

THE RELATIVE STABILITY OF STORED EXTRACT WITH AND WITHOUT INOCULATION WITH HIGH LEVELS OF DIFFERENT MICROBIAL TYPES

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INTRODUCTION

At the 2003 ASSBT Meeting I presented a paper on "Microbes and Extract Storage" and referred to microbial issues we had in one of the American Crystal Sugar molasses desugarization (MDS) facilities at Hillsboro. Also the remedial measures we had taken to circumvent these problems (1 and 3).

About the same time Willems et al. (5) had been working on microbial issues and spoilage in thick juice in Belgium. In their studies they had found two microbial types, namely mesophilic anaerobes and fastidious microbes to be the causative agents of thick juice spoilage. American Crystal Sugar Company at the time did not monitor for these two particular microbial types on a routine basis in stored extract from our MDS plants. Therefore, we wanted to find out what level of fastidious and mesophilic anaerobic microbial loading was in extract from our MDS facilities and what effect they had on long-term storage. This resulted in routine monitoring for these two microbial types for a period of 13-14 months in extract from both of our MDS facilities at Hillsboro (HLB) and East Grand Forks (EGF). In addition, we carried out challenge studies with inoculation of extract with high loading of different microbial types and observed the stability of this extract during long-term storage. These studies involved the preparation of large amounts of inoculum for initial challenge of extract. This in itself was a challenge and resulted in some interesting findings which will be discussed.

MATERIALS AND METHODS

A) Microbiology

1) Sample collection

Samples of material were obtained aseptically in sterile screw cap containers from each location (extract to storage or different points on extract tanks) at Hillsboro (HLB) and East Grand Forks (EGF) molasses desugarization (MDS) facilities. The samples were boxed and sent to the ACS Technical Services Center Microbiology Lab via UPS next day service. Microbiological analyses were carried out the same day or on the following day the samples were received. For storage trials extract was obtained aseptically in clean 5 gal. pails from the respective MDS facilities.

2) Mesophilic and thermophilic counts

Appropriate serial dilutions were made, and decimal dilutions of samples were pipetted into labeled sterile Petri plates. A pour plate technique with tempered plate count agar

(PCA) was used. The plates were incubated at 35°C for Mesophiles and 55°C for thermophiles per 48 hrs.

3) Lactic acid bacteria

Appropriate serial dilutions were made, and decimal dilutions of samples were pipetted into labeled sterile Petri plates. A pour plate technique with tempered MRS Agar (DeMan, Rogosa, Sharpe) was used. The plates were incubated at 30°C for 72 hrs. in a 5% CO₂ incubator and observed for growth and counts made.

4) Mesophilic anaerobes

Appropriate serial dilutions were made, and decimal dilutions of sample were pipetted into labeled sterile Petri plates. A pour plate technique with tempered Reinforced Clostridial Agar (RCA) was used. The inverted plates were placed in an anaerobic jar with an anaerobic gas generator sachet and anaerobic indicator pill. The closed jars with plates were incubated at 30°C for 48-72 hrs and counts made.

5) Regular and osmophilic yeast and mold counts

Appropriate serial dilutions were made using Butterfield's phosphate buffer for regular yeasts and mold and the same buffer with 40% sucrose for osmophilic yeast and mold. The microbial counts were obtained using the Hydrophobic Grid Membrane Filter (HGMF) method or Iso-Grid Method, with use of 0.45 µm membrane filters and YM-11 agar with chlortetracycline-HCl supplement for regular yeast and mold. The medium used for Osmophilic yeast and mold had 40% sucrose added to the YM-11 agar with chlortetracycline-HCl. The YM-11 plates were incubated at 28°C for 48 hrs and the YM-11 sucrose plates at 30°C for 72 hrs and counts were made.

6) Flat sours and total thermophilic spore counts

The dilution used for mesophiles and regular yeast and mold analyses was boiled for 5 minutes and cooled. Decimal dilutions were then plated with Brom dextrose tryptone agar using a pour plate technique. Plates were incubated at 55°C for 48 hrs and counts were made.

7) Thermophilic anaerobes producing H₂S

The remaining content of heated storage juice solution from test 6) above was used in thermophilic anaerobic analyses. A 20 ml portion of the boiled solution was divided equally among 6 tubes of sulfite agar and the tubes were cooled rapidly. The 6 tubes per sample were then incubated at 55°C for 24 and 48 hrs and counts were made.

8) Thermophilic anaerobes not producing H₂S

The remaining content of heated storage juice solution from test 6) above was used in this test. Another 20 ml portion of the boiled solution was divided equally among 6 tubes of Brom PE-2 medium (pea tube test). Each tube was then stratified on the surface with 2% agar and cooled rapidly. The 6 tubes per sample were incubated at 55°C per 72 hrs and counts made.

- 9) Fastidious bacteria
Pre-poured plates of Columbia Agar with 5% sheep blood (Hardy Diagnostics #A 1b), level and not dehydrated, were warmed to room temperature. Appropriate serial dilutions were made, and decimal dilutions of sample were plated in a spiral pattern using an Autoplate 4000 Spiral Plater. Inverted plates were incubated at 35°C for 72 hrs. or longer, if required. The colonies were counted manually over the entire plate and the calculation in the Autoplate User Guide was used to determine cfu/g.
- 10) Inoculum preparation for microbial challenge of EGF-MDS extract in the Challenge Study I begun in September 2005. The EGF #3-4 extract was used for inoculum preparation for the microbial challenge and storage study of extract. This EGF extract was used to prepare four different cultures by adding 1 ml of extract to approximately 100 ml of each type of broth.
- i) Thermophilic culture in plate count (PC) broth and incubated at 55°C.
 - ii) Mesophilic culture in plate count (PC) broth and incubated at 35°C.
 - iii) Mesophilic anaerobic culture in Reinforced Clostridial (RC) medium and incubated at 30°C.
 - iv) Fastidious microorganism culture in Columbia broth (CB) and incubated at 35°C.

The above cultures were grown for ~ 48 hrs. and 1 ml of each of these cultures was transferred to 30 ml of the appropriate broth in seven centrifuge tubes for each culture. These centrifuge tubes (7 per each microbial type) were incubated at the appropriate temperatures given above, overnight (~18 hrs). Six of the culture tubes for each microbial type were centrifuged at 19,000 rpm (SS-34 rotor) in a Sorvall RC-5B refrigerated super speed centrifuge from DuPont instruments for 20 minutes. The 7th tube of each of the culture types was plated to obtain a viable cell count. The supernatant from each tube was discarded, and the six pellets of each microbial type were consolidated into one tube using as little 40% sucrose as needed. Each of the combined pellet tubes were then centrifuged again as before to obtain one large pellet of cells for each microbial type. Each large pellet (thermophiles, mesophiles, mesophilic anaerobes, fastidious microbes) was then added to separate screw cap plastic containers containing one liter of EGF extract (#3-4). See Fig. 3 for detail. The inoculated extract samples and controls were then swirled fairly vigorously for even distribution of the inoculum and then incubated at 30°C for a period of nine months (9/13/05 to 6/26/06). Sample aliquots were obtained from each container and microbial counts, pH, and brix measurements were made throughout the storage period.

- 11) Inoculum preparation for microbial challenge of MDS extract in Challenge Study II begun in November/December 2006. In this study
- i) for initial preparation of inoculum Hillsboro (HLB) extract from Tank C (mixture of St. 7 sample from 5/22/05 and St. 6 sample from 5/29/05) were used. East Grand Forks (EGF) extract from Tank #3 (5/31/06) and a 1:1 mix of the above HLB and EGF extract was made up and used. These three extract samples (EGF, HLB, and EGF/HLB mix) were diluted to 20 RDS with sterile water and incubated at 35°C for

72 hrs. Sample aliquots were obtained at periodic intervals (16½ hr, 24 hr, 42 hr, 44 hr, 46 hr, 48 hr, 72 hr) for mesophilic microbial assessment. See Fig. 6 for detail.

- ii) The second set of inoculum for this trial was prepared using only EGF Tank #3 extract from 5/31/06 (RDS ~70.82) and only the mesophilic microbial population was cultured. The extract was made to 20 brix with sterile water. After 48 hr. growth at 30°C, 10 ml of inoculum was transferred to a new 20 RDS bottle and incubated for 24 hrs. Two more similar transfers were made every 24 hrs to new 20 RDS bottles to increase microbial population. The 4th transfer was made into two 20 RDS bottles and incubated for 24 hrs. The inoculum from the two bottles were mixed together and used to inoculate the extract in the challenge study. See Fig. 8 for detail.
- iii) For the Challenge Study II EGF extract from the evaporators (RDS of 68.45) taken on 11/2/06 was used. For this study: Set I of the EGF extract was concentrated to 74.26 RDS and 45 g. of the 20 RDS inoculum was added to it giving a final RDS of 68.93. Four bottles of the microbial challenged 68.93 RDS extract were made up, and duplicate bottles were incubated at 20°C and 30°C respectively. To Set II of EGF extract at 68.45 RDS, 45 g. of the 20 RDS inoculum was added giving a final RDS of 64.45 RDS. Four bottles of the microbial challenged 64.45 RDS extract were made up, and duplicate bottles were incubated at 20°C and 30°C. One control of the starting extract (68.45 RDS) was incubated at each temperature (20°C and 30°C).

