of (20 ml/min) and eluted compounds were detected by flame ionization detector (FID). Hydrogen was used as fuel gas, with flow rate 40 ml/min, along with air at a flow rate of 400 ml/min. Secondary butyl acetate was used as internal standard.

Specific gravity method: 
Ethanol was determined by specific gravity method (Anon, 1984). In this method, one hundred ml of sample along with 40 ml distilled water was distilled to collect 100 ml distillate on ice bath. The specific gravity of the distillate was determined at 20°C. The percentage (v/v) of ethanol was obtained from the alcohol tables published in AOAC (1984).

2.2.14 Glycerol estimation: An appropriate amount of cell culture (usually 1.5 ml) was rapidly sampled from fermenting defined very high gravity medium and centrifuged in an Eppendorf centrifuge at 5000 rpm at 4 °C for 10 min. The clear supernatant containing the extracellular glycerol was transferred to a water bath and boiled for 10 min together with a parallel sample of the uncentrifuged cell culture. The latter sample containing extracellular plus intracellular (total) glycerol was cleared by centrifugation, and both samples were frozen until analyzed. Intracellular glycerol was determined by subtracting the extracellular glycerol from that of the total. Glycerol was determined enzymatically by the glycerol kinase method (Megazyme: Glycerol assay method K-GCROL) (Wieland, 1988; Klopper, 1986).

2.2.15 Total lipid extraction: Lipids were extracted by treating a portion (200–300mg dry weight) of the cells with 20 ml of 80% (v/v) ethanol at 80 °C for 15 min to deactivate lipolytic enzymes. The suspension was filtered, and the residue was re-extracted with chloroform-methanol (1: 1, v/v) at room temperature for 3hr and then again for a further 2 h. After filtration, the extracts were combined and subjected to washings with the addition of a 0.25 volume of 0.88% (w/v) KC1 solution. Fatty acids analysis was carried out by gas chromatograph using DB 225 column.
2.2.16 Yeast cells for trehalose, glycogen estimation

For high gravity fermentation studies, yeast cells were harvested at regular intervals from the fermenting defined very high gravity complete medium. Glucose concentrations were altered accordingly, without altering other nutrients concentrations in case of 20% and 25% high gravity complete media.

2.2.16.1 Trehalose extraction and determination

Trehalose was detected according to Sharma and Trevelyan’s method (Sharma, 1997; Trevelyan and Harrison, 1956). 5 ml of yeast suspension was added to a test tube. Yeast cells were washed twice with cold sterile distilled water and all the test tubes were placed on ice. To each test tube, 4 ml of cold tri-chloro acetic acid (TCA, 0.5 M) was added, and the contents in tubes were mixed at 10 min intervals up to 30 min. Centrifugation (4000 rpm for 15 min) was performed and supernatant was collected in a clean 50 ml volumetric container. After adding TCA solution, centrifugation and supernatant collection were repeated. The final volume of collected TCA solution was adjusted to 50 ml with cold sterile distilled water and 1 ml of it was transferred to another clean test tube containing 5 ml of Anthrone reagent solution (0.8 g of anthrone in 500 ml of 27.4N sulfuric acid). All the tubes were placed on boiling water bath for 10 min and then the O.D. was taken at 620 nm. The trehalose content of each sample was compared with the standard curve and recorded.

2.2.16.2 Glycogen determination

Cells (10 mg [dry weight]) were collected by centrifugation (3,000 × g; 2 min) from very high gravity fermenting media (200-300 g/l glucose) at several stages and were washed with cold distilled water. Cells were stored at -20°C. Then, they were thawed, resuspended in 0.25 ml of 0.25 M Na2CO3, transferred to screw-cap tubes, and incubated at 95 °C for 4 h. Cell extracts were neutralized by adding 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M Na acetate (pH 5.2). And then, the samples were processed for enzymatic determination of glycogen as described (Parrou, 1997).

2.2.17 Determination of invertase activity of yeasts

The invertase activity was measured by the dinitrosalicylic reagent (DNS) colorimetric test as described in Ekunsanmi and Odunfa. Yeast was grown on YPD agar slants for 48 hours, the cells were diluted in sterile water, washed by
centrifugation, and 0.1 g wet weight was resuspended in 10 ml of acetate buffer, pH 5.0. One milliliter of cell suspension was added to 2 ml of 4% sucrose solution in the same buffer and incubated for 5 min at 30°C. One unit of invertase activity was defined as the amount of enzyme which liberated one µmol/min of reducing sugars under these conditions.

