Chapter - 5
CHAPTER-5.0
Effect of added malts on ethanol formation under VHG fermenting conditions

5.1 Introduction

In recent years, malting of cereals other than hulled barley has been in research domain (Dewar et al., 1997; Suhasini and Malleshi, 1995; Hammond and Ayernor, 2001). The main reasons are being economic considerations and local availabilities. In African countries, various cereals such as sorghum, rice, maize and finger millet (ragi) have been malted for use as sources of enzymes in sugar and brewing industries. The primary objective of malting is to promote the development of hydrolytic enzymes. These hydrolytic enzymes convert reserve complexes of seeds to simple forms during germination. Cereal malts are important sources of α-amylases for fermentation process in brewing industry. During malting, the seeds undergo various changes of modification such as increase in quantities of α and β amylases, partial degradation of reserve substances like cell wall, gums, protein and starch. It also helps in conversion of protein reserves in favor of assimilable nitrogenous materials essential for adequate yeast growth and fermentation (Jones and Pierce, 1964; Baxter, 1981; Pierce, 1982). Barley malt is preferred because of production of relatively large amounts of both α and β amylases during malting. These amylases are able to convert starch adjuncts to simple fermentable sugars during mashing process of beer production. Other than barley, wheat and rye malts are known to produce higher amounts of amylases (Egwin and Oloyede, 2006). The combination of both amylolytic enzymes results in a more rapid and complete degradation of starch to fermentable sugars.

Ethanol and organic acids fermentation using malts was studied previously by many researchers. The use of a water extract of barley malt sprouts as a substitute for expensive yeast extract was demonstrated successfully (Hujanen and Linko, 1996) for lactic acid production with Lactobacillus casei. While a pepsin digest of barley malt sprouts successfully stimulated biomass growth and ethanol productivity by three cider yeasts (Iliev et al., 1992). Tryptic digests of sorghum malt sprouts was successfully used in VHG fermentation media to generate vast improvements in ethanol yields and production efficiency (Ezeogu et al., 2005).
Malts are sources of amylases, therefore can be used in starch to ethanol fermentation for conversion of starch to fermentable simple sugars. On the other hand, malts also contain sufficient amounts of nitrogen in the form of free amino nitrogen (FAN), the one which is lacking in native starches. The FAN is the important component of fermenting media. Cecil (1995) reported maltose, glucose and higher polymers in sugar syrup from cassava using rice malt as enzymes source. These fermentable sugars can be used to produce alcohol by yeast. During alcoholic fermentation, various changes are observed as a result of the metabolic activities of the yeasts.

The objectives of the present study are as follows:

1. To evaluate the suitability of malts other than barley for conversion of starch to fermentable sugars and further fermentation to ethanol by yeasts.

2. To study the role of cereal and millet malts (as source of α and β amylases and free amino nitrogen) in starch to ethanol fermentation process.

3. To assess the effects of malt addition on yeast growth, fermentation efficiency, ethanol productivity and cell viability in high gravity fermenting medium.

5.2 Materials and Methods

5.2.1 Germination/malting of seeds

Viable finger millet seeds, paddy, sorghum grains were sorted manually to remove broken kernels and foreign materials, and washed thoroughly in water and soaked in a volume of water three times the weight of the seeds for 24 h, individually. The soaked seeds were placed on moist cloth in a basket and kept under ambient temperature (25-35°C) and watered 2-3 times a day to enhance germination process.

5.2.2 Preservation of enzymes (green malt)

A portion of the germinated seeds of all the three were solar dried (36-38°C) for 20-25 h. The dried malt samples were milled, packaged and stored in a refrigerator at 4°C.
5.2.3 Malt processing by kilning

Germinated seeds were taken out every 24 h and dried at 50 °C in a hot air oven for 12 h. The process is known as kilning. After that, vegetative portions (sprouts) were removed manually. De-vegetated seeds were weighed, powdered and stored at 4°C.

5.2.4 Fermentation of ethanol using kilned malts

Pre-cultured *Saccharomyces bayanus* yeast was used for fermentation of a typical high gravity fermentation medium containing 300 g/l glucose as carbon source along with nutrients such as peptone-0.5%, yeast extract-0.3% and malt extract-0.3%. pH was maintained at 5.0 and temperature at 30°C. All the powdered grain malts (from 5th day of germination) were added in the range of 1-4% w/v concentrations to the above typical high gravity fermenting medium for supplementation studies and fermentation was carried out for 72 h under stationary conditions.