B) Chemistry

- 1) Sample – on completion of collection of aseptic samples, a small volume of sample was poured off aseptically in the factory lab for pH, brix, and L-lactic acid measurements.
- 2) pH – measurements were made using pH meter (Orion, Beckman, or Accumet basic).
- 3) Brix – measurements were made using an Index GPR 10-23 or 1045 refractometer.
- 4) L-lactic acid – Measurements were made using a YSI Model 1500 or YSI model 2700 biochemistry analyzer from Yellow Springs Instrument Company.

C) GR&R Studies for Repeatability and Reproducibility

In Test 1 of the GR&R test, two analysts (1 & 2) tested the same EGF extract sample for aerobic thermophiles, mesophiles, mesophilic anaerobes, and fastidious microorganisms 10 times over giving 20 readings for these microbes on the same sample.

In Test 2 of the GR&R test, each of the two analysts (1 & 2) tested 10 different EGF extract samples for aerobic thermophiles, mesophiles, mesophilic anaerobes, and fastidious microbes giving 20 readings (duplicate sample readings) for the 10 different samples.

RESULTS AND DISCUSSION

At American Crystal Sugar (ACS) Company we have been carrying out microbial testing of extract from both our molasses desugarization plants for a long time. The number of tests we

carry out on each extract sample also has increased over the years. As such in May and June of 2005 we began assessing mesophilic anaerobes and fastidious microbes in extract as well. This was after Willems et al. (5) published findings of degradation of thick juice by these two microbial types.

The fastidious microbes are termed as such as they are more difficult to detect and require a more nutritive medium for growth. Therefore a nutritively rich medium called Columbia agar with 5% sheep blood for growth and detection of these microbes same as in (5) was used. The test results for these microbes obtained over a period of 13-14 months, which is longer than one beet campaign for both East Grand Forks (EGF) and Hillsboro (HLB) facilities, show that the fastidious microbial counts tend to be higher than the mesophilic counts (Fig. 1 and Fig. 2) in extract with pH of 9.8-10.2 and brix of 68.5-71.13 range in these samples. This would be expected as the Columbia agar with sheep blood is a more nutritive medium than the standard methods agar used for the cultivation of mesophiles.

Willems et al. in their paper state that the ratio of fastidious to mesophilic counts is an ideal ratio for monitoring degradation of thick juice. According to them the fastidious bacteria coevolve with mesophilic microbes in non infected thick juice and the fastidious component shifts to dominance at the onset of a serious pH fall and increase of invert sugar. This is contrary to our finding in desugared extract where the fastidious microbial population is higher than the mesophilic population throughout the campaign with no drop in pH. In the Willems studies with thick juice, they observed a peak for mesophilic and the fastidious group about 35 days after storage at log 4-log 6 cfu/g. Since we were looking at microbial counts as per 10 g DSE this would be relative to a pH drop at log 6-log 8 cfu/10 g DSE range. After about 70 days of storage they observed a switch in populations with the mesophiles decreasing rapidly in numbers to log 1 cfu/g while the fastidious counts increased rapidly further to log 5-log 6 cfu/g after 80 days of storage.

Willems et al. (5) also found that once mesophilic anaerobic populations reach peak microbial loading of log 7 cfu/g (relative to \approx log 9 cfu/10 g DSE) it could cause spoilage in thick juice within 24 hrs. Therefore, in May of 2005 we began monitoring for mesophilic anaerobes in MDS extract from both EGF and HLB facilities. Soon after we started this testing we observed counts of log 6 cfu/10 g DSE in one of our EGF tanks (Tank #3) in regions just below the surface. See Table 1 for detail. During 3 successive weeks of testing of this extract (5/7, 5/14, and 5/21/05), it seemed as though the mesophilic anaerobic infection was going deeper into the tank. Therefore, EGF started processing Tank #3 first (around 5/24/05). However, as soon as we began pulling extract out of this tank, the mesophilic anaerobic counts decreased rapidly to zero. Therefore, we did not see the same decay observed by Willems and co-workers in desugared extract and we were not able to give an explanation for the sudden change in counts to zero.

Lactic acid bacteria were also monitored in extract on MRS agar plates incubated in 5% CO₂. However, as we obtained hardly any growth on plates, we discontinued testing for this microbe.

Some other workers who have reported on problems in thick juice storage were Sargent et al. (4). They observed deterioration of thick juice in some storage tanks at British Sugar at fairly low

mesophilic counts (log 3 cfu/10g range) and osmophilic yeast counts (log 2 cfu/10 g range). See Table 2. However, our MDS extract tanks stored during the beet campaign 2005/2006 at both HLB and EGF and in previous campaigns (3) typically show higher microbial loading. See Table 3.

The main problem area during extract storage at ACS facilities has been the surface of the tank. As shown in Table 4, when increase in microbial loading over consecutive weeks was observed, we managed to quell the infections by applying 25% caustic solution on the surface of the tank (3).

In addition we have had no degradation of MDS extract with a drop in pH for the past 4-5 years. The following Table 5 gives the length of storage of extract in some HLB and EGF tanks from 2003 to 2006. This table shows we have stored extract from \approx 6.5 to 14.5 months in HLB and 8-11 months at EGF from start of filling the tanks to end of processing. Therefore, the long length in storage time of extract without degradation concerns is quite significant.

Inoculum Preparation and Microbial Challenge Studies of Extract:

Two microbial challenge studies were carried out with EGF extract. The purpose of these studies was to see what type of microbial populations would have the greatest impact on degradation of extract when inoculated at high levels. Two different approaches were used for the preparation of inoculum for the two challenge studies.

Challenge Study I – This study was begun in September 2005. The EGF extract was used for inoculum preparation for the microbial challenge and storage study of extract. Four different cultures (mesophiles, thermophiles, mesophilic anaerobes, and fastidious microbes) were prepared using this EGF extract and appropriate broth and incubation temperatures required for growth of these microbes were used. See Material and Methods Section A-10 and Fig. 3 for details of inoculum preparation and extract microbial challenge. The inoculated extract samples and controls were monitored over a period of nine months (Sept. 13, 2005 – June 6, 2006). Fig. 4 shows the level of microbes in extract throughout the storage period. Fig. 5 gives the microbial levels in the controls. These graphs show that we did not increase the microbial levels very much more than was originally there in the extract except for the level of fastidious microbes. However, all these populations decreased to the log 3-4 cfu/10 g DSE range in 24 hrs and close to the original microbial levels in the extract. After this the different microbial levels kept fairly steady for about 4 months and a further decrease in counts was observed closer to 8 months storage time similar to the control microbial loading. See Table 6. In addition Table 7 gives the range of pH and brix values in separate challenged extract containers and controls throughout the storage period. This shows if extract is stored at high pH (9.9-10.5) and Brix (68.6-70.8), it will remain stable even if inoculated with high levels of microbes.

Challenge Study II – (Nov./Dec. 2006) Here a different approach for inoculum preparation (by lowering brix of extract) was utilized. Also in this study instead of using a large number of different microbial types we focused only on the mesophilic population. In the initial inoculum preparation in this study (See Materials and Methods section A-11a and Fig. 6) we used Hillsboro (HLB) extract, East Grand Forks (EGF) extract, and a 1:1 mix of the above HLB and EGF extract. These three extract samples (EGF, HLB, and EGF/HLB mix) were diluted to 20

RDS with sterile water and incubated at 35°C for 72 hrs. Sample aliquots were taken at periodic intervals over a 72 hr period for mesophilic microbial assessment. This gave us some very interesting results.

We expected both HLB and EGF extract at 20 RDS to give high growth, but instead only the EGF 20 RDS extract gave a high mesophilic count (log 8.23 cfu/g) after 72 hrs of incubation. The HLB 20 RDS extract remained at a low log 1 cfu/g range throughout the incubation period. The EGF/HLB 1:1 (20 RDS) mix started off initially at a low count and then caught up to the EGF microbial loading (log 8 cfu/g) at ≈48 hrs of incubation. See Table 8 and Fig. 7 for detail. This showed us that the remedial measures taken at HLB during 2001/2002 were certainly producing a very stable extract which was almost sterile and was difficult to deteriorate even if we intentionally wanted to. This added further credence to Table 6 referred to earlier as to why we could store HLB extract in our tanks for 14.5 months without any problems.

Since it was almost impossible to obtain a large amount of inoculum for challenge studies from HLB extract we had to use the EGF extract for inoculum, preparation and microbial challenge of extract in Study II. See Materials and Methods section A-11b and Fig. 8 for inoculum preparation for Challenge Study II. Also Materials and Methods section A-11c for the protocols used in the set up and inoculation of extract in Challenge Study II.

This study is continuing and the results obtained so far (2-2 ½ months after inoculation) do not show differences in mesophilic counts in extract samples of 68.45 RDS and 64.45 RDS incubated at 20°C or 30°C which was surprising. See Table 9 and Fig. 9 for detail. This study again goes to show that if extract is produced under sufficiently stringent measures, it probably would take a lot of mesophilic inoculum to deteriorate the extract even if there was a drop in RDS or increase in ambient temperatures.

