

INVESTIGATIONS INTO PROBLEM ETHANOL FERMENTATIONS

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Abstract

Problems were experienced at Triangle's fuel ethanol plant with extended and incomplete fermentations. Direct lactic acid, residual sugars and heavy metal analyses were done on plant samples to isolate the cause of the sub-optimal fermentations. Laboratory fermentation experiments were done using Triangle yeast and nutrients. Tests were done at various pHs and temperatures to ascertain levels of stress on the yeast. Copper was found in toxic levels in the problem fermentations, and the most probable source was the fertilizer-grade diammonium phosphate used as nutrient. Laboratory tests incorporating copper at varying concentrations were done to investigate the effect on yeast population levels and on the fermentative capability of the yeast. Laboratory tests showed an effect on yeast populations at 16 ppm copper, and the level affecting ethanol fermentations was between 20 and 40 ppm. Laboratory tests confirmed that the problem of extended fermentations and incomplete sugar utilization was due to copper toxicity.

Introduction

Fuel ethanol is produced by the fermentation of sugars in high purity molasses feedstock by inoculation with the yeast *Saccharomyces cerevisiae*. Urea and a fertilizer-grade diammonium phosphate (DAP) were added as a nitrogen and phosphate source, respectively. Laboratory fermentation yields average 62 l/100 kg total fermentable sugars as sucrose (TFAS). Under industrial conditions a plant yield of 95% of laboratory yield (59 l/100 kg TFAS) is considered a production target easily achieved. Triangle uses a batch fermentation system and has an annual production capacity of 40 million litres of ethanol. The plant has previously produced fermentations using the same yeast supply for a three month period; a yeast footing is continually held back from a freshly prepared pre-fermenter (PF) and diverted into a yeast storage tank. Further fermentations are started with this charge in the yeast storage tank. During the problem period at the plant, yeast had to be changed every five days due to poor fermentation. Yeast performance was sub-optimal, with fermentations sluggish and requiring extended time periods. Yeast morphology and viability (% live yeast cells) were affected. Incomplete fermentation can occur because of insufficient inoculum or low viability. The result can be either non-consumption of the sugars, or diversion of the sugars to form by-products other than ethanol. Yeast viability is affected by its surrounding environment, by chemical and physical factors such as the presence of toxins or heavy metals, pH, temperature, aeration and nutrient status.

Procedures

Direct analysis

Plant fermentation mash samples were taken during incomplete or slow fermentation processes and analysed for:

- individual residual sugar levels to obtain sugar usage profiles

- lactic acid levels to determine whether the sugars were consumed by contaminant bacteria resulting in the formation of lactic acid instead of ethanol.

Determination of sugars

Only gas chromatographic (GC) measurements of sugars are considered accurate and reliable, especially in low purity feedstocks.

Stress tests on yeast viability

The effects of low pH and temperature levels on yeast viability were evaluated, together with results from previous investigations on the effects of these factors on ethanol yield.

Laboratory mash was made using Triangle molasses and Triangle dry yeast, and divided into six equal portions. These were acidified to various pH levels using Triangle sulphuric acid, and viabilities were checked 30 to 60 minutes later. The samples were then frozen and stored overnight. The next day they were thawed and the cells counted, then left for six hours at room temperature and the cells again counted.

Laboratory mash was made as before, the pH adjusted to 3,6 and small volumes placed in incubators for 30 or 60 minutes at various temperature settings. Temperature of the mash at the time of cell counting was recorded as the test temperature.

The effect of DAP and sulphuric acid on ethanol yield

Previous yield tests were performed using different DAP samples obtained from Triangle (Cazalet, 1986). Fermentation experiments using the current supply of sulphuric acid were done for this study.

Analyses of heavy metals

Analyses of heavy metals were done on three Triangle pre-fermenter samples (two 'bad' samples and one 'good' sample), Triangle sulphuric acid and old Triangle DAP, by the Technikon Natal using atomic absorption spectrophotometry.

The effect of copper and chromium on yeast growth

Copper and chromium were incorporated into agar growth media to assess their effect on the growth characteristics of the yeast *Saccharomyces cerevisiae*, isolated from Triangle's pre-fermenters.

The effect of copper on yeast performance

Copper was added to a final concentration of 0, 20, 40, 60, 80 and 100 ppm in mash. Mass loss as a measure of the rate of fermentation was monitored with time. GC analyses were done to determine ethanol levels and individual residual sugars (see Figure 1). Yeast populations before and after fermentation in the presence of copper were counted microscopically, using a haemocytometer.