5.2.5 Starch hydrolysis by malt enzymes

Starch flours namely cassava, sorghum and finger millet (*ragi*) flours were selected for enzymatic hydrolysis using green malts. Thousand grams of each of the flours was mixed with 3500 ml of water to form slurry, separately. The mixtures were allowed to boil until gelatinization at 70 °C and allowed to cool. About 250 g finger millet (*ragi*) malt was added to each gelatinized mash, stirred and the mixer was allowed to cool gradually to 50 °C for the amylase in the malt to convert the gelatinized starch to sugars. Thinned slurry was then heated to 70 °C and the last batch of 250 g finger millet (*ragi*) malt added for further conversion of unhydrolysed starch to sugars. The mixture was boiled briefly and filtered using muslin cloth. The three hydrolysates were collected and fermented separately.

5.2.6 Fermentation of the starch hydrolysates

Ethanolic fermentation of cassava, sorghum and finger millet hydrolysates were carried out by the yeast *Saccharomyces bayanus*. The inocula were prepared by cultivating the strain in a 250 ml Erlenmeyer flasks having 100 ml individual hydrolysate medium at 30°C on a rotary shaker (130 rpm) for 24 h. The media
contained (in %) sugar, 5; urea, 0.2; MgSO₄, 0.05 and yeast extract, 0.1. It was used in order to pre-condition the cells to their substrates.

For fermenting medium, pH was adjusted to 5.0, and temperature was maintained at 30°C. Fermentation was allowed to take place for 72 h duration. Fermenting medium was supplemented with various yeast nutrients such as urea (1.8 g/l), yeast extract (1.2 g/l) and magnesium sulphate (0.5 g/l). The sugars and other nutrients were sterilized separately before inoculation. An amount of preculture having a final concentration of 2.5×10⁷ yeast cells ml⁻¹ was added to the fermenting medium. Experiment was conducted in triplicate flasks.

5.2.7 Analytical methods

Moisture content of starch flours were analyzed using AOAC Method no. 925.10 (Air Oven Method) (AOAC, 2000). Total reducing sugars concentration was measured by using the DNS method (Miller, 1959). The diastatic activity was determined by using Association of America Cereal Chemists method 22-15 (AACC, 2003). Free amino nitrogen (FAN) content in the malts was estimated by the ninhydrin method (Lie, 1973). Specific gravity of the fermented hydrolysates was determined using a hydrometer.

a) Determination of % starch content: Three grams of flour was taken in 500 ml stopper iodine flask, added 150 ml water and then added 22 ml of conc. HCl. Mixed and kept in a water bath at 85°C-90°C for 2 hour 30 minutes. After-that, cooled to room temperature, added 2 drops of phenolphthalein, neutralized with 6 N NaOH till slight pink color. Made up to 500 ml, filtered through cotton and taken the filtrate in burette, titrated against Fehling’s solution (Lane and Eynon, 1923). Starch content was calculated from total reducing sugar(s) × 0.9.
5.3 Results and Discussion

5.3.1 Ethanol ic fermentations of a simple VRG glucose medium using kilned malts

5.3.1.1 Starch content

Starch contents in finger millet, paddy and sorghum grains were decreased during course of malting (Figure 5.1). This is mainly due to its degradation by amylolytic enzymes, both α-amylase and β-amylases. The energy for growing embryo is derived from starch degradation. In vivo hydrolysis of starch is a very slow process and it depends on the level of amylases and the granular organization of starches (Witt and Sauter, 1996). All the three grain starches had shown almost similar pattern of starch reduction, from initial 60-70% to 35-40% after 4 days of malting.

![Graph showing starch content changes in finger millet, paddy and sorghum grains](image)

Figure 5.1 Malting time dependent starch content changes in finger millet, paddy and sorghum grains.