GR&R Studies:

During the past 13-14 months of evaluation of fastidious and mesophilic anaerobic tests on extract we observed large variation in counts especially for fastidious microbes. Therefore we questioned the reliability of these counts and other microbial tests we were carrying out. As such it was decided to carry out some GR&R or repeatability and reproducibility testing for these microbial tests to check variation (2).

Repeatability looks at the variation between measurements of the same part when measured by the same analyst with the same measurement device. Reproducibility is the difference in measurements between analysts. Therefore the R&R test will tell us the total variation in the measurement system that comes from Repeatability and Reproducibility together.

In Test 1 of the GR&R test the two analysts (1&2) tested the same EGF extract sample for aerobic thermophiles, mesophiles, mesophilic anaerobes, and fastidious microorganisms 10 times over, giving 20 readings for these microbes on the same sample. See Table 10. The results here show that the mesophilic and thermophilic counts obtained by the 2 analysts were close while the fastidious and mesophilic anaerobic counts showed variation.

In Test 2 of the GR&R test each of the two analysts (1&2) tested 10 different EGF extract samples for the same 4 microbes as in Test 1, giving 20 readings (duplicate sample readings) for the 10 different samples. See Table 11. Here again the same type of results for the 4 microbial tests were obtained as before.

Statistical analysis was carried out on these tests and the confidence interval was found to be very broad for the fastidious and mesophilic anaerobic tests (7 and 5 log units respectively) on a single sample while for the mesophilic and thermophilic tests the confidence interval was narrow (0.5 and 1.1 log units respectively). See Table 12 for detail. The mean of the test was 5.5 – 6.5. In the analysis of variance for the analysts the p factor was 0.7 – 0.9. This shows that there was no difference between the analysts and the variation was in the fastidious and mesophilic anaerobic tests.

Therefore due to the very variable results and the lack in reliability of these numbers, we decided to discontinue testing for the fastidious and mesophilic anaerobic microbes from the beginning of this campaign.

CONCLUSIONS

These studies have shown that:

- 1) The HLB storage intervention strategies taken in 2002 have produced a very stable extract. For instance, lowering the HLB extract to 20 RDS and incubating the same extract at 35°C for 72 hrs hardly increased the microbial loading from the initial level (log 1 cfu/g range). This was further demonstrated by the HLB MDS facility's ability to store extract for 14.5 months or longer.
- 2) The microbial challenge studies carried out with EGF extract using mesophilic, aerobic thermophilic, fastidious, and mesophilic anaerobic microbes show that degradation of extract will not occur if it had been produced under sufficiently stringent measures. That is microbes inoculated at high levels die out and are maintained at levels close to those found in uninoculated control extract samples.
- 3) The GR&R tests for repeatability and reproducibility have shown large variation in numbers for the fastidious and mesophilic anaerobic tests. Therefore routine assessment of these two types of microbes in MDS extract was discontinued at American Crystal Sugar Co. from the beginning of this campaign after 13-14 months of routine testing.
- 4) These studies have given us some indication of the frequency of testing required for these microbial types.

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Fig. 1 EGF Extract Tank Samples(7-5-05/7-7-05) Fastidious & Mesophilic Microbial Count

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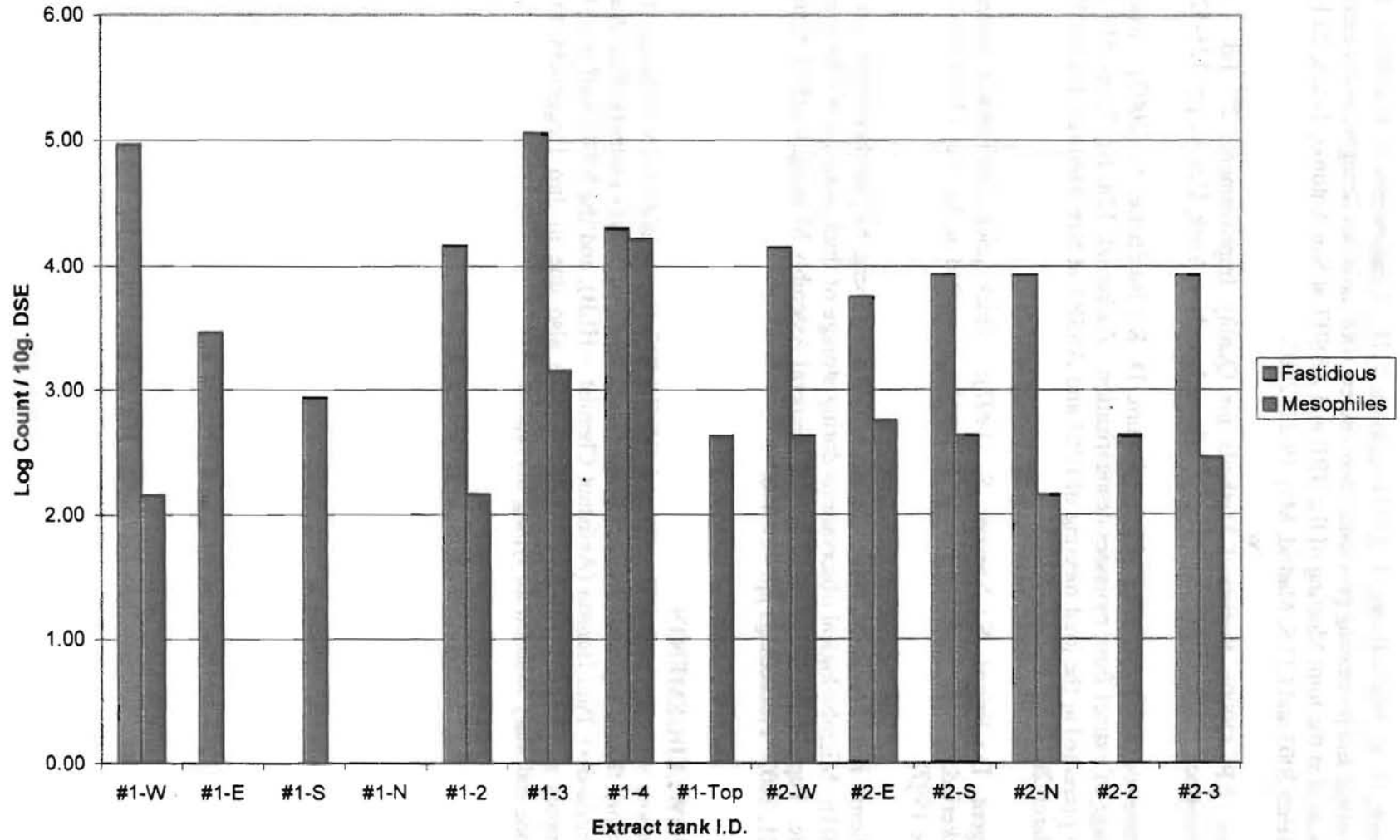


Fig. 2 HLB Extract Tank Samples (6-19-05/6-20-05) Fastidious and Mesophilic Microbial Count