Results and Discussion

Direct analysis

Table 1
GC residual sugar analyses on Triangle plant fermentation mashes

Sample	% Fru	% Glu	% Suc
Mash with nutrients and yeast	5,10	5,27	0,28
PF109. B175. SA yeast 12/07	1,93	0,34	0,05
PF107. B179. SA yeast 19 hours 13/07	0,53	0,12	6,62
PF108. Triangle yeast	0,13	0,13	6,08
PF. B217	0,86	0,54	2,02

A similar pattern of non-inversion of sucrose is evident in three residual sugar results (see Table 1). Yeast supplies from both South Africa and Zimbabwe tended not to utilize the sucrose fraction. The same problem thus occurred at two different times, in two different pre-fermenters and with two different yeast supplies. This indicates that the yeast supply was not defective. The South African yeast fermented normally on its first run on 12 July, but deteriorated the following day.

During the fermentation process some environmental condition, physical or chemical, caused either reduced cell viability or inactivation of the enzyme invertase, and resulted in non-consumption of the sucrose fraction.

Contamination by lactic acid bacteria

Table 2
Lactic acid levels

Sample	Lactic acid, ppm
PF109. B175. Brewers yeast 12/07	900
PF107. B179. Brewers yeast - 18 hours	680
Mash with nutrients and yeast	740
MF105. B175	1 800

Based on the conversion of sugar to lactic acid, the levels of lactic acid were not high enough to account for the large drop in ethanol yields that were experienced (see Table 2).

Stress tests on yeast viability

Table 3
The effect of low pH on yeast viability

pH	% Yeast viability after:		
	initial pH adjusted	frozen overnight and thawed	further 6 hours at room temperature
3,6	94	—	—
2,6	99	84	—
2,0	99	92	80
1,5	99	85	85
1,0	94	93	89
0,7	100	94	77

Yeast viability was not greatly affected by low pH level (see Table 3). No massive death effect occurred, as was experienced at Triangle. A study done by Tongaat-Hulett Sugar Technology Department (STD) showed that the result of lower pH on the fermentation process was a much longer lag phase (Cazalet, 1985). Another study by STD found that yeasts were not adversely affected by conditions of extreme acidity, i.e. pH 1,5 (Cazalet, 1984).

Table 4
The effect of temperature on yeast viability

Temperature °C	Time (min)	% Viability
14	30	91
22	30	89
42	60	86
50	60	79*

*Cell size affected - cells much smaller

The yeasts were able to withstand a temperature of 40°C without much adverse effect (see Table 4). This agrees with data obtained by other workers who found that it was possible to grow yeast reasonably well in the range 20-40°C (Burrows, 1970). At temperatures above 35°C, loss of yeast biomass and fermentative activity may occur. Temperature recorder charts from the time of the fermentation problem indicated no extreme temperatures.

The effect of Triangle DAP samples on ethanol yields

Table 5
Ethanol yields for fermentation trials using Triangle DAP samples

Test	Yield l ethanol /100 kg TFAS
Yeast extract as nutrient	61,9
Triangle DAP grey	57,5
Triangle DAP brown	58,6
Triangle DAP black	58,2
NCP DAP (Holpro)	60,2

Higher yields were obtained using yeast extract or the better grade NCP-DAP supplied by Holpro as nutrient (see Table 5). All the fertilizer-grade DAP samples used by Triangle at various times gave depressed yields. The low grade DAP samples inhibited yeast fermentation and were detrimental to yields. This suggested the possible presence of heavy metals in the DAP. Arsenic contamination of sulphuric acid was initially suspected as the cause of the fermentation problems. However, fermentations using Triangle sulphuric acid showed normal ethanol yields.

Heavy metal analyses on Triangle nutrients and mash samples

The extremely high levels of heavy metals found in the DAP might account for the depressed ethanol yields (see Tables 6 and 7).

