Acidification of distillery spentwash using different nutrient media propagated microbial consortia
Acidification of distillery spentwash using microbial consortia

2.1. Introduction

2.1.1. Anaerobic digestion

2.1.2. Two phase anaerobic digestion

2.1.3. Significance of two phase anaerobic digestion

2.1.4. Acid phase consortium

2.2. Methodology

2.2.1. Fabrication of Two phase anaerobic digestion system

2.2.2. Development of consortium

2.2.3. Propagation of consortium in different media

2.2.4. Physico-chemical analysis of treated and untreated distillery Spentwash

2.3. Results and Discussion

2.3.1. Monitoring of acid phase digester

2.3.2. Screening of media for cultivation of acidogenic consortium

2.3.3. Cultivation of Acidogenic conosortium in nutrient broth and spentwash

2.3.4. Effect of inoculum size on the volatile fatty acids production

2.3.5. Effect of set pH on the volatile fatty acid production in spentwash inoculated with spentwash propagated consortium

2.4. Conclusion

2.5. References

2.1. Introduction

2.1.1. Anaerobic digestion:

Anaerobic Process: Direct application of anaerobic processes to waste waters has substantial benefits over aerobic processes (Gosh et al., 1975). The anaerobic process consists of three stages, where the first two, hydrolysis and acidification are performed by a group of relatively fast growing acid forming bacteria. Depending on the fermentation products, methanogenesis is then carried out by one group of bacteria (methanogens; CO₂/H₂, ethanol, acetate consuming bacteria) or by two groups of bacteria living in symbiosis (acetogenic microbes) coupled to methanogenic
phase if the products are more complex, eg. ethanol, propionate and butyrate (Mah et al., 1979). The volatile substances are converted by methanogens into CH₄ and CO₂ (Fig 2.1).

**Fig 2.1. Anaerobic Digestion:**

**Acidogenesis:** Biodegradable organic matter is liquefied under aerobic or microaerophilic conditions. Macromolecules viz. polysaccharides, proteins, nucleic acids, lipids etc. and/or their oligomers are hydrolysed respectively by cellulolytic or amylolytic, proteolytic, nucleaolytic and lipolytic bacteria, into their monomers. The monomeric constituents are fermented then by a wide variety of chemoheterotrophic non-methanogenic bacteria (e.g. *Clostridium propionicum, C. acetobutylicum, Eubacterium limosum*, and members of the coliform group) to acetate, propionate, butyrate, valarate, caproate, heptanoate, octanoate, alcohols, H₂ and CO₂ (Mah, 1980). This results in lowering the pH of the system to values below pH 5.

**Methanogenesis:**
Methane formation is carried out by methanogenic archea at pH 6.5 to 9. These microorganisms possess a unique biochemistry, which enables them to derive metabolic energy from methanogenic pathway in the absence of O₂ (Whitman et al.,
1992 and Thaeur, 1998). Most of the described species of methanogens are rather specialized. *Methanobrevibacter* spp. uses only H\(_2\) and CO\(_2\) for growth, whereas *Methanosaeta* spp. only use acetate as their energy and carbon source. On the other hand, *Methanosarcina* spp. are more versatile: they can use H\(_2\)+CO\(_2\), acetate, methanol, methylated amines and pyruvate for growth and methane production (Whitman *et al.*, 1992 and Jetten *et al.*, 1992). Therefore, propionate, butyrate and higher fatty acids have to be anaerobically oxidized to methanogenic substrates prior to further conversion to methane and CO\(_2\). Proton reducing acetogens like *Syntrophobacter* spp. are able to degrade propionate but not butyrate, while *Syntrophomonas* and *Syntrophospora* are able to degrade butyrate and some higher fatty acids but do not degrade propionate. Recently, *Smithella propionica* was described as a bacterium, which oxidizes both propionate and butyrate (Liu *et al.*, 1999). Under moderate conditions, about 70% of the methane is formed by cleavage of acetate, while about 30% methane is derived from H\(_2\) + CO\(_2\) or formate as shown in Fig 2.2 (Gujer and Zehnder 1983).

**Fig 2.2. Methanogenesis**
2.1.2. Two-phase anaerobic digestion:

Since the anaerobic digestion process is essentially a two-phase process liquefaction followed by acidogenesis and methanogenesis, instability may often be due to imbalances occurred between these groups of microorganisms achieving the initial liquefaction and acidification, and those affecting the final conversion of the substrate to methane. This may lead to the accumulation of intermediary products and a sub-separation of these groups of bacteria.

Methane formation is a rate limiting step of the overall process, the advantage of stage separation might be useful as described by Cohen et al. (1979) i.e. acidogenesis and methanogenesis performed in two separate reactors arranged in series. In earlier experiments, acidogenesis was described, using sewage as inoculum and glucose as carbon limited source (Zoetemeyer et al. 1982 a, b). The influence of pH and temperature was described.