2.3. Results and Discussion

2.3.1 Evaluation of ethanol tolerance

The ethanol tolerance of *S. cerevisiae CFTRI 101* and *Schizosaccharomyces pombe NCIM 3457* as well as the self-flocculating yeast strain, *Saccharomyces bayanus*, was examined by applying 15% and 18% ethanol shocks to their cultures for two hours, followed by the count of their viable cells. Higher the percentage of the viable cells, better the ethanol tolerance of the strain will be. The experimental results are illustrated in the table 2.1.

**Table 2.1** Ethanol tolerance of *S. cerevisiae CFTRI 101*, *Schizosaccharomyces pombe* and *S. bayanus* in comparison with *S. cerevisiae* (baker's yeast) control.

<table>
<thead>
<tr>
<th>Strains</th>
<th>conditions</th>
<th>viability</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em> (baker's yeast)</td>
<td>15% ethanol shock for 2 hours</td>
<td>87.7%</td>
</tr>
<tr>
<td></td>
<td>18% ethanol shock for 2 hours</td>
<td>0.0</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> CFTRI 101</td>
<td>15% ethanol shock for 2 hours</td>
<td>95.4%</td>
</tr>
<tr>
<td></td>
<td>18% ethanol shock for 2 hours</td>
<td>12.8%</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>15% ethanol shock for 2 hours</td>
<td>92.7%</td>
</tr>
<tr>
<td><em>NCIM 3457</em></td>
<td>18% ethanol shock for 2 hours</td>
<td>5.0%</td>
</tr>
<tr>
<td><em>S. bayanus</em></td>
<td>15% ethanol shock for 2 hours</td>
<td>97.6%</td>
</tr>
<tr>
<td></td>
<td>18% ethanol shock for 2 hours</td>
<td>28%</td>
</tr>
</tbody>
</table>

The experimental data indicate that no significant difference in ethanol tolerance was observed among the four yeast strains when 15% ethanol shock was applied. However, when the concentration of ethanol was increased to 18%, *S. bayanus* and *S. cerevisiae CFTRI 101* with their viable cell percentages of 28% and 12.8% exhibited better ethanol tolerance, and no viable cells were detected in *S. cerevisiae* (baker's
yeast) culture. These experimental results were in good agreement with generally expected high ethanol tolerance of *S. bayanus* than that of the rest, as the strain is self-flocculent.

### 2.3.2 Growth under high gravity conditions

Prolonged lag phase in growth curve was observed (6-12 h) after inoculation under high sugar concentration. An increased sugar concentration from 20% to 30% has decreased the cell growth of all the selected yeast strains.

![Growth curves of Schizosaccharomyces pombe NCIM 3457](image)

**Figure 2.3** Growth curves of the yeast *Schizosaccharomyces pombe NCIM 3457* under various increased sugar concentrations (High gravity) of the Wickerham medium at 30°C.
Figure 2.4 Growth curves of the yeast Saccharomyces cerevisiae CFTRI 101 under various increased sugar concentrations (High gravity) of the Wickerham medium at 30°C.

Figure 2.5 Growth curves of the yeast Saccharomyces bayanus under various increased sugar concentrations (High gravity) of the Wickerham medium at 30°C.

Growth of all the three yeast strains were continued up to 42 h under all the tested high gravity concentrations. At 200 g/l sugar concentration, both Saccharomyces cerevisiae 101 and Saccharomyces bayanus strains have shown equal growth rates (Figure 2.4 & 2.5) compared to Schizosaccharomyces pombe (Figure 2.3). However, increased sugar concentration of the media to 250 g/l has shown negative effect on the growth of S. cerevisiae 101; whereas in case of S. bayanus
growth was extended. The lag of these two strains was prolonged for 12-18 h in the media containing 30% glucose. Increased growth rate was observed with *Saccharomyces bayanus* at this high sugar concentration. Growth of the yeast *Schizosaccharomyces pombe* has shown least growth among the three tested strains under high gravity conditions.

### 2.3.3 Growth of strains in high sucrose media

Instead of glucose as carbon source, 30% sucrose was utilized in preparation of very high gravity medium. The three ethanol tolerant yeast strains were tested for their growth in the modified Wickerham medium (as described earlier). pH was adjusted to 5.0 and incubated on a rotary shaker (130 rpm) at 30°C.