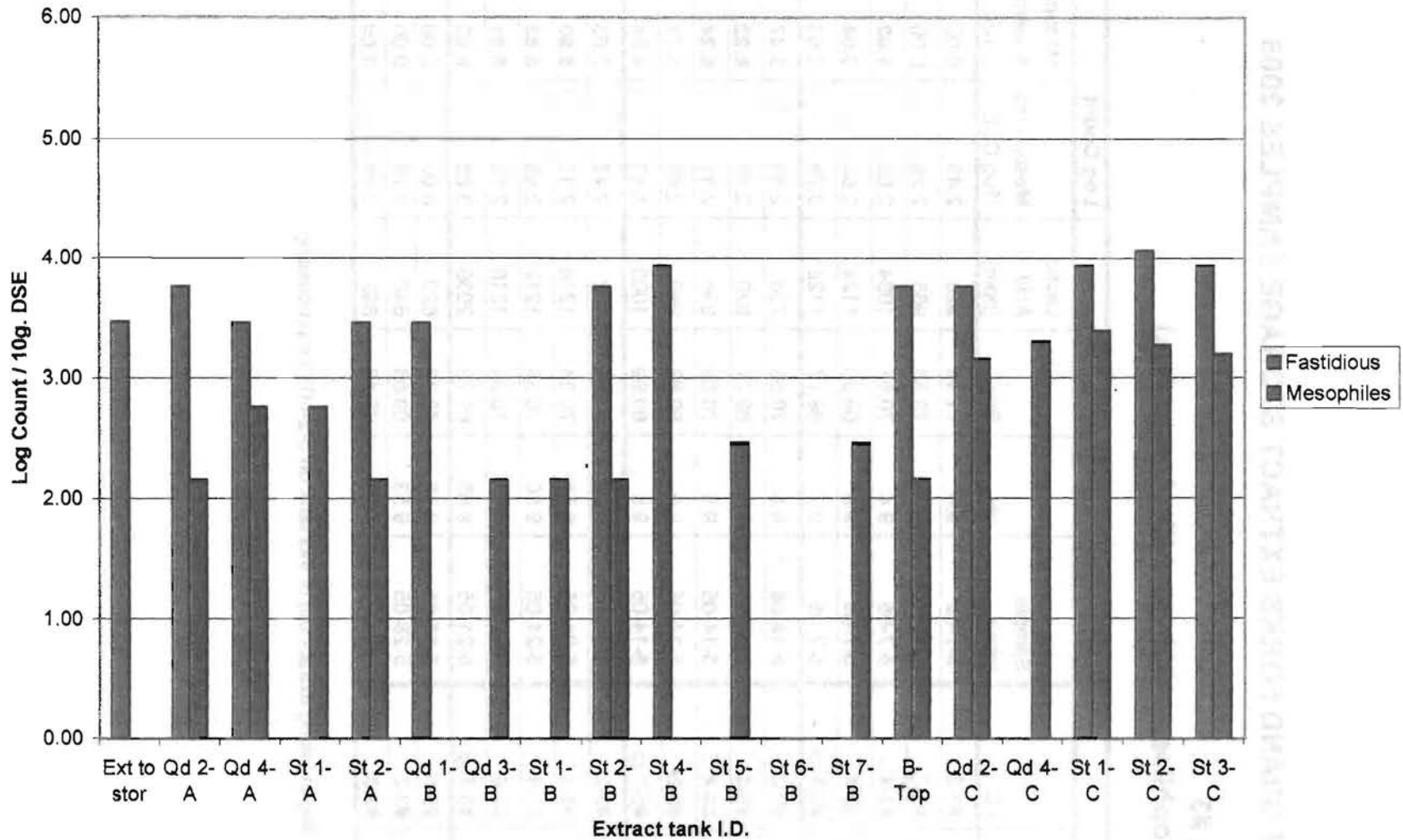


Table 1

EAST GRAND FORKS EXTRACT STORAGE SAMPLES 2005

**Tank #3
(Mesophiles and Mesophilic Anaerobes)**

| # | I.D. | Sample taken | pH | Brix | Lactic Acid (ppm) | Log Count | |
|---|--------|--------------|------|-------|-------------------|---------------------|---------------------------------|
| | | | | | | Mesophiles /10g.DSE | Mesophilic Anaerobes / 10g. DSE |
| 1 | #3-2 | 5-7-05 | 9.4 | 71.40 | 928 | 2.45 | 0.00 |
| 2 | #3-3 | 5-7-05 | 9.4 | 70.83 | 968 | 2.85 | 0.00 |
| 3 | #3-4 | 5-7-05 | 9.4 | 70.61 | 1084 | 2.63 | 6.02 |
| 4 | #3-5 | 5-7-05 | 9.6 | 69.86 | 1124 | 2.63 | 6.04 |
| 5 | #3-Top | 5-7-05 | 9.7 | 69.77 | 1128 | 3.74 | 2.63 |
| 1 | #3-2 | 5-14-05 | 9.5 | 70.56 | 736 | 2.15 | 3.47 |
| 2 | #3-3 | 5-14-05 | 9.5 | 69.77 | 820 | 2.16 | 6.22 |
| 3 | #3-4 | 5-14-05 | 9.5 | 70.08 | 936 | 2.15 | 6.24 |
| 4 | #3-5 | 5-14-05 | 9.6 | 69.82 | 980 | 2.63 | 5.77 |
| 5 | #3-Top | 5-14-05 | 9.6 | 69.99 | 1000 | 4.45 | 4.54 |
| 1 | #3-2 | 5-21-05 | 9.56 | 70.52 | 992 | 2.45 | 2.63 |
| 2 | #3-3 | 5-21-05 | 9.56 | 70.34 | 1216 | 2.15 | 5.90 |
| 3 | #3-4 | 5-21-05 | 9.60 | 70.39 | 1212 | 2.85 | 5.83 |
| 4 | #3-5 | 5-21-05 | 9.63 | 70.34 | 1316 | 2.85 | 5.81 |
| 5 | #3-Top | 5-21-05 | 9.65 | 69.73 | 2036 | 3.86 | 5.53 |
| 1 | #3-N | 5-28-05 | 9.56 | 70.78 | 620 | 0.00 | 0.00 |
| 2 | #3-2 | 5-28-05 | 9.53 | 69.86 | 640 | 2.76 | 0.00 |
| 3 | #3-3 | 5-28-05 | 9.54 | 69.12 | 892 | 2.86 | 0.00 |

Note: Began taking extract out of this tank on 5-24-05 for processing

Table 2

**THICK JUICE QUALITY IN TWO BRITISH SUGAR FACTORIES
(1994/95 Campaign)**

Table 15/2: Development of thick juice quality in two British Sugar factories during the 1994/95 campaign (Sargent et al. 1997) **NOTE: Log counts were added for comparison purposes**

| Days after tank filled | pH value | DS content in % | Lactate in mg/kg | Mesophiles counts per 10 g | Mesophiles LOG count per 10 g | Osmophilic yeasts counts per 10 g | Osmophilic yeasts LOG count per 10 g |
|------------------------|----------|-----------------|------------------|----------------------------|-------------------------------|-----------------------------------|--------------------------------------|
| Factory 1 | | | | | | | |
| 40 | 8.80 | 68.9 | 1,307 | 1,400 | 3.15 | 44 | 1.64 |
| 76 | 8.89 | 68.7 | 834 | 670 | 2.83 | 342 | 2.53 |
| 104 | 8.90 | 68.5 | 1,739 | 660 | 2.82 | 60 | 1.78 |
| 132 | 9.03 | 68.4 | 1,545 | 980 | 2.99 | 11 | 1.04 |
| 160 | 8.87 | 68.6 | 1,854 | 190 | 2.28 | 5 | 0.70 |
| 187 | 8.79 | 68.9 | 1,644 | <1,000 | <3.00 | 591 | 2.77 |
| 216 | 8.80 | 68.5 | 1,905 | 323 | 2.51 | 485 | 2.69 |
| Factory 2 | | | | | | | |
| 40 | 8.87 | 69.0 | 1,345 | 9,787 | 3.99 | 9,873 | 3.99 |
| 63 | 9.13 | 68.5 | 1,133 | 5,893 | 3.77 | 6,467 | 3.81 |
| 99 | 9.07 | 68.3 | 1,587 | 2,533 | 3.40 | 160 | 2.20 |
| 127 | 9.09 | 68.6 | 1,444 | 5,253 | 3.72 | 193 | 2.29 |
| 155 | 9.02 | 68.3 | 1,233 | 3,440 | 3.54 | 19 | 1.28 |
| 184 | 8.32 | 67.2 | 2,500 | 72,400 | 4.86 | 1 | 0.00 |
| 204 | 7.09 | 68.6 | 3,256 | 6,667 | 3.82 | 240 | 2.38 |
| 212 | 7.11 | 68.4 | 3,527 | 4,667 | 3.67 | 0 | |
| 239 | 7.07 | 68.4 | 3,770 | 1,540 | 3.19 | 353 | 2.55 |

Table 3**EXTRACT MICROBIAL RANGE – Campaign 2005/2006**
LOG COUNTS

| MICROBIAL TYPES | EGF EXTRACT (cfu/10g DSE) | | HLB EXTRACT (cfu/10g DSE) | |
|---------------------------|------------------------------|-----------|------------------------------|-----------|
| | Tank (range) | Top (max) | Tank (range) | Top (max) |
| Mesophiles | 0 - log 4.39 | log 5.26 | 0 - log 3.78 | log 5.63 |
| Thermophiles | 0 - log 4.12 | log 3.41 | 0 - log 3.30 | log 2.16 |
| Fastidious microbes | 0 - log 5.16 | log 5.64 | 0 - log 5.10 | log 5.25 |
| Mesophilic anaerobes | 0 - log 3.48 | log 4.98 | 0 - log 3.61 | log 5.93 |
| Osmophilic yeast | 0 - log 4.22 | log 5.25 | 0 - log 3.27 | log 2.56 |
| Osmophilic mold | 0 - log 4.63 | log 1.84 | 0 - log 4.12 | log 1.93 |
| Thermophilic flat sours | 0 - log 3.37 | log 2.15 | 0 - log 2.16 | log 1.16 |
| Total thermophilic spores | 0 - log 3.37 | log 2.15 | 0 - log 2.17 | |

Table 4

**EAST GRAND FORKS SURFACE SAMPLE WITH HIGH YEAST COUNTS
DECREASING TO ZERO WITH THE ADDITION OF CAUSTIC**

Tank #2 Surface Sample: 1-31-02

| | | | | | LOG COUNT | | | | | | | |
|------|-------|-------------------|-----------------------------|---------------------------------------|------------------------------------|-------------------------------------|----------------------|------------------------|----------------|---------------|---------------------------|---------------------------|
| pH | Brix | Lactic Acid (ppm) | Anaerobes Not Producing H2S | Anaerobes Producing H2S (# of spores) | Thermo-philic Flat Sours /10g. DSE | Total Thermo-philic Spores /10g.DSE | Meso-philic /10g.DSE | Thermo-philic /10g.DSE | Yeast /10g.DSE | Mold /10g.DSE | Osmophilic Yeast /10g.DSE | Osmo-philic Mold /10g.DSE |
| 9.89 | 70.43 | 484 | 3/6 | 50 | 0.00 | 0.00 | 4.12 | 3.75 | 3.77 | 0.00 | 4.11 | 0.00 |