Notable differences in heavy metals between the two 'bad' pre-fermenters (PF107 and PF108) and the 'good' pre-fermenter (PF109) appear to be the levels of copper and chromium. The difference in levels between the two bad fermenters is probably linked to the number of times the

Table 6
Heavy metal analyses

Sample metal	Units: mg/kg (ppm)				
	1	2	3	4	5
Copper	203	5,4	13	37	7
Chromium	5 850	5,3	2,6	2,3	0,2
Arsenic	44	18	0,3	0,4	0,4
Zinc	6 480	63	57	73	70
Lead	16	0,7	0,4	0,4	0,2
Molybdenum	8,4	1,0	1,6	1,8	1,1
Selenium	0,6	2,5	<0,005	<0,005	<0,005
Mercury	<0,005	<0,005	<0,005	<0,005	<0,005

Key to samples

1. Grey granular DAP.
2. Concentrated sulphuric acid
3. PF107. Using SA yeast 13/07. 6% residual sucrose.
4. PF108. Using Triangle yeast 31/07. 6% residual sucrose.
5. PF109. Using SA yeast 12/07. 0,05% residual sucrose.

Table 7

Summary of the effect of heavy metals on yeast (White and Munns, 1951).

Group	Toxic concentration* (ppm)	Element
Very toxic	0,4 - 10,0	Cadmium, copper, silver mercury, palladium, osmium
Moderately toxic	115-400	Cobalt, lithium, fluoride tin
Slightly toxic	500-600	Selenium, thallium
Very slightly or non-toxic		Lead, iron, halogens as potassium salts, antimony strontium, barium

* Toxic concentrations refer to yeast growth in synthetic media.

yeast has been used. Some micro-organisms have active transport systems for uptake of these elements. Plankton, for example, concentrates copper 7 000 times, zinc 65 000 times and iron 87 000 times (Kushner, 1978).

This toxicity may manifest itself in various ways, e.g. altered cell morphology, altered cell metabolism, bacteriostasis (cells are viable but do not multiply) or lethality. Resistant strains may arise that are more tolerant to the metal, i.e. they may require higher concentrations than the parent culture before being affected.

Copper is bound mainly on the cell surface and therefore does its damage in the precise location of the invertase enzyme. Invertase is found closer to the external than the internal surface of the cell wall. Trace quantities of heavy metals are needed by the yeast for enzyme function, but high levels are known to be detrimental.

The effect of copper and chromium on yeast growth in solid media

The control with no copper showed no growth inhibition of the plates seeded with yeast. Zones of inhibition of yeast growth were clearly visible in the plate containing 16 ppm copper.

No reference has been found on a toxicity level for chromium. No inhibitory effect on yeast growth was seen with chromium as a constituent of growth media up to 10 ppm. (The chromium level in problem fermentation mashes was 2 ppm.)

The effect of copper on yeast performance

Table 8

Yeast population levels before fermentation in the presence of copper

Flask No.	× 10 ⁶ cells/ml			% Viable	% Budding
	Viable	Dead	Budding		
1	139	5	8	97	6
2	148	2	18	99	12
3	123	9	11	93	9
4	158	4	17	98	11
5	134	2	8	99	6
6	145	6	11	96	8

Table 9

Yeast populations after fermentation in the presence of copper

Flask No.	[Cu] ppm	10 ⁶ cells/m			% Viable	% Budding
		Viable	Dead	Budding		
1	0	589	18	183	97	31
2	20	253	45	77	85	30
3	40	71	52	3	58	4
4	60	2	164	0	1	0
5	80	2	142	0	1	0
6	100	2	139	0	1	0

Table 10

Yeast morphology after fermentation in the presence of copper

Flask No.	[Cu] ppm	Shape	Size	Condition	Bacterial contamination
1	0	Round	Large	Healthy	Slight
2	20	Round	Large	More dead cells	Slight
3	40	Irregular	Small	Starved	Moderate
4	60	Ellipsoid	Small	Clumping	Severe
5	80	Ellipsoid	Small	Clumping	Severe
6	100	Shrunken	Small	Clumping	Severe

Before fermentation commenced, yeasts in the test flasks were all healthy and in sufficient numbers (Table 8). However, in the presence of copper, cell morphology, viability and budding were affected (Tables 9 and 10). With the addition of 20 ppm copper, cell viability was reduced by 12%. There was a four-fold population increase in yeast grown in the absence of copper and less than a two-fold increase in yeast in the presence of 20 ppm copper. As was seen at Triangle, bacterial contamination increased when there was a decrease in yeast viability.