![Growth curves of the three yeast strains under very high gravity (300 g/l) sucrose medium at 30°C.](image)

Figure 2.6 Growth curves of the three yeast strains under very high gravity (300 g/l) sucrose medium at 30°C.

Figure 2.6 depicts the growth pattern of three selected yeast strains under very high gravity (30% sucrose) condition. Even with using sucrose as carbon source, *Saccharomyces bayanus* yeast strain has shown better growth in the Wickerham medium containing minimal nutrients.

Growth rate was accelerated after initial 6-12 h lag phase, a continuous increase of cell count was observed up to 42 h under constant shaking condition. Whereas in case of other two strains, viz. *Saccharomyces cerevisiae* 101 and
Schizosaccharomyces pombe NCIM 3457, decreased growth rates were observed at this concentration of sucrose medium.

2.3.4 Yeast extract supplementation

In an un-supplemented typical high gravity medium, the cell number increased to $2 \times 10^8$ cells per milliliter of medium within first 48 h. Upon the addition of 1.5% yeast extract, there was a continuous growth throughout the fermentation period and a maximum cell number as high as $4.2 \times 10^8$ cells/ml of medium was reached after 72 h duration.

Figure 2.7 A comparison of effect of yeast extract addition on growth of Saccharomyces bayanus under high gravity (200 g/l glucose) Wickerham medium at 30°C.

In very high gravity (VHG) fermenting medium maximum cell count was reached to $3.4 \times 10^8$ cells per milliliter upon supplementation with yeast extract 15 g/l, whereas in case of un-supplemented medium, maximum count was not exceeded beyond 120 millions/ml during 72 h growth.
2.3.5 Effect of temperature

Temperature has shown significant effect on the growth of the *S. bayanus* strain under high gravity conditions. A steady increase in total cell count was observed with increase of temperature from 15-30 °C in the modified Wickerham's high gravity media (Figure 2.9).

*Figure 2.8* A comparison of effect of yeast extract addition on growth of *Saccharomyces bayanus* under very high gravity (300 g/l glucose) Wickerham medium at 30°C.

*Figure 2.9* Effect of incubation temperature on growth of *Saccharomyces bayanus* under very high gravity (300 g/l glucose) Wickerham medium at 36 h of growth.
Beyond 30 °C, raise in incubation temperature has shown negative effect on growth rate. Thus, 30 °C temperature found to be optimal. More than 65% increase in the cell number was observed with raise of temperature from 15-30°C.

2.3.6 Ethanolic fermentations of defined very high gravity medium

Increased ethanolic fermentations were observed when various yeast nutrients were supplemented to the defined high gravity medium (control). In the present study, an un-supplemented medium of 300 g/l of glucose initial concentration was incompletely fermented by all the tested strains (Figure 2.10), and final concentration of ethanol did not exceed 11% (v/v). Addition of 15 g/l yeast extract as nutrients supplement has shown a significant increase, final ethanol concentration >15.5% (v/v), with S. bayanus yeast strain (Figure 2.11).

![Figure 2.10 Profiles of sugar utilization and ethanol formation by selective yeast strains in a defined very high gravity (control) medium.](image-url)
Supplementation has decreased the time required for the maximum conversion of sugar to ethanol in the process. Fermentation period was decreased from 72 to 48 hours upon supplementation with yeast extract as well as other nutrient sources. Thus, overall ethanol productivity has exceeded 2.5 g/l/h, nearly 50% of final ethanol concentration was formed within 24 h in the supplemented media.

2.3.7 Influence of various yeast nutrients on ethanol production

Besides yeast extract, other nutrients, namely ammonium sulphate, yeast autolysate, skim milk solids and urea were tested for their stimulating effect on ethanol fermentation activity. All these supplemented nutrients are rich sources of yeast assimilable nitrogen, helps in yeast growth and multiplication, thereby growth dependent higher ethanol yield.
Maximum ethanol concentration as high as 123 g/l was observed within 48 h of fermentation duration; representing 80% fermentation efficiency. Yeast extract was found to be suitable yeast nutrient under very high gravity conditions as it has shown better ethanol productivity and final yield, among the tested nutrients (Figure 2.12)

2.3.8 Fermentation by-products:

2.3.8.1 Glycerol:

![Image of glycerol concentration over fermentation time](image)

**Figure 2.13** Time dependent accumulation and release of glycerol during fermentation of defined very high gravity medium with *Saccharomyces bayanus*. 