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Tank #2 Surface Sample: 2-5-02 - After addition of Caustic

| | | | | | LOG COUNT | | | | | | | |
|-------|-------|-------------------|-----------------------------|---------------------------------------|------------------------------------|-------------------------------------|----------------------|------------------------|----------------|---------------|---------------------------|---------------------------|
| pH | Brix | Lactic Acid (ppm) | Anaerobes Not Producing H2S | Anaerobes Producing H2S (# of spores) | Thermo-philic Flat Sours /10g. DSE | Total Thermo-philic Spores /10g.DSE | Meso-philic /10g.DSE | Thermo-philic /10g.DSE | Yeast /10g.DSE | Mold /10g.DSE | Osmophilic Yeast /10g.DSE | Osmo-philic Mold /10g.DSE |
| 10.69 | 70.52 | 452 | 3/6 | 43 | 0.00 | 0.00 | 2.15 | 4.15 | 0.00 | 0.00 | 0.00 | 0.00 |

Table 5**STORAGE LENGTHS OF MDS EXTRACT IN TANKS AT HLB AND EGF (2003 TO 2006)**

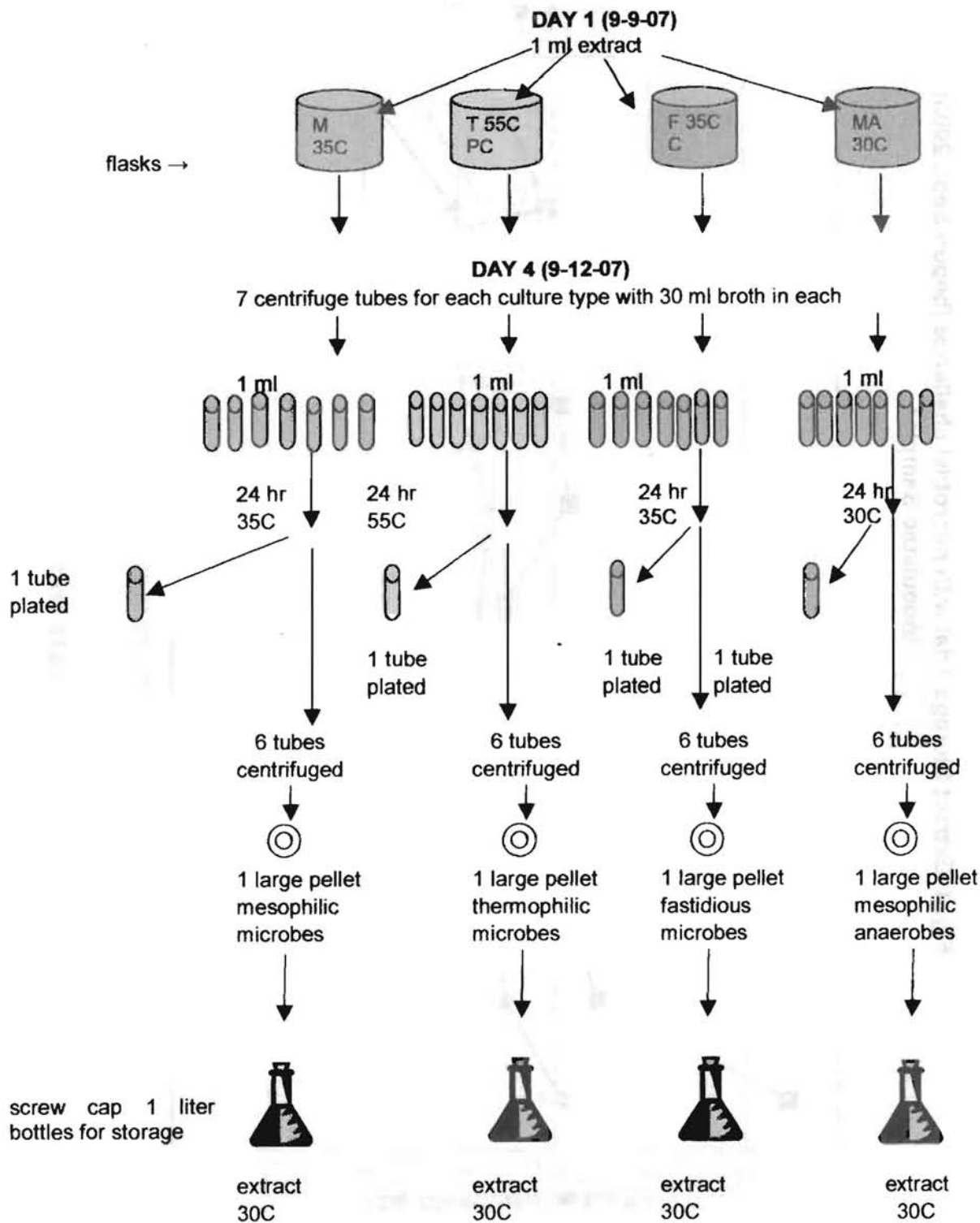
| HLB TANKS | Fill Start Date | Processing End Date | Days | Months |
|------------------|------------------------|----------------------------|-------------|---------------|
| B | 9-11-03 | 6-16-04 | 279 | 9 |
| B | 9-26-04 | 12-6-05 | 437 | 14.5 |
| C | 12-23-04 | 7-7-05 | 196 | 6.5 |
| C | 10-1-05 | 5-16-06 | 228 | 7.5 |

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| EGF TANKS | Fill Start Date | Processing End Date | Days | Months |
|------------------|------------------------|----------------------------|-------------|---------------|
| TANK #1 | 1-19-03 | 9-20-03 | 216 | 8 |
| TANK #3 | 9-5-03 | 6-15-04 | 284 | 9 |
| TANK #2 | 1-7-06 | 10-8-04 | 279 | 9 |
| TANK #1 | 3-10-05 | 2-18-06 | 346 | 11 |
| TANK #3 | 9-30-05 | 6-13-06 | 256 | 9.5 |

Fig. 3

**FLOW DIAGRAM FOR INITIAL CULTURE GROWTH (4 Microbial Types)
AND INOCULATION INTO EXTRACT (September 2005)
EGF #3-4 (5-7-05) extract used to make up cultures**