When two identical acidification systems are compared presuming the input to be different with respect to the concentration of the carbon source, difference may be observed due to product inhibition. Inhibition of the bacterial growth rate, resulting in a lower maximum dilution rate and a lower yield at higher carbon input concentrations might be caused by the inorganic salts or by the volatile fatty acids produced.

In the literature Kugelman and Chin (1971) reported synergism and antagonism effects caused by nutrient increase in anaerobically growing cultures. Depending on their concentration, volatile fatty acids may have a stimulating or inhibiting effect on bacterial growth. Sometimes, stimulation at low concentration and inhibition (at higher concentrations) has been found with the same bacteria (Stewart, 1975: Roche et al., 1973). According to data (Zoetemeyer et al., 1982a) the products of the acid fermentation are butyric, propionic, acetic, formic and lactic acid and ethanol. The relative amounts of the products depend on the dilution rate and, more strongly, on the culture pH value. Thus by selecting appropriate pH value, selectivity in the yield of major product can be achieved with the exception of propionic acid. The products formed can be considered as normal products of carbohydrate fermentation (Hobson et al., 1974).

It is presumed that volatile fatty acids in their undissociated form may freely permeate the cell membrane. Rao and Berger (1970) reported an increase in acid concentration in the cell if the culture pH was lowered. If undissociated volatile fatty
acids are pumped into the culture, they will penetrate the plasma membrane and will
dissociate intra-cellularly, depending on the pH inside the cell; thus the pH inside the
cell will be lowered. To prevent unfavorable physiological conditions within the
bacterial cell, the excess protons will be exchanged against potassium ions (Tempest
et al., 1970; Tempest and Meers, 1968). Because this so called “proton/potassium
pump” is an energy consuming process, less energy will be available, per unit of
substrate consumed, for the synthesis of biomass and therefore the growth rate will be
lowered.

2.1.3. Significance of Volatile fatty acid (VFA) relationships:
VFA relationships are important in the anaerobic digestion of animal wastes, as they
(acetic, propionic and butyric) are direct precursors of methane, either through direct
conversion of acetate through the intermediate formation of hydrogen and carbon
dioxide. Thus, they are essential compounds in the biological conversion of
heterogeneous to useable products. VFAs are also known inhibitors in the biological
conversion process if their concentrations are sufficiently high. Thus, VFAs are
simultaneously essential for the process and can be toxic agents should they be
present in excess quantities. This relationship makes quantifying VFAs in modeling
studies essential to accurately predicting digester failure or success. A highly
correlated relationship between the level of acetic and / or the propionic acid to acetic
acid ratio in digesters that were successful and in digesters that failed has been shown.
These data have been used to calibrate an original comprehensive methanogenesis
model and along with the addition of dual-use substrate kinetics for the simultaneous
catalysis of propionate and butyrate, have produced a much improved prediction of
the VFA relationships observed in operating anaerobic digesters.

VFAs are intricately involved in methane production, both as intermediate
substrates and as inhibitory agents (Hill, 1982). The successful operation of a digester
involves a delicate balance of VFA levels.

Hill (1982) has described conceptually a four culture dynamic mathematical model of
the methanogenesis of animal waste. This model proposes conversion of
heterogeneous organic matter to CO₂ and CH₄ through a four-stage process involving
hydrolysis, acetogenesis, hydorgenogenesis and methanogenesis.

Correlation of VFA relationships of 70 digester studies (Hill et al., 1986) has
been incorporated into the original methanogenesis model with a mathematical
representation of dual use substrate kinetics.
In the original model, the hydrogenogenesis stage (conversion of propionate and butyrate to acetate and \((CO_2 + H_2)\) was modeled using a “proportionate” uptake of propionate and butyrate by hydrogenogenic bacteria (Hill, 1982).

The proportional uptake was based on the ratio of propionate and butyrate to the total propionate and butyrate present. This was known at the time to be an unverified approach and was stated to be a new concept in the representation of biological kinetics (Hill, 1982).

The original model also used one inhibition constant for all microbial cultures for ammonia. The VFA relationships elucidated by Hill and coworkers (1986) have shown that to simulate those relationships mathematically, one must provide different inhibition levels for both VFA and total VFA (TVFA) for the hydrogenogenic bacteria. This is necessary to produce propionate to acetate ratios (P/A) greater than 1.4, as observed in operating digesters that are undergoing failure.

The particular relationships observed by Hill and coworkers (1986) for digesters that are undergoing failure (methane productivity (?) < 0.25 L CH₄/g VS added) had a P/A ratio of > 1.4.

Hydrogenogenic bacteria, by virtue of their ability to metabolize propionate and butyrate to hydrogen, carbon dioxide, and acetate, play a critical role in determining digester ‘health’.

The original digestion model (Hill, 1982) partitioned growth of the hydrogenogenic culture on propionate and butyrate according to the ratio of propionate and butyrate levels to the total amount of substrates present. This proportional approach to describing relative substrate utilization rates by hydrogenogenic bacteria allowed the model to accurately predict methane production rates, but proved inadequate in terms of predicting VFA relationships in digesters operating at near failure.