5.3.1.2 Amylase activity

Table 5.1 Amylase activities of malts

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Amylase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 h</td>
</tr>
<tr>
<td>Sorghum malt</td>
<td>45</td>
</tr>
<tr>
<td>Paddy rice malt</td>
<td>82</td>
</tr>
<tr>
<td>Finger millet (Ragi) malt</td>
<td>96</td>
</tr>
</tbody>
</table>

*Amylase activity- equivalent to mg maltose released by the extract from 1 g malt acting on soluble starch at 37 °C and pH 5.8.
Higher levels of amylases were found synthesized in the malts of finger millet compared to the sorghum and paddy malts at 48 and 96 h of malting. So, the β-amylases of finger millet malt were found suitable to degrade the starches into simple sugars in other two substrates.

5.3.1.3 Free amino nitrogen

![Graph showing concentrations of free amino nitrogen (FAN) during malting of finger millet, paddy, and sorghum grains.]

Figure 5.2 Concentrations of free amino nitrogen (FAN) during malting of finger millet, paddy, and sorghum grains.

Among the tested three grain types, free amino nitrogen was increased gradually up to five days of germination, and then decreased with increased malting time (Figure 5.2). Free amino nitrogen content as high as 280 mg% was found in grain sorghum at 5 day of malting period. In finger millet, lower levels of FAN and in paddy still lower amounts were found when compared to sorghum at this malting time.

VHG fermentation often results in sluggish or stuck fermentation, especially in media with large proportions of materials such as unmalted grain or added sugar, which are deficient in yeast usable nitrogen (Thomas and Ingledew, 1996). Assimilable nitrogen (N) is an important component of fermentation media. It influences the yeast ethanol tolerance and the rate of ethanol production during VHG fermentation. Germination (sprouting) of grains develops the enzymes that are required to modify the grain's stored complex nutrients to yeast assimilable form.
Therefore, supplementation of medium with malted flours yielded better yeast growth and viability leading to higher ethanol concentration.

5.3.1.4 Supplementation of malts

Glucose was incompletely fermented by *Saccharomyces bayanus* in an unsupplemented medium and the final concentration of ethanol did not exceed 10% (v/v) as in case of traditional fermentation. However, the addition of 1-4% w/v malt flours led to a significant increase in the amount of glucose fermented and remarkably high final ethanol concentrations, reaching as high as 15.9% (v/v) (Table 5.2). Such unusual concentrations of ethanol were obtained after 60h of fermentation.

Supplementation of malts of sorghum, finger millet and paddy even at 1.0% concentration led to increased consumption of glucose, thereby final ethanol concentration was increased by 1.25 fold. Table 5.2 represents the ethanol concentrations in the medium at 48 and 72 h of fermentation period. All the malts at 2.0% concentration were shown better in terms final concentration, productivity of ethanol production. Higher amounts than this did not show any significant effect on ethanol yield. So the 2% addition was found to be optimal.

Table 5.2 Effects of various concentrations of sorghum malt, finger millet (*ragi*) malt and paddy malts addition on ethanol formation in very high gravity (300 g/l) glucose medium.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Ethanol concentration</th>
<th>% IMP at 48h</th>
<th>Fermentation efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (w/v)</td>
<td>At 48h</td>
<td>At 72h</td>
</tr>
<tr>
<td>Un-supplemented</td>
<td>5.9</td>
<td>8.6</td>
<td>---</td>
</tr>
<tr>
<td>Sorghum malt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>7.2</td>
<td>9.8</td>
<td>22</td>
</tr>
<tr>
<td>2.0</td>
<td>9.1</td>
<td>11.8</td>
<td>54</td>
</tr>
<tr>
<td>4.0</td>
<td>9.3</td>
<td>11.5</td>
<td>57</td>
</tr>
<tr>
<td>Finger millet (<em>Ragi</em>) malt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>8.8</td>
<td>11.3</td>
<td>49</td>
</tr>
<tr>
<td>2.0</td>
<td>9.4</td>
<td>12.4</td>
<td>59</td>
</tr>
<tr>
<td>4.0</td>
<td>9.3</td>
<td>12.6</td>
<td>57</td>
</tr>
<tr>
<td>Paddy malt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>7.9</td>
<td>10.5</td>
<td>34</td>
</tr>
<tr>
<td>2.0</td>
<td>8.6</td>
<td>11.7</td>
<td>45</td>
</tr>
<tr>
<td>4.0</td>
<td>8.3</td>
<td>11.6</td>
<td>40</td>
</tr>
</tbody>
</table>

IMP, improvement
The supplementation of malts at optimal concentrations led to an increased rate and extent of growth (Figure 5.3). After 18-24h of fermentation, yeast growth was stopped in un-supplemented medium, whereas in malt supplemented medium yeast growth extended up to 30 h and the maximum cell count was above 300 million cells per ml of medium. After the cessation of growth, death occurred, and the death rate increased with the concentration of ethanol accumulated.