Table 11
Mass loss profiles

Flask No.	[Cu] ppm	g mass loss/100 g molasses			
		Day 1	Day 2	Day 3	Total
1	0	23,4	0,3	0,2	23,9
2	20	23,1	0,6	0,2	23,9
3	40	2,3	1,5	0,7	4,5
4	60	0,3	0	0	0,3
5	80	0,1	0	0	0,1
6	100	0	0	0	0

Mass loss (Table 11) can be seen as a measure of the rate of fermentation. Flasks 1 and 2 were almost completely fermented in 24 hours. This effect would have been even more marked if a small number of cells was inoculated, as at Triangle. There was a critical effect with copper between 20 and 40 ppm, with a marked fall-off in fermentation rate even in the presence of 70×10^6 viable cells. This type of extended or stuck fermentation is basically what characterized the problem experienced at Triangle.

Table 12
GC sugars data

Flask No.	[Cu] ppm	% Residual			g TFAS		ml EtOH expected	ml EtOH short-fall
		Fru	Glu	Suc	Residual	Utilized		
1	0	Nil	Nil	Nil	Nil	10,27	6,40	0,3
2	20	Nil	Nil	Nil	Nil	10,27	6,40	0,3
3	40	4,53	3,31	Nil	7,45	2,82	1,76	0,3
4	60	5,00	4,67	Nil	9,19	1,08	0,68	0,5
5	80	5,33	5,28	Nil	10,08	0,19	0,12	0,04
6	100	5,47	5,26	Nil	10,19	0,08	0,05	0

GC sugars on molasses (Table 12) were 39,38% sucrose, 6,95% fructose and 5,66% glucose, totalling a per cent TFAS of 51,36. The quantity of TFAS available in each flask was 10,27 g.

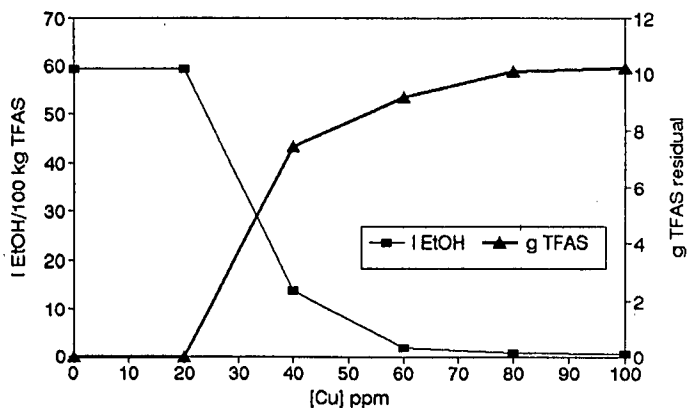


FIGURE 1 The effect of copper on EtOH yield.

The critical level for copper affecting ethanol yield was between 20 and 40 ppm (see Figure 1). This agreed with levels found in mashes of stuck fermentations at Triangle. However, in the case of a smaller inoculum it is possible that the effect would be seen with a lower level of copper. It is assumed that, due to the large number of yeasts at the start of fermentation, all the sucrose was successfully inverted into monosaccharides, as invertase was present at 100 times or more than the maximum required to complete fermentation.

Effect on Triangle plant fermentation yields

During the period of fermentation problems, ethanol yields were generally below 90% of laboratory yield. When copper was identified as the cause of the problem, fertilizer-grade DAP addition was stopped. Subsequent ethanol yields have returned to previous levels giving overall plant yields (fermentation and distillation) of 95-100% of laboratory yield. Copper concentrations in fermentation mashes have been regularly monitored and are generally less than 2 ppm. Although plant performance is again optimal, toxic heavy metal analyses of fermentation mashes are continuing as a precautionary measure, since the effects of adding poor quality nutrients can be more hazardous than nutrient deprivation.

Conclusions

The problem experienced at Triangle ethanol plant appeared to be the non-utilization of sugars, particularly sucrose, and not diversion to other end-products. The levels of contamination by bacteria or unwanted yeasts found in the problem pre-fermenters did not account for the severe drop in ethanol yields that occurred. Yeasts are quite resilient under highly acidic conditions and temperatures up to 40°C. Recorder charts from the time of the problem fermentations did not indicate a major change in temperature. Fertilizer-grade DAP samples adversely affected ethanol yields. Copper was found in toxic levels in the problem fermentation mashes. The most probable source appeared to be the DAP. In laboratory tests copper was found to affect yeast growth, morphology and viability. The amount of copper affecting yeast population levels was between 16 and 20 ppm for these laboratory investigations. Copper severely depressed the rate of fermentation in yield tests. This type of extended or stuck fermentation is what characterized Triangle's problem fermentations.

Acknowledgements

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