2.1.4. Significance of two-phase anaerobic digestion:
Disposal of wastewater from various agro-based industries like distillery is a problem. Microbial abatement of such wastes with high organic load has been practiced using conventional anaerobic systems throughout India. However, there are problems with these methods of pollution abatement. These problems are due to fluctuations in gross composition of waste, and lack of uninterrupted supply of wastewater, which in turn depends on invariable supply of raw material for industry. The methanogenic
microorganisms have been replaced which results in increased retention period due to inability of the microbes to acclimatize to wastewater different in composition or microorganisms responsible for acidogenesis. Methanogens are also subjected to shock loading with existing anaerobic systems.

Designing of two-phase wastewater treatment system that enables to control two different microbial systems independently. In first stage, it will be possible to develop microbial consortia targeted to spentwash that enable to control two different microbial systems independently. Developed microbial consortia targeted at spentwash will be mass propagated, characterized and used directly for acidogenesis (stage-1) whenever required.

There are reports on two-phase system of anaerobic waste treatment process. The aim of this was to protect the methanogens from abrupt pH change, shock loading to reduce retention period of the wastewater. Anderson et al. (1994), worked on dairy wastewater treatment and Rintala and Ahning (1994) worked out feasibility of a two-phase thermophilic anaerobic process for the treatment of household solid waste.

Several workers in India have attempted to use the two-phase anaerobic waste treatment process. A modification of two-phase system had also been suggested for abatement of pollution due to plant material (Joshi et al., 1986). Subsequently Verrier et al. (1987) studied disposal of solid vegetable waste using two-phase anaerobic process. Kalia et al. (1992), attempted to reduce pollution load due to plant material especially dumped wheat grains. The two-phase waste treatment process had been attempted for market wastes by Ranade et al. (1987). The aim of these authors was to protect the methanogens and study prevalence of different methanogens in presence of different acids.

2.1.5. Acid phase consortium:

Morgan et al. (1991) studied microbial ecology of the two-phase UASB reactor. In this study acidogens were enumerated in the anaerobic digesters used for treatment of ice-cream wastewater.

There are no reports directed towards development of microbial consortia suited to specific organic liquid wastes that fluctuate from time to time in their composition or provision of microbial inocula for starting discontinued plants due to interrupted supply of wastewater. Therefore, in the present work we have segregated acidogens and methanogens in two-phase anaerobic digester and acidogenic micro-
organisms were propagated in nutrient broth, spentwash, serum and whey. The volatile fatty acid profiles and amount produced by these propagated consortia were compared with that of acid phase consortia in the two-phase system.

2.2. Methodology:

2.2.1. Fabrication of two-phase anaerobic digestion system:
Two-phase anaerobic digester comprising of 5L methanogenic phase reactor and 1L acid phase reactor (with 500 ml spentwash) was kept at 40°C. Anaerobic reactor was connected to gas collecting assembly comprising of two 2L capacity bottles with the help of tubing having sampling port for gas analysis. Gas was collected on acidified water in 2 L calibrated bottle attached at bottom with a water-adjusting bottle of same capacity (Plate 2.1.).

2.2.2. Development of consortium:
Acid phase reactor was initially fed with 50 ml spentwash from acid phase of 5 L two-phase stabilized anaerobic digester and 50 ml fresh spentwash, pH- 8.0 to 8.5 and COD- 1,64,000 mg /L. After 15 days of incubation only fresh spentwash of pH-8.0 was fed to the acid phase reactor. Sampling for volatile fatty acid analysis and measurement of pH was done before feeding the reactor. 250ml acid phase broth of pH-8 was fed to methane phase reactor. Acid phase reactor was fed with fresh spentwash of pH-8. Acidogenic microbial consortium from this stabilized digester was used for further experiments.

Gas collecting assembly consisted of two 500 ml bottles. One of these bottles contained acidified water (pH-2) and attached with methane digester through rubber tubing with gas sampling septum in between. Second bottle partially filled with acidified water was connected with first bottle at the bottom.
2.2.3. Propagation of consortium in different media:
One ml culture was centrifuged at 10,000 rpm for 20 min and the pellet washed with saline three times. Washed pellet of the biomass was inoculated in the 10 ml spentwash (pH-8), serum, nutrient broth, carbohydrate fermentation medium (CHO fermentation medium) and whey separately. Volatile fatty acids produced were monitored as described in 2.2.2 -I.

2.2.4. Cultivation of biomass in nutrient broth and spentwash:

A) Nutrient broth: Nutrient broth (pH 7.2) was used for the cultivation of acid phase consortium. Spentwash culture (5% v/v) from acid phase digester was centrifuged at 10,000 rpm for 5 min. Pellet was used for inoculation of 300 ml nutrient broth in 500ml conical flask. The flask was incubated at 40°C under stationary state for 48 hrs. Five aliquots of 10 ml of nutrient broth culture were centrifuged at 10,000 rpm for 20 min. and the pellet washed with sterile distilled water. Washed biomass was filtered through Watman filter paper No. 1. Weight of the biomass (g/100 ml) was determined using a precision balance.