**Fig. 4 Extract Storage Trial with microbial challenge (began Sept. 2005)
Inoculated Samples**

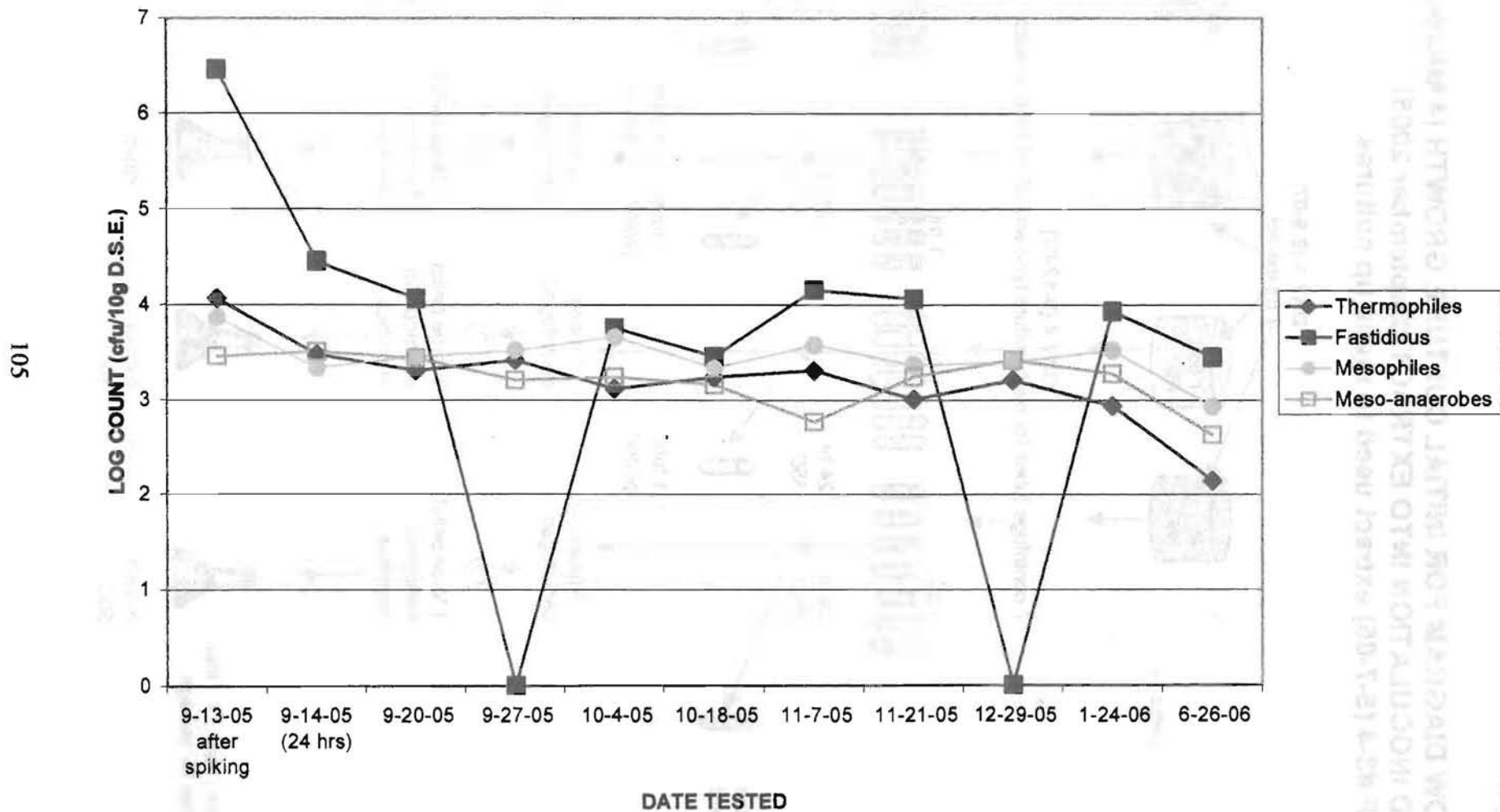


Fig. 5 Extract Storage Trial
Control Sample

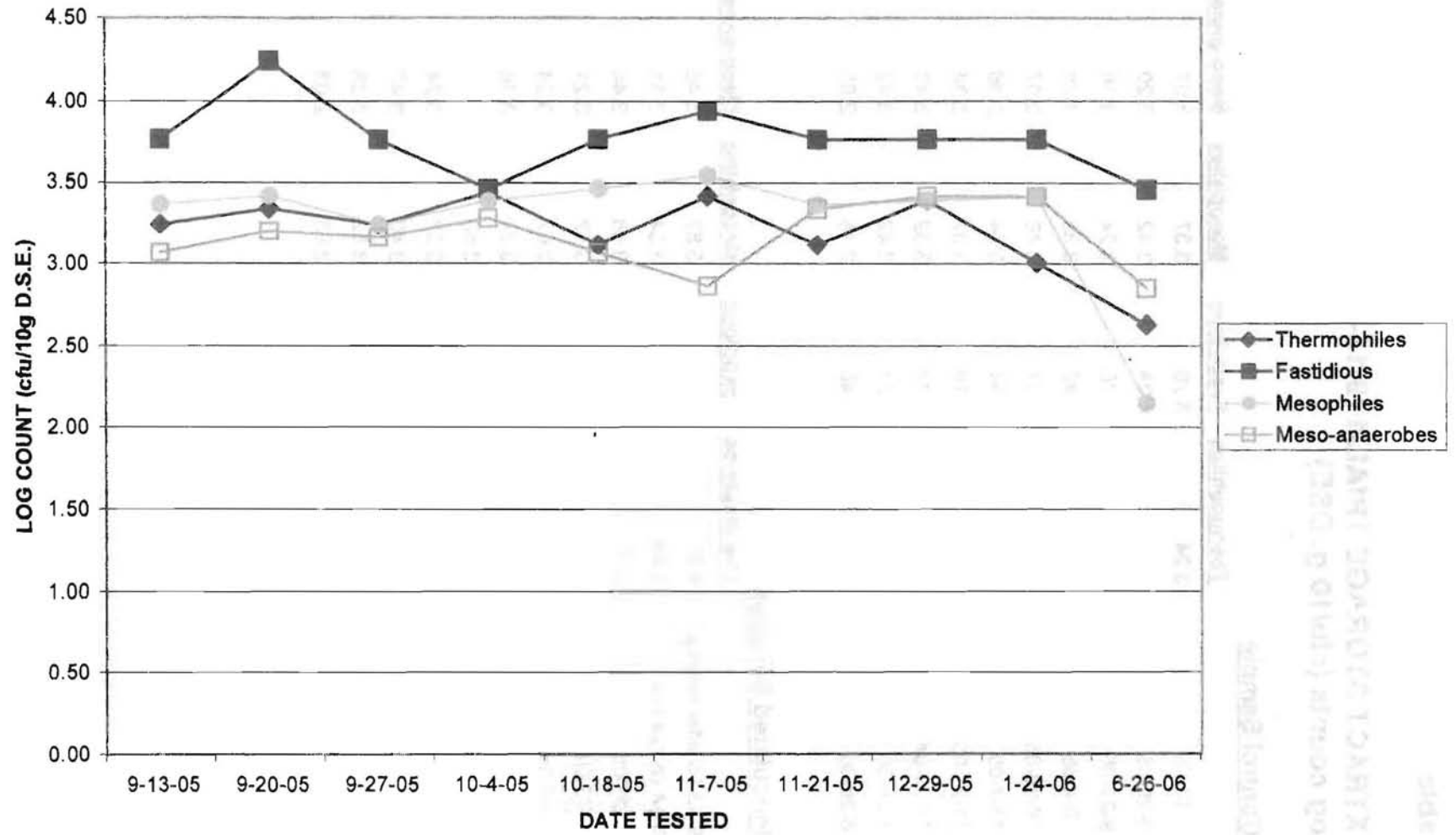


Table 6**EXTRACT STORAGE TRIAL-1 (9-13-06 to 6-26-06)
Log counts (cfu/10 g. DSE)****Control Sample**

| | <u>Thermophiles</u> | <u>Fastidious</u> | <u>Mesophiles</u> | <u>Meso-anaerobes</u> |
|----------|---------------------|-------------------|-------------------|-----------------------|
| 9-13-05 | 3.24 | 3.76 | 3.37 | 3.07 |
| 9-20-05 | 3.34 | 4.24 | 3.42 | 3.20 |
| 9-27-05 | 3.24 | 3.76 | 3.24 | 3.16 |
| 10-4-05 | 3.44 | 3.46 | 3.39 | 3.28 |
| 10-18-05 | 3.12 | 3.77 | 3.46 | 3.07 |
| 11-7-05 | 3.42 | 3.94 | 3.54 | 2.86 |
| 11-21-05 | 3.12 | 3.76 | 3.37 | 3.34 |
| 12-29-05 | 3.39 | 3.76 | 3.39 | 3.42 |
| 1-24-06 | 3.01 | 3.76 | 3.42 | 3.42 |
| 6-26-06 | 2.63 | 3.46 | 2.15 | 2.85 |

Inoculated Samples

| | <u>Thermophiles</u> | <u>Fastidious</u> | <u>Mesophiles</u> | <u>Meso-anaerobes</u> |
|-----------------------|---------------------|-------------------|-------------------|-----------------------|
| 9-13-05 after spiking | 4.07 | 6.46 | 3.87 | 3.46 |
| 9-14-05 (24 hrs) | 3.48 | 4.46 | 3.34 | 3.51 |
| 9-20-05 | 3.31 | 4.07 | 3.46 | 3.44 |
| 9-27-05 | 3.42 | 0.00 | 3.52 | 3.21 |
| 10-4-05 | 3.12 | 3.76 | 3.67 | 3.24 |
| 10-18-05 | 3.24 | 3.46 | 3.34 | 3.16 |
| 11-7-05 | 3.31 | 4.16 | 3.58 | 2.77 |
| 11-21-05 | 3.01 | 4.07 | 3.37 | 3.24 |
| 12-29-05 | 3.21 | 0.00 | 3.40 | 3.42 |
| 1-24-06 | 2.94 | 3.94 | 3.52 | 3.28 |
| 6-26-06 | 2.15 | 3.45 | 2.93 | 2.63 |

Table 7

**EXTRACT STORAGE STUDY I (at 30°C)
9-13-05 through 6-26-06**

| MICROBIAL TYPES | pH Range during Storage | Brix Range during Storage |
|--|--------------------------------|----------------------------------|
| Thermophiles | 9.9 – 10.5 | 67.8 – 70.6 |
| Fastidious Microbes | 9.9 – 10.5 | 68.2 – 70.8 |
| Mesophiles | 10.0 – 10.5 | 67.8 – 70.5 |
| Mesophilic Anaerobes | 10.0 – 10.5 | 67.8 – 70.1 |
| Controls for each of the 4 microbial types | 10.0 – 10.5 | 68.6 – 70.1 |

Fig. 6

**INITIAL INOCULUM TRANSFERS AT 35C (August 2006) for Challenge Study II
(Egf = 20 RDS, Hib = 20 RDS, Egf/Hib Mix = 20 RDS)**

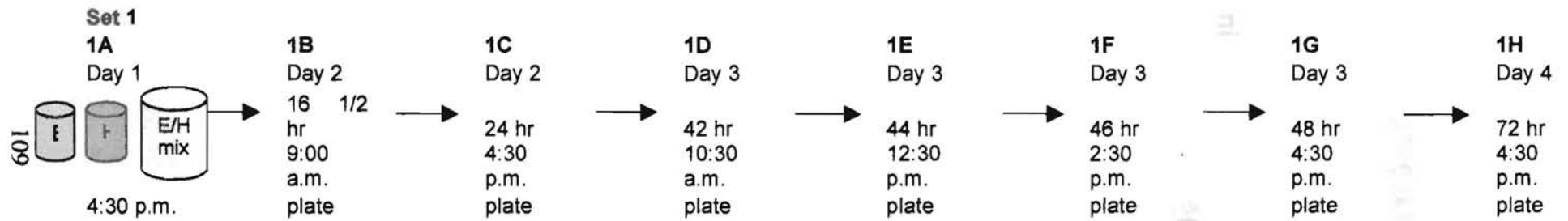


Table 8

**INITIAL 20 RDS INOCULUM
Mesophilic log counts (cfu/g)**

| | Time hrs. | EGF | HLB | EGF/HLB |
|----|------------------|------------|------------|----------------|
| 1B | 16.5 | 4.82 | 1.30 | 1.00 |
| 1C | 24 | 6.15 | 1.95 | 2.54 |
| 1D | 42 | 7.46 | | 7.57 |
| 1E | 44 | 6.45 | | 7.59 |
| 1F | 46 | 6.54 | 1.30 | 7.62 |
| 1G | 48 | 6.26 | 1.78 | 8.00 |
| 1H | 72 | 8.23 | 1.48 | 7.37 |