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Figure 2.13 shows intra- and extra-cellular levels of major ethanol fermentation by-product, glycerol, in very high gravity (defined) fermenting medium. The intracellular accumulation of glycerol was observed under high osmolarity condition due to activation of HOG synthesis pathway. The glycerol content of cells increased in linear proportion with increase of external osmolarity during initial lag phases and early logarithmic growth phases. Nearly a 2 fold increase was observed within 12 h after inoculation. During later stages of log phase and stationary phases of fermenting yeast, the intracellular accumulated glycerol released into the surrounding medium.

2.3.8.2 Other by-products
Figure 2.14 Congeners (by-products) profile during VHG defined (control) medium fermentation a) chromatogram b) concentrations of individual by-products.

Total fusel oils concentrations in the distillate of very high gravity glucose medium was found to be low, and was due to lower concentrations of amino acids in the medium. High methanol concentrations were observed under nutrients insufficient high gravity conditions.

2.3.9 Fatty acids composition:
The most abundant fatty acids were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1) and linoleic (18:2) acids, which formed over 90% of the total fatty acid pool. Other fatty acids identified in this study, include linolenic (18:3) and eicosanoic (20:0) acids.

Table 2.2 Effect of yeast extract supplementation on fatty acid composition of yeast.

<table>
<thead>
<tr>
<th>Fatty Acids Composition (wt. %)</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (24 h)</td>
<td>8.4</td>
<td>25.4</td>
<td>9.6</td>
<td>35.2</td>
<td>7.4</td>
<td>1.9</td>
<td>0.8</td>
<td>11.3</td>
</tr>
<tr>
<td>Supplementation</td>
<td>6.4</td>
<td>30.1</td>
<td>24.1</td>
<td>26.0</td>
<td>6.1</td>
<td>1.1</td>
<td>1.0</td>
<td>11.3</td>
</tr>
<tr>
<td>(Yeast extract)-24 h</td>
<td>12.3</td>
<td>27.1</td>
<td>18.9</td>
<td>21.8</td>
<td>6.0</td>
<td>1.7</td>
<td>0.8</td>
<td>11.4</td>
</tr>
<tr>
<td>At 48 h fermentation(YE-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplemented)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are the average of three assays

16:0 (Palmitic acid); 16:1 (Palmitoleic acid); 18:0 (Stearic acid); 18:1 (Oleic acid); 18:2 (Linoleic acid); 18:3 (Linolenic acid)
Increased concentrations of unsaturated fatty acids were detected in yeast without additional yeast extract supplementation. Whereas, degree of unsaturation decreased upon yeast extract supplementation, especially oleic acid concentration. Increased synthesis of saturated fatty acids in supplemented medium was the indication of resistance of cells to the ethanol induced stress.

2.3.10 Trehalose metabolism under high gravity conditions

Trehalose metabolism was monitored in fermenting S. bayanus yeast cells under high gravity (>200 g/l dissolved solids) and very high gravity (~300 g/l dissolved solids) media at regular intervals between 0-72 h. Figure 2.15 depicts the pattern of trehalose concentrations over the time course of all fermentations. At normal gravity as in case of inoculum development, the yeast cells were not subjected to much osmotic stress and therefore lower trehalose content at start of the fermentation. Under high gravity conditions, trehalose content was rapidly accumulated inside the cells during lag phase of growth.

Table 2.3 Intracellular accumulation of trehalose at initial stages of fermentation under very high gravity conditions.

<table>
<thead>
<tr>
<th>Substrate concentration</th>
<th>Intracellular trehalose content mg/g cells dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>200 g/l</td>
<td>7±2</td>
</tr>
<tr>
<td>250 g/l</td>
<td>4±1</td>
</tr>
<tr>
<td>300 g/l</td>
<td>9±2</td>
</tr>
</tbody>
</table>

Values represent means of triplicates ± SD

At normal gravity, the maximum accumulated trehalose was 1.5% on dry weight basis (dwb), and no trehalose was detected at the end of fermentation. At high gravity conditions, yeast cells accumulated much higher amounts of trehalose compared to normal gravity fermentations. The maximum concentration found was 7.8% dwb (a 5.2-fold increase over the maximum level found in yeast fermenting normal gravity) at initial 0-6 h fermentation. Even at the end of fermentation (48-72 h), more trehalose was found accumulated. Increased trehalose concentrations in cells of 25% glucose medium than that of 20% medium was likely due to increased osmotic stress induced at this higher gravity. Initial accumulated trehalose was degraded, and a comparatively low trehalose levels were found at 24-48 h duration. These cells were
still under glucose repression and trehalose synthesis was blocked until medium glucose was consumed. During the initial 48 h of fermenting period there was maximal yeast growth and the fastest fermentation rate. A similar result was observed by Majara et al., 1996.