B) Spentwash: Spentwash (pH 7.2) was used for cultivation of acid phase consortium. Spentwash culture (5% v/v) from acid phase digester was centrifuged at 10,000 rpm for 5 min. The pellet was used for inoculation of 300 ml spentwash in 500 ml conical flask. Flask was incubated at 40°C under stationary state for 48 hrs. Five aliquots of 10 ml of spentwash culture were centrifuged at 10,000 rpm for 20 min. and the pellets washed with sterile distilled water. Washed biomass was filtered through Watman filter paper No. 1. Weight of the biomass were determined in terms of g/100 ml.

2.2.5 Effect of spentwash concentration on the biomass production: Centrifuged pellet of acid phase digester spentwash (5% V/V) was used for inoculation of 300 ml of 25%, 50%, 75% and 100% sterile spentwash (pH 8.0) in 500 ml conical flasks. Flasks were incubated under stationary condition at 40°C for 48 hrs. Five aliquots of 10 ml of spentwash were centrifuged at 10,000 rpm for 20 min. and the pellets washed with sterile distilled water. Washed biomass were filtered through Whatman filter paper No. 1. Weight of the biomass was determined as earlier.
2.2.6. Effect of inoculum size and source on volatile fatty acid profile
The inocula from 2.2.4 (A and B) were used to inoculate 10 ml spentwash (pH 8.0) and inoculated with consortium in the range of $10^5$-$10^8$ cfu/ml. Tubes were incubated at 40 °C for 3 days. Volatile fatty acids and TVC of consortium were monitored for 3 days as described in 2.2.8.

2.2.7. Effect of pH on volatile fatty acid profile of spentwash inoculated with spentwash propagated microbial consortium:
Spentwash (10 ml) aliquots were taken in tubes, was adjusted to varying pH values ranging from 4-11. After sterilization at 120°C for 15 min each tube was inoculated with 10% (v/v) spentwash propagated consortium (10^8 cfu/ml). Tubes were incubated at 40°C and volatile fatty acids and pH were monitored everyday.

2.2.8. Physico-chemical Analysis:
Volatile fatty acids, biogas and pH were monitored daily for the two-phase anaerobic digester. Volatile fatty acids and biogas were analyzed with the help of Nucon Gas chromatograph-5700. Measurement of pH was done with microprocessor based pH analyzer, PHAN, Labindia.

i) Volatile fatty acid:
Sample preparation:
0.8 ml sample from acid phase reactor was taken in 1.5 ml Eppendorf tube. It was acidified with 0.2 ml conc. O-phosphoric acid. Samples were thoroughly mixed for 10 min., allowed to settle for 15 min. and centrifuged at 10,000 rpm for 10 mins. (Roderick and Mackie, 1981).

Standard Volatile fatty acids:
Standard VFA mixture containing 500 mM concentration of Na-salt of acetate, propionate and butyrate each, was prepared in distilled water. Out of this, 0.8ml mixture was acidified with 0.2 ml O-phosphoric acid, mixed thoroughly and used for analysis.

The VFAs were analyzed on the GC by injecting 1 μl sample with the help of 10μl syringe (Top micro-syringe). For the analysis, a 2m long SS Chromosorb, WHP column having liquid phase sp 2300-2400 was used. Injector, oven and detector temperatures were kept at 220, 150 and 250°C, respectively. Nitrogen was used as a carrier gas at a flow rate of 30 ml/min. FID was used as a detector. Hydrogen was used as a fuel and air as an oxidant at 25 ml/min. Individual VFAs and their
concentration was calculated by comparing with standard VFA with the help of Aimil software on the basis of area under the curve.

**ii) Gas Analysis:**
Total volume of gas was measured with calibrated bottle. CH₄, CO₂ and H₂S were analyzed on Poropaq QS column and TCD was used as detector. Injector, oven and detector temperatures were kept at 80, 60 and 90°C respectively. H₂ was used as carrier gas at 25ml/min flow rate. Individual gases were measured as % gas in biogas (Kalai et al., 1992).

**2.3. Results and Discussion:**

2.3.1. Monitoring of two-phase anaerobic digester:

![Fig 2.3. VFA profile of Acid phase digester at 40°C](image)

**Variation in pH:**
The general trend of the reactor pH was that it decreases from 1st day to 18th day. There were some variations in pH due to failure in feeding and feeding of high pH spentwash. The pH of the acid phase reactor was adjusted after every 24 hours.

During first week the acid phase pH decreased from 7.5 to 6.5. On the sixth day feeding was not done. Therefore, the pH on the 7th and 8th day was 6.4. There was
increase in pH from 8th to 13th day due to increase in pH of the feed. As feeding was stopped after 14th day there was again a decrease in pH (Fig 2.3).