Fig. 7 - Challenge Study II (Mesophilic growth)
Initial Inoculum

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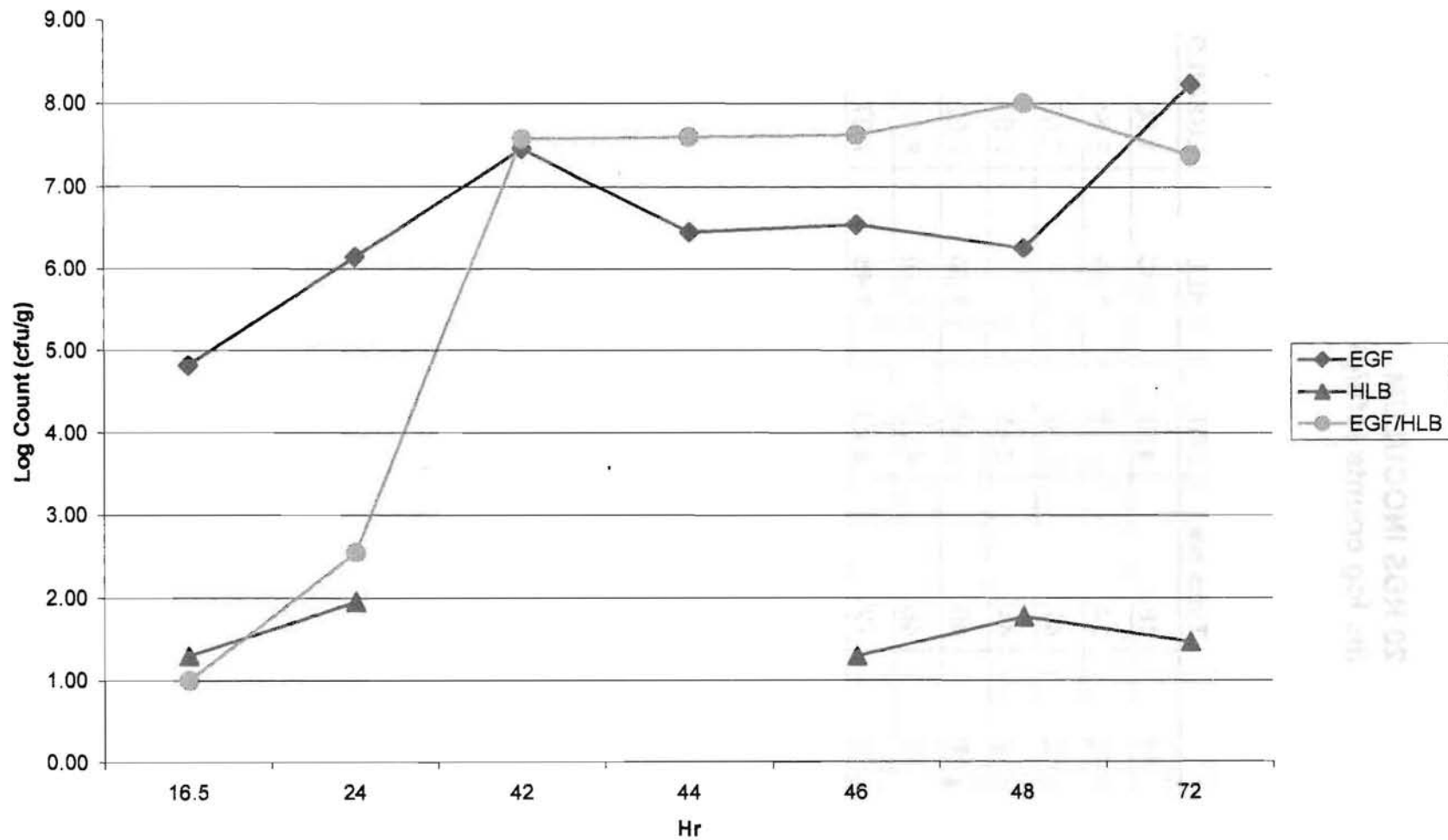


Fig. 8
Mesophilic Inoculum @ 35C (EGF = 20 RDS)

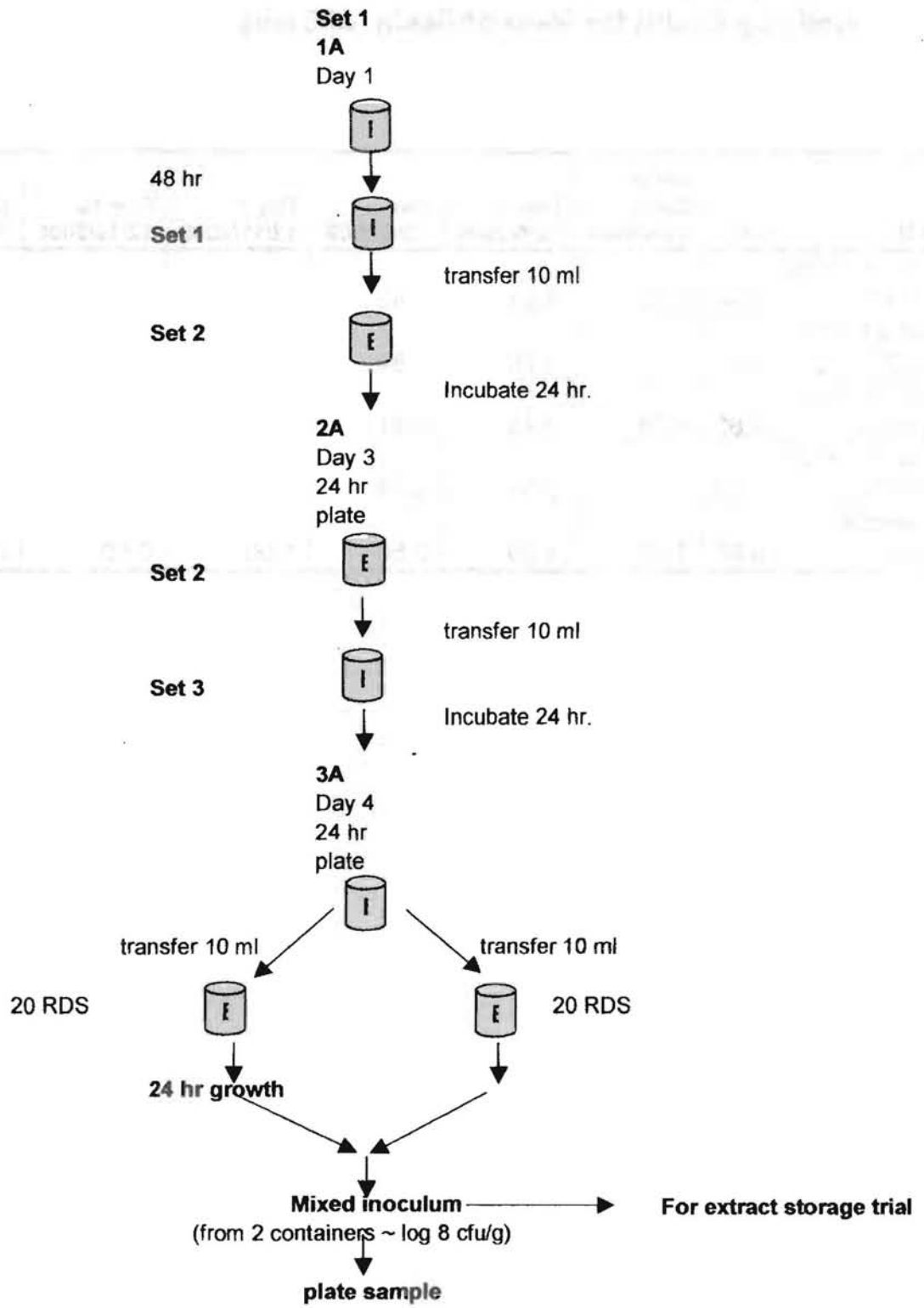


Table 9**CHALLENGE STUDY II EGF EXTRACT
Averaged Log Count for Mesophiles/g. of Sample**

| I.D. | pH | Initial Day 0 12/4/2006 | Day 1 12/5/2006 | Day 2 12/6/2006 | Day 7 12/11/2006 | Day 15 12/19/2006 | Day 45 1/19/2007 | Day 81 2/23/2007 |
|--------------------|------|-------------------------------|--------------------|--------------------|---------------------|----------------------|---------------------|---------------------|
| 68.45 RDS (20C) | 9.64 | 3.80 | 3.91 | 3.82 | 3.78 | 3.82 | 3.95 | 3.82 |
| 68.45 RDS (30C) | 9.64 | | 3.78 | 3.84 | 3.79 | 3.78 | 3.90 | 3.84 |
| 64.45 RDS (20C) | 9.63 | 3.78 | 3.45 | 3.57 | 3.47 | 3.44 | 3.45 | 3.37 |
| 64.45 RDS (30C) | 9.63 | | 3.54 | 3.57 | 3.43 | 3.45 | 3.45 | 3.46 |
| Control (20C) | 9.67 | 1.30 | 1.00 | 0.50 | 1.00 | 0.50 | 0.00 | 0.50 |