![Figure 5.3](image_url)

**Figure 5.3** Effect of sorghum, finger millet and paddy malts addition (2% w/v) on viability of cells in very high gravity (300 g/l) glucose medium.

The viability percentage of yeast cells in malts supplemented media were greater than in control medium. There was a significant decrease in the viable cell count from $100 \times 10^6$ to $30 \times 10^6$ in control medium with an increase of ethanol concentration even to 7.5% (v/v) in 48 h. But in the supplemented medium the cell viability was above 100 millions even up to 12% (v/v) ethanol. Even after 40 h, glucose fermentation continued very slowly till the number of viable cell count diminished. Significant increase in viable cell concentration in supplemented medium clearly indicates the important role of nutrients in the fermentation.

Ethanol productivity was increased significantly from 1.3 g/l/h to 2.1 g/l/h in 2% finger millet malt supplemented medium when compared to un-supplemented medium. It is well known for beer production that to get a rapid and full fermentation it is necessary to extend the growth phase, because non-growing cells yeast ferments slowly, whereas rapidly growing yeast ferments rapidly (Kirsop, 1978). Finger millet and other malts acting to overcome nutritional deficiencies, allowed more growth.

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From this study, among the three tested malts, the superior performance of malted finger millet was observed in terms of high Diastatic power (starch degradation capacity), free amino nitrogen content and in provision of nutrients/osmoprotectants for high yeast viability during very high gravity (VHG) sugar fermentations. So, finger millet malt was used as the source of enzyme for starch hydrolysis as well as nutrient source for yeast.

5.3.2 VHG fermentations of starchy substrates using finger millet malt

5.3.2.1 Reducing sugar, ethanol content, and specific gravity

![Graph a](image1.png)

![Graph b](image2.png)
Figures 5.4 (a), (b), (c) shows the changes in reducing sugar contents and alcohol contents during fermentation. Initial reducing sugar concentrations of finger millet, cassava and sorghum hydrolysates were 16%, 14.5% and 13.4%, respectively. There was a rapid decrease in the reducing sugars concentration in all the media from 0-48 h duration, with concomitant increase in the alcohol content. According to Webb (1964), it is evident that as the alcohol content of the fermenting medium increases, the sugar content decreases by a proportionate amount. A steady decrease in reducing sugars was observed up to 48 h in all the three hydrolysates, this was due to a rapid multiplication of yeast cells after a prolonged lag phase of cells, up to 6-12 h, under high gravity conditions. Final ethanol concentrations 13.4%, 12.2% and 11.1% (v/v) were observed in fermenting media of finger millet, cassava and sorghum, respectively. More than 70% of total ethanol volume was produced within 48 h fermentation period. Low ethanol productivity was due to slow and continuous release of fermentable sugars by the malt amylases action on gelatinized starch. Whereas in normal gravity fermentations, more than 50% conversion takes place within 24 h fermentation period. Fermentations were allowed to continue up to 96 h duration.
About 3-4% residual reducing sugars were left unfermented in all the three media after 96 h. The incomplete fermentation may be due to inhibitory effect of ethanol on yeasts, which is high under high gravity conditions compared to normal gravity. It may also be due to fermentation inhibitors in substrates itself such as cyanogenic compounds from cassava, phytes and tannins from sorghum.

Figure 5.5 Changes in dissolved-solid contents during high gravity fermentation of finger millet, sorghum and cassava hydrolysates (prepared by malt enzymes).

Specific gravity changes are illustrated in figure 5.5, initial specific gravities 1.142, 1.128 and 1.137, which corresponds to 30-33% dissolved solids, were observed in finger millet, cassava and sorghum media, respectively. During 0 to 48 h of fermenting period, the fall in the specific gravities can be correlated with the reduction in the reducing sugars of all the fermentation media. Final specific gravities around 1.04-1.05 were observed after 96 h, equivalent to 15-16% dissolved solids content.
Figure 5.6 Changes in free amino nitrogen (FAN) contents during high gravity fermentation of finger millet, sorghum and cassava hydrolysates (prepared by malt enzymes).