**Volatile fatty acid pattern in Acid phase reactor:**

Total VFA (Acetate+Propionate+Butyrate):

Fig 2.3 indicates that the maximum VFA concentration in the reactor was found on 18th day (654 mM), while minimum was 287 mM on 5th day. There was decrease in total VFA concentration during first five days. From sixth day there was sharp increase in total VFA concentration up to 9th day and sudden drop on 10th day. Gradual increase in concentration was observed from the 11th day. Initial decrease in the total VFA may be due to increased utilization of VFA for the growth of microorganisms. After sixth day increase in concentration may be due to increase in population as well as non-feeding of the acid phase reactor. Sudden increase on the day nine may be due to sudden increase in the feed of the reactor. There was increase in the total VFA as pH decreased. But during first six days pH decreased with no increase in total VFA. This may be due to the ongoing hydrolytic processes and higher fatty acids produced which were not detected (Fig 2.3.).

**Acetate:**

Trend in acetate production in the acid phase reactor was exactly as that of total VFA. During first six days acetate concentration decreased slowly from 245 mM to 131 mM. Later it increased up to 289 mM on ninth day and again there was decrease on 10th day and then increased equivalent to that on 9th day. Maximum acetate was detected on 9th day when pH was 6.4.

**Propionate:**

Propionate concentration decreased from 125 mM to 87 mM during first five days. From 6th day to 9th day it increase up to 170 mM. Concentration falls on 10th day and increases to 195 mM on 18th day. Trends of acetate and propionate production were similar, only difference was in the rate of increase and decrease. The reactor total VFA and acetate production was higher than that of the propionate. Maximum concentration of total VFA and propionate was detected on 18th day while that of acetate on 9th day.

**Butyrate:**

Butyrate concentration decreased from 64 to 52 mM i.e. lowest on the third day. Increase in concentration found up to 175 mM during 3 to 9 day and drop to 160 mM on 13th day. Again it increased up to 183 mM on 16th day and drop on 18th day.
The butyrate concentration starts increasing when acetate and propionate concentrations decrease. Maximum butyrate concentration was detected two days earlier than that of acetate and total VFA concentration (Fig 2.3.).

![Fig 2.4. Dynamics of fatty acid production at 40°C.](image)

Fig 2.4 indicates that the percentage of acetate in the acid phase reactor varied from 40 to 60% during the period of monitoring. Relative acetate concentration was more on first two days. From third day it was relatively constant up to ninth day. From 13th day it was relatively lower up to 18th day. The concentration of propionate was 24 to 30 %, which was relatively constant, while butyrate concentration was 14 to 13 %. The concentration of butyrate in the acid phase reactor increases with increase in incubation. When the acetate percentage was low, propionate and butyrate were found to be in equimolar concentration i.e. 1:1 proportion (Fig 2.4.).

Variations in pH and P/A ratio of acid phase reactor at 40°C:

P/A ratio of the acid phase digester ranged from 0.5 to 0.7(Table 2.1), which is well below 1.4. Therefore, microbial consortium in the acid phase digester is producing volatile fatty acids indicating good health of the digester. Hence acidogenic consortium from this digester was used for further experiments.
Table 2.1. Variations in pH and P/A ratio of acid phase reactor at 40°C:

<table>
<thead>
<tr>
<th>Incubation (Days)</th>
<th>pH</th>
<th>Propionate: Acetate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.2</td>
<td>0.51</td>
</tr>
<tr>
<td>2</td>
<td>7.4</td>
<td>0.47</td>
</tr>
<tr>
<td>3</td>
<td>7.12</td>
<td>0.52</td>
</tr>
<tr>
<td>4</td>
<td>6.91</td>
<td>0.64</td>
</tr>
<tr>
<td>5</td>
<td>6.84</td>
<td>0.66</td>
</tr>
<tr>
<td>6</td>
<td>6.86</td>
<td>0.63</td>
</tr>
<tr>
<td>7</td>
<td>6.4</td>
<td>0.61</td>
</tr>
<tr>
<td>8</td>
<td>6.42</td>
<td>0.55</td>
</tr>
<tr>
<td>9</td>
<td>6.72</td>
<td>0.59</td>
</tr>
<tr>
<td>13</td>
<td>6.73</td>
<td>0.71</td>
</tr>
<tr>
<td>14</td>
<td>6.59</td>
<td>0.74</td>
</tr>
<tr>
<td>15</td>
<td>6.2</td>
<td>0.64</td>
</tr>
<tr>
<td>16</td>
<td>6.09</td>
<td>0.71</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>0.68</td>
</tr>
</tbody>
</table>

Biogas production in single and two-phase anaerobic digesters:

Feeding of the single and two-phase anaerobic digesters that were working steady was terminated till the gas production was completely ceased. Both digesters were fed with the spentwash (pH 8). Biogas production was monitored for 13 days and CH₄ percentage was analysed.