![Figure 2.15 Intracellular accumulation of trehalose over 72 h fermenting period in the Saccharomyces bayanus strain under high gravity conditions.](image)

Even greater concentrations of trehalose were accumulated in cells fermenting very high gravity medium. Maximum concentration of 26% dwb trehalose was accumulated with in 24 h after the onset of fermentation. This corresponds to a 3.3 fold increase over the maximum level observed in 20% medium. In spite of cell growth and vigorous fermentation, cells tend to accumulate greater amounts of trehalose at 24 h duration. Later, intracellular concentration rapidly decreased to low levels at the end of fermentation.

2.3.11 Glycogen metabolism under high gravity fermenting conditions

Under normal gravity or low gravity conditions, glycogen accumulation is initiated in yeast cells when glucose level depletes in the medium; whereas under high gravity fermenting conditions, initial accumulation of glycogen and trehalose was observed. Glycogen levels decreased with the progression of yeast growth.
As shown in the figure 2.16, low glycogen depositions were observed in yeast *S. bayanus* fermenting 20% defined high gravity medium at start of the fermentation, compared to 25 and 30% glucose media. Glycogen accumulation was initiated after 36 h when the sugar concentration lowered in the medium. However, under osmostress conditions (25-30% glucose), initial rapid accumulation and slight reduction in later stages of fermentations were observed. Over 50% reduction in cellular glycogen concentrations were observed in osmo- and ethanol-stressed cells as compared to cells fermenting 20% glucose medium at 72 h duration. Due to low glycogen reserves, these stressed yeasts are not suitable for yeast recycling fermentations.

### 2.3.12 Invertase activity of sugar and ethanol tolerant strain

*Saccharomyces bayanus* yeast strain was tested for its invertase activity as the strain has showed better growth under very high gravity conditions. Initially, the strain was grown and invertase activity was determined in low gravity medium. For determination of invertase activity under high sucrose concentration, the sucrose concentration was increased to 300 g/l and yeast extract, peptone concentrations were 2.0 g/l, 5.0 g/l respectively at pH 5.0. The flasks were autoclaved at 121 °C for 15 min, and then cooled to room temperature and inoculated. Flasks were then incubated in a rotary shaker (130 rev/min) at 30 °C for 48 h, one milliliter cell suspensions were
drawn at regular time intervals inorder to determine invertase activity as described previously.

![Graph showing invertase activity under low and high gravity conditions.](image)

**Figure 2.17** A comparison of invertase activity of yeast under low and high gravity fermentation conditions.

### 2.3.13 Effect of high sugar content on invertase activity

Increased sugar concentrations of the growth medium is inversely proportional to the sucrase or invertase activity of the yeast. In the present study, as shown in the figure 2.17, there was a marked decrease in the invertase activity upon growth in the high gravity medium. Almost two fold decrease was noted, invertase synthesis was initiated in the medium after a six hour lag phase in the very high gravity medium. The reason was generation of a high concentration of invert sugar in the medium resulting in glucose induced repression of invertase (Elorza *et al.*, 1977; Vitolo *et al.*, 1995)

Many factors influences the activity of invertase in yeast cells such as cultivation conditions, pH temperature, many metal ions and nitrogen sources in the medium. Nitrogen sources and their concentrations have a major effect on enzyme yield because sucrose metabolism shows a specific physiological response to the presence of nitrogen source (Silveira *et al*. 2000).
2.4 Conclusion: From this study it can be concluded that *Saccharomyces bayanus* yeast has better ethanol tolerance (up to 18%) among the tested yeast strains. It showed growth and fermenting ability under high gravity conditions. Yeast extract supplementation shows profound impact on its growth and fermenting ability. Ethanolic concentrations as high as 15.6% (v/v) with simple VHG medium is a good achievement by using this strain and further studies needed for industrial scale applicability.

Due to its better ethanol tolerance and growth under in very high gravity conditions, *S. bayanus* was selected and used in further studies of present thesis work.