Figure 5.6 depicts the changes in free amino nitrogen content in all the three media during fermentation. A steep decrease in FAN content was observed in all the hydrolysates up to 48 h period. It was due to continuous multiplication of yeast cells in exponential growth phase, which require assimilable nitrogen for growth and regeneration process. After an initial decline, there was an increase in the FAN levels for all the VHG fermentations. The increase in FAN may be due to the release of nutrients by lysing yeast cells. Yeast cell lysis may be due to exposure to high concentrations of ethanol for long periods of time and osmotic stress exerted by the unfermented sugars (Jones and Ingledew, 1994a). Though the media was supplemented with urea and yeast extract as nitrogen sources, fermentation did not occur to completion. Similar results have been reported for VHG fermentation of wheat at 30 °C (Jones and Ingledew, 1994a). Under normal conditions, an initial decrease in FAN levels was observed up to 24 h then depletion of FAN occurred for the rest of the fermentation (Bvochora et al., 2000). FAN levels may have limited the fermentation to completion of media under normal fermentation conditions. However,
yeast cell lyses might not have occurred under normal fermentation conditions as the increase in FAN levels observed under VH G conditions was not observed.

5.3.2.3 pH and total acidity

![Figure 5.7 Changes in pH and acidity levels during high gravity fermentation of finger millet, sorghum and cassava hydrolysates (prepared by malt enzymes).](image)

Changes in pH and the total acidity of three hydrolysates during the fermentation are illustrated in the figure 5.7; the initial pH and total acidity of the hydrolysates were 4.7-4.9 and 0.2-0.3 g/100 ml, respectively. There was a slow decrease in the pH, from 4.8 to 4.4 at the end. There was a gradual increase in total acidity from 0.2 to 0.55 g/100 ml till 72 h, where it remained till 96 h. The rise in the total acidity also corresponded to the fall in the reducing sugar content and increase in alcohol concentrations. Though the acidity of the media was increasing gradually up to 72 h, the pH remained almost constant within the range 4.6-4.7. This phenomenon might probably be due to unionized weak acids such as acetic acid, which caused buffering action in the fermenting media. It could also be due to other buffering systems in the medium. According to Mark et al. (1963), the pH of a fermenting medium must be adjusted to between 5.28-4.8 to inhibit bacterial development. They also reported that these pH ranges provide buffering action during the fermentation cycle, which is important, since secondary conversion won’t takes place if the fermenting pH drops below 4.1 in the intermediate stages of fermentation.
5.3.2.4 Total cell count and viability

Figure 5.8 Changes in viability and total yeast count during high gravity fermentation of finger millet, sorghum and cassava hydrolysates (prepared by malt enzymes).

The total cell count increased to about $2.6 \times 10^8$ cells/ml from $2.5 \times 10^7$ per milliliter at start, after 96 h fermentation duration, in finger millet hydrolysate medium. In cassava and sorghum hydrolysates slow yeast growth was observed (Figure 5.8). Viability percentages of yeasts in these media were also varied. Finger millet fermentation medium had shown highest viability, not below 75% percent throughout the 96 h period. Whereas in cassava and sorghum media, increased viability losses were observed after 48 h, thereby decreased growth coupled final ethanol yield.

5.4 Conclusions

The results show that, among the tested malt nutrients finger millet malt shows superior performance in terms of ethanol yield, theoretical yield near to 85% was obtained and there was a 1.47 fold increase in final ethanol concentration when compared to un-supplemented medium. In malt supplemented media, yeast growth and viability was increased, so that a significant increase in final ethanol concentration $>15\%$ (v/v) was achieved.

Using finger millet malt amylases and other nutrients, the final ethanol concentration $>15\%$ (v/v) was not observed in fermentations in all the three tested
starch fermentations. It may be due to low specific activity and thermostability compared to commercial enzymes. Whereas thermostable and high specific active enzymes can work well in high dissolved solids medium, can withstand their activity under thermo-mechanical disintegration steps in starch liquefaction and saccharification. However, malts supply yeast nitrogen sources and other nutrients, which help in yeast growth and viability. But, rapid and higher final concentrations are the prime target for any ethanol industry. So, the cereal malt enzymes may not be suitable for effective starch degradation to fermentable sugars, but these additions can provide yeast nutrients during high gravity ethanol fermentations.