Biogas production was observed on second day in single as well as two-phase digesters. Biogas production increased during initial 7 days, then a fall in production was observed for two days and finally it reached maximum i.e. 780ml on 13th day.

Biogas production in single stage anaerobic digester was observed with peaks and falls, which is the peculiarity of batch fed reactor. Maximum production of biogas that was observed on 6th day was barely half of the biogas produced in two-phase reactor (Fig 2.5.).
CH$_4$ percentage in two-phase anaerobic digester increased steadily up to 65% on 12$^{th}$ day except for a drop on the 7$^{th}$ day, which may be due to failure in spentwash feeding. However, in case of single-stage digester, the CH$_4$ content was 20%, which remained constant throughout (Fig 2.6). The results depict that start-up period during restarting of two-phase anaerobic digester is significantly less than that of single stage anaerobic digester. This may be due to efficient volatile fatty acid production by acclimatized acidogenic consortium in acid phase reactor. Therefore, it will be worthwhile to explore economical media for multiplication of acclimatized acidogenic microbial consortium for restarting of anaerobic digester. In the next experiment, acclimatized consortium was propagated in serum, whey, nutrient broth and spentwash and their volatile fatty acid patterns were compared with volatile fatty acid patterns generated by acclimatized microbial consortium of acid phase reactor.
2.3.2. Screening of media for the cultivation of acidogenic consortium:

Volatile fatty acid profiles generated in spentwash by acidogenic consortium propagated in different media and sourced from cow dung are as follows:

**Acetate:**

Average 250 mM acetate level was observed in acid phase reactor at steady state. Maximum acetate level was observed in spentwash inoculated with consortium
sourced from cow dung and propagated in CHO fermentation medium, nutrient broth and spentwash on fifth day. However, acetate level in the spentwash inoculated with spentwash and nutrient broth propagated consortium was as observed in the acid phase digester, whereas, it is lower in case of microbial consortium propagated in whey, serum and sourced from cowdung (Fig 2.7).

Propionate:

NB: Nutrient Broth

175 mM propionate level was observed in acid phase reactor during steady state. Maximum propionate level was observed in spentwash inoculated with propagated and sourced from cowdung consortium except serum and whey-propagated consortium. Propionate level in the spentwash, inoculated with spentwash propagated consortium was near to propionate level in acid phase digester (Fig 2.8.). This may be due to decrease in pH below 5.15 or suppression of propionate generating microbial community.
Butyrate:

170 mM butyrate level was observed in acid phase reactor during steady state (Figure 2.9). Butyrate was produced in all tubes inoculated with different consortia under observation. Highest rate of butyrate production and butyrate level was observed in spentwash inoculated with spentwash-propagated consortium. Same level of butyrate was observed in the spentwash inoculated with whey, CHO fermentation medium and NB propagated consortia. Rate of butyrate production was lower in the spentwash inoculated with whey-propagated consortium than the spentwash wash propagated consortium. Consortium propagated in CHO fermentation medium and NB showed initial lag of 3 days and 4 days respectively. The rate of butyrate production was lower than with whey-propagated consortium, CHO fermentation medium propagated than the NB propagated consortia. Butyrate level was constant after first day in spentwash inoculated with consortia propagated in serum and sourced from cow dung, which is significantly lower than observed in acid phase digester (Fig 2.9).

NB: Nutrient Broth

Fig. 2.9. Butyrate profile of spentwash inoculated with different media propagated consortia at 40 °C

Total volatile fatty acid: (Acetate + Propionate + Butyrate)

As seen in Figure 2.10, in the acid phase digester on an average 600 mM total volatile fatty acid level was observed. Total volatile fatty acid level observed in the spentwash inoculated with NB and spentwash propagated consortia was similar to that
of acid phase digester on 5th day. Total volatile fatty acid level observed in the spentwash inoculated with CHO fermentation medium and whey propagated consortia was 2/3rd of the total volatile fatty acids observed in acid phase digester, whereas it was 1/3rd in the spentwash inoculated with consortium sourced from cowdung. Negligible total volatile fatty acids level was detected after 24 hr in spentwash inoculated with serum-propagated consortium (Fig 2.10).

Data depicts that although the same acidogenic microbial consortium from the acid phase reactor was used for the propagation in different media, the volatile fatty acid pattern produced by individual propagated consortium was different i.e. quantity and proportion of individual volatile fatty acid was different than observed in acid phase digester. These alterations in the volatile fatty acid patterns may have occurred due to the synergism or antagonism effects in anaerobically growing cultures caused by nutrient increase or alteration in metabolic pathways during propagation due to different propagation media (Kugelman and Chin, 1971). The normal products of the acid fermentation of carbohydrates are formic, acetic, butyric and lactic acid and ethanol (Hobson et al., 1974). The relative amounts of the products depend strongly on the pH of the culture media (Zoetemeyer et al., 1982a).
As the concentrations of the volatile fatty acids in the acid phase digester and in the spentwash inoculated with spentwash and nutrient broth propagated consortia are similar, the spentwash and nutrient broth can be used for the development of starter cultures for the start-up of anaerobic digester treating spentwash.