Fig. 9 SURVIVAL OF MESOPHILES IN EXTRACT AFTER MICROBIAL CHALLENGE (STUDY II)
AVERAGED COUNTS (Log count cfu/g)

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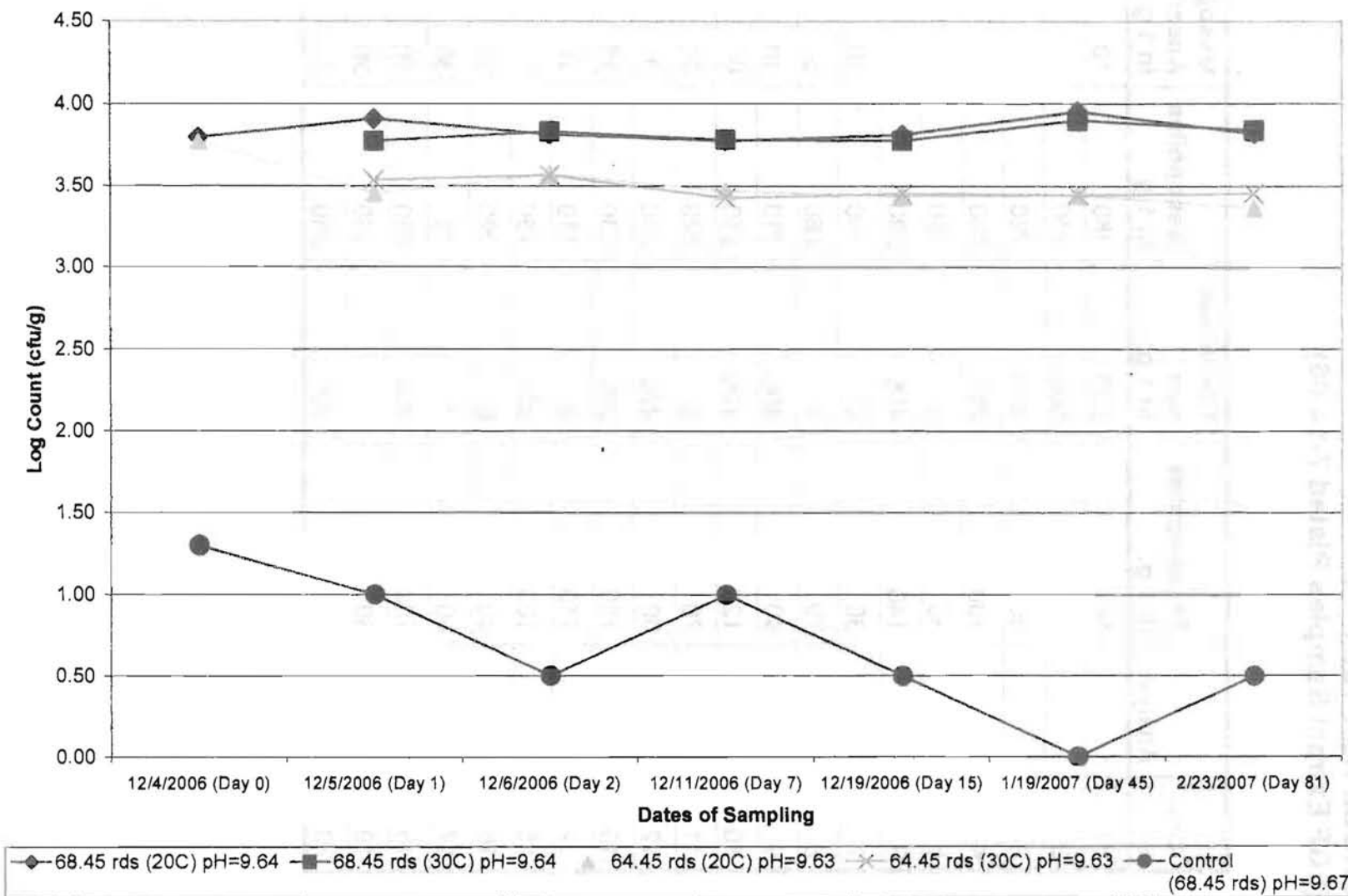


Table 10

**GAUGE R&R TEST – 1
(EGF Extract Samples Plated 7-14-06)**

| Lab # | Analyst | Thermophiles in 1 g. | Fastidious m/o in 1 g. | Mesophiles in 1 g. | Mesophilic Anaerobes in 1 g. |
|--------------|----------------|-----------------------------|-------------------------------|---------------------------|-------------------------------------|
| 1 | 1 | 50 | 200 | 280 | 10 |
| 2 | 1 | 70 | 600 | 230 | 20 |
| 3 | 1 | 80 | 600 | 220 | 0 |
| 4 | 1 | 100 | 200 | 230 | 20 |
| 5 | 1 | 50 | 0 | 380 | 10 |
| 6 | 1 | 140 | 400 | 290 | 20 |
| 7 | 1 | 30 | 0 | 190 | 20 |
| 8 | 1 | 70 | 0 | 280 | 0 |
| 9 | 1 | 40 | 400 | 310 | 10 |
| 10 | 1 | 120 | 400 | 250 | 0 |
| 11 | 2 | 70 | 0 | 320 | 20 |
| 12 | 2 | 60 | 400 | 230 | 0 |
| 13 | 2 | 150 | 200 | 230 | 10 |
| 14 | 2 | 140 | 0 | 310 | 10 |
| 15 | 2 | 100 | 800 | 190 | 0 |
| 16 | 2 | 40 | 0 | 290 | 20 |
| 17 | 2 | 80 | 0 | 28 | 30 |
| 18 | 2 | 100 | 400 | 260 | 20 |
| 19 | 2 | 60 | 0 | 190 | 20 |
| 20 | 2 | 80 | 200 | 250 | 0 |

Table 11

**GAUGE R&R TEST – 2
(EGF Extract Samples Plated 7-14-06)**

| Lab # | # | Analyst | Sample I.D. | Thermophiles in 1 g. | Fastidious m/o in 1 g. | Mesophiles in 1 g. | Mesophilic Anaerobes in 1 g. |
|-------|----|---------|---------------|----------------------|------------------------|--------------------|------------------------------|
| 21 | 1 | 1 | #2 (4-29-06) | 210 | 800 | 80 | 20 |
| 22 | 1 | 2 | #2 (4-29-06) | 100 | 400 | 100 | 30 |
| 23 | 2 | 1 | #14 (4-29-06) | 10 | 0 | 20 | 0 |
| 24 | 2 | 2 | #14 (4-29-06) | 20 | 0 | 0 | 0 |
| 25 | 3 | 1 | #16 (4-29-06) | 0 | 600 | 90 | 0 |
| 26 | 3 | 2 | #16 (4-29-06) | 10 | 0 | 30 | 10 |
| 27 | 4 | 1 | #9 (5-20-06) | 10 | 400 | 30 | 0 |
| 28 | 4 | 2 | #9 (5-6-06) | 40 | 200 | 20 | 0 |
| 29 | 5 | 1 | #7 (5-13-06) | 20 | 0 | 30 | 0 |
| 30 | 5 | 2 | #7 (5-13-06) | 20 | 200 | 20 | 0 |
| 31 | 6 | 1 | #8 (5-13-06) | 30 | 0 | 40 | 0 |
| 32 | 6 | 2 | #8 (5-13-06) | 20 | 0 | 50 | 0 |
| 33 | 7 | 1 | #9 (5-20-06) | 20 | 600 | 30 | 0 |
| 34 | 7 | 2 | #9 (5-20-06) | 0 | 200 | 30 | 10 |
| 35 | 8 | 1 | #3 (6-3-06) | 40 | 200 | 10 | 10 |
| 36 | 8 | 2 | #3 (6-3-06) | 50 | 0 | 50 | 0 |
| 37 | 9 | 1 | #8 (6-3-06) | 30 | 400 | 120 | 110 |
| 38 | 9 | 2 | #8 (6-3-06) | 50 | 400 | 200 | 190 |
| 39 | 10 | 1 | #3 (6-24-06) | 20 | 0 | 40 | 20 |
| 40 | 10 | 2 | #3 (6-24-06) | 20 | 0 | 20 | 10 |

Table 12

CONFIDENCE INTERVALS FOR MICROBIAL TESTING

| Microbial Types | Confidence Interval (CI) / 10 g. DSE (on a single sample) |
|------------------------|---|
| Mesophiles | 0.5 log units |
| Thermophiles | 1.1 log units |
| Fastidious microbes | 7 log units (to tighten CI to 1 log unit, need to run a minimum of 6 samples) |
| Mesophilic anaerobes | 5 log units (to tighten CI to 1 log unit, need to run 5 samples) |

Range of means 5.5-6.5