2.3.3. Cultivation of acidogenic conosortium in nutrient broth and in the spentwash:
Significantly higher amount of biomass was obtained in spentwash than in the nutrient broth. Therefore, spentwash is a better medium for the development of starter culture for the anaerobic digester (Table 2.2).

Table 2.2. Biomass production in nutrient broth and spentwash:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Cultivation medium for acidogenic consortium</th>
<th>Wet wt. of biomass (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Nutrient broth</td>
<td>0.310</td>
</tr>
<tr>
<td>2.</td>
<td>Spentwash</td>
<td>0.450</td>
</tr>
</tbody>
</table>

The biomass increased with increase in spentwash concentration in the cultivation medium (Table 2.3). Maximum 0.460% biomass was obtained in the medium with 100% spentwash. This indicates that undiluted (100%) spentwash is the optimum medium for the biomass production followed by nutrient broth.

Table 2.3. Effect of spentwash concentration on biomass production:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Spentwash concentration (% v/v)</th>
<th>Wet wt. of biomass (g/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>25</td>
<td>0.210</td>
</tr>
<tr>
<td>2.</td>
<td>50</td>
<td>0.280</td>
</tr>
<tr>
<td>3.</td>
<td>75</td>
<td>0.330</td>
</tr>
<tr>
<td>4.</td>
<td>100</td>
<td>0.460</td>
</tr>
</tbody>
</table>

However, this may be due to the fact that the acid phase consortium was well adapted to the nutrients in spentwash than any other media used in the present studies.
2.3.4. Effect of inoculum size on the volatile fatty acids production:

*Nutrient broth propagated consortium:*

![VFA profile of spentwash inoculated with NB propagated consortium (Inoculum size: 2.57 X 10^5 cfu/ml)](image)

The pH of spentwash inoculated with 10^5 cfu/ml NB propagated consortium decreased below 5.3 on 1st day later increased to 5.75 up to 3rd day. Acetate level increased to max. on 1st day and decreased later. Propionate level was below detectable limit during first two days, which appeared on third day. Butyrate production started from first day and increased gradually on 2nd and 3rd day. Maximum total VFA level was observed on 2nd day and decreased on 3rd day. The VFA production pattern is quite different as compared to the pattern of acid phase digester (Fig 2.11.).

The TVC of bacteria increased relatively faster upto 2nd day, which slowed down subsequently in the spentwash. Total VFA increased up to second day then it was constant for next 24 hrs. Sharp increase in the rate of VFA production was observed during 4th and 5th day. Acetate and butyrate level increased gradually after 48 hrs incubation and maximum level was detected on 5th day. Propionate level decreased on 4th day and maximum level was attended on 5th day. Propionate: Acetate ratio was well below 1.4 (Fig 2.12).
There was an increase in TVC of the spentwash inoculated with NB propagated consortium with inoculum size $10^8$ cfu/ml with two plateaus during 2$^{nd}$-3$^{rd}$ day and 4$^{th}$-5$^{th}$ day. The TVFA curve was found to run parallel to the TVC curve. Acetate, propionate and butyrate levels increased with increase in incubation period as in earlier case, however concentration of acetate, propionate and butyrate were less than those in the spentwash inoculated with NB propagated consortium (inoculum size $10^6$ cfu/ml). Propionate level was higher than the acetate level but P/A was less than 1.4 (Fig 2.13.).
Spentwash:
Decrease in pH of the spentwash inoculated with spentwash-propagated inoculum (10^5 cfu/ml) was more than the spentwash inoculated with NB- propagated consortium (Fig 2.14.).

Acetate level was highest on 1st day compared to NB- propagated consortium on 2nd day, which later decreased. Similarly decrease in total volatile fatty acids level was observed. Propionate appeared on 3rd day (Fig 2.14).

Increase in TVC is similar to that with nutrient broth propagated consortium. Although the maximum concentrations of TVFA are same in spentwash inoculated with spentwash and nutrient broth propagated consortia, production rate in spentwash inoculated with spentwash propagated consortium with inoculum size 10^6 cfu/ml was more and peak level was reached one day earlier than with nutrient broth propagated consortium with same inoculum size. Propionate and butyrate levels were proportionately higher than before (Fig 2.15.).
Total viable count in the spentwash inoculated with spentwash-propagated consortium is similar to that in spentwash inoculated with nutrient broth propagated consotium i.e. two plateaus during 2\textsuperscript{nd}-3\textsuperscript{rd} and 4\textsuperscript{th}-5th days. However, maximum TVFA level in this experiment was detected on 3\textsuperscript{rd} day which was achieved on 5\textsuperscript{th} day in spentwash inoculated with nutrient broth propagated consortium. Acetate, propionate and butyrate levels were highest on 3\textsuperscript{rd} day. Propionate concentration increased after 3\textsuperscript{rd} day. These two plateaus and increase in propionate level from first plateau indicate that in first phase acetate producers are dominant and later propionate producers (Fig 2.16.).
Data depicts that amount of volatile fatty acids produced in the spentwash inoculated with 1X $10^6$ cfu/ml inoculum size is more than in the spentwash inoculated with 1X $10^8$ cfu/ml size. However, period for generation of maximum amount of volatile fatty acids is less in the spentwash inoculated with inoculum size 1X $10^8$ cfu/ml than other inocula sizes. Volatile fatty acid pattern of spentwash inoculated with spentwash-propagated consortium resembles more to the volatile fatty acid pattern of acid phase digester than spentwash inoculated with nutrient broth propagated consortia. The reason for this is that spentwash propagated consortia is acclimatized to the spentwash. This is in agreement with the statement sufficient bacterial selection during reactor start-up also significantly effect the rate of acidification and methanogenesis (Morgan, et al., 1991). When commissioning a reactor for the first time on a particular effluent stream, it is advantageous to utilize sludge from a reactor treating similar waste. If this is not possible, the sludge will have to be acclimatized to the specific effluent (Hicky et al., 1991).

2.3.5. Effect of set pH on the volatile fatty acid production in spentwash inoculated with spentwash-propagated consortium:

In the earlier experiments propagation media and inoculum size were varied. As a consequence of it, volatile fatty acid patterns and amount of volatile fatty acids varied. However, data from the inoculum size experiments reveals that there is another factor than propagation media composition.

Volatile fatty acid patterns in the spentwash inoculated with nutrient broth and spentwash propagated consortia (Inoculum size: 1X $10^5$ cfu/ml) are quite similar with some minor variations. In case of spentwash, there was a decrease in pH below 5.30 with sharp increase in acetate level and presence of initial acetate, propionate and butyrate. Therefore, there must be indigenous microbial flora developed in the spentwash during storage at site, contributing in acetate production and decrease in pH level below 5.30 which in turn inhibits propionate production.

To verify these observations, effect of pH ranging from 4.13 to 11 on volatile fatty acid production in spentwash by spentwash propagated consortium was studied in this experiment.

Observed pH of spentwash with set pH 4.13-11.00 remained constant at 4.5 up to 3rd day, whereas, it increased to pH 5.7 on 4th and 5th day in with set pH 8-11 (data not shown).
Sharp increase in acetate level in the spentwash of set pH 4.13 and slight increase in spentwash of rest of experimental tubes was observed. High level of acetate was detected exclusively in the spentwash of set pH 4.13 – 7 and observed pH below 5.20. Exceptionally butyrate was observed on 2\textsuperscript{nd} and 3\textsuperscript{rd} day in the spentwash of set pH 6 with increase in observed pH above 5.20.

Acetate and butyrate was in increasing concentration with incubation period in the tubes with spentwash of pH 8-11 and observed pH>5.10. However, propionate could be detected on 3\textsuperscript{rd} day in the spentwash with set pH 10-11 and observed pH>5.7 (Fig 2.17.).

These results are in agreement with the statement, stable operation of the acidogenesis of carbohydrates in a single as well as two-phase anaerobic process is hardly possible in the pH range 6.0-8.0 (Zoetemeyre et al., 1982a). However, it seems reasonable to state that up to pH 6 fermentation of the butyric type predominates; in the pH range 6-7 fermentation of the coliform and/or heterolactic type take over, while at the highest pH values acetic bacteria also could play a role (Thimann, 1962). Zoetemeyer et al., (1982a) concluded that running the acid reactor at high dilution rate in the pH range 5.7-6.0 offers a stable and more favourable substrate. In this range high level of butyrate is produced and butyrate is converted at faster rate to methane than acetate and more faster than propionate.

In Propionibacterium regulation of organic acid formation depends on whether oxygen or fumarate, serves as an electron acceptor in the respiratory chain reaction. No TCA cycle was found to exist in this species grown under anaerobic conditions. It is evident that the randomizing pathway worked in a reversed direction in the presence of oxygen, through which the propionic acid is oxidized to pyruvate and then acetate. It has also been demonstrated that propionibacteria are sensitive to the excess oxygen (Vries et al., 1972) and the cell growth rate will significantly decrease if cells are exposed to oxygen for long time (Ye et al., 1996). Therefore, oxygen could be one of the physical factors in presence and absence of propionate in earlier experiments which in turn create acidic environment by producing more acetate.
Fig 2.17. Effect of spentwash acidification on VFA production
2.4. Conclusions:
From the above discussion it can be concluded that spentwash and nutrient broth can be used as media for cultivation of acidogenic microbial consortia. These consortia can be used as start-up cultures for the biogas digester for treating distillery spentwash. Feed of spentwash with $10^5$ cfu/ml density of acidogenic microorganisms will help in reducing the initial lag phase of volatile fatty acid production. For the stable operation, pH of acid phase digester should be maintained in the range of 5.7-6.0.

2.5